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The Crystal Structure of Dimethanesulphonyl Disulphide

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The chemistry and preparation of dimethanesulphonyl disulphide have been described and discussed by Foss [1], who has also pointed out that chemical properties speak in favour of an unbranched sulphur chain. No detailed studies, however, have yet been reported in the literature of the structure of this compound, or any other tetrathionic compound. Lattice dimensions and space group were determined for potassium tetrathionate by Tunell, Merwin and Ksanda [2], but no structure details are available. On the other hand, extensive analyses of the structures of the trisulphides have been reported by Zachariassen [3], Ketelaar and Saunders [4], Robertson *et al.*[5,6] and Donohue [7,8]. Robertson *et al.* have also given some data and a discussion of possible structures for pentathionates. From cell dimensions extended, unbranched sulphur chains are likely in the structures of pentathionates, and such structures are also the most probable on chemical grounds. The same point of view applies to the tetrathionic compounds, thus to dimethanesulphonyl disulphide $(CH_3SO_2S)_2$, for which some preliminary X-ray data and possible structural arrangement were reported by Sörum and Foss [9].

The present paper concerns the results of a detailed analysis of the crystal structure of this compound by Fourier and least squares methods.

Unit cell and space group

The monoclinic crystals are usually found as needles or plates elongated in one direction. The specimen used in this analysis was obtained from one such plate by cutting and grinding it to approximately circular cross-section of about 0.2 mm diameter. Absorption of the X-rays in the specimen should therefore be negligible. All the X-ray photographs were obtained with Cu-K-radiation. Rotation and Weissenberg photographs were prepared with the crystal rotating around the needle axis (a-axis) and around an axis perpendic-

ular to the most prominent crystal face (b-axis). In the first case Weissenberg photographs were also obtained of the first and the second layer-line. The unit cell dimensions, determined from these photographs, are:

$$a = 5.52 \pm 0.02 \text{ Å} \qquad c = 10.05 \pm 0.02 \text{ Å}$$
$$b = 15.78 \pm 0.02 \text{ Å} \qquad \beta = 97.6° \pm 0.5°$$

corresponding to an axial ratio $a : b : c = 0.349 : 1 : 0.637$, volume $V = 866$ Å^3, specific density of 1.71 g/cm^3 and four molecules per unit cell. The following systematic absences of reflexions are recorded:

$0k0$ when k odd,
$00l$ when l odd,
$h0l$ when l odd,

indicating that the b-axis is a screw axis and the (010) plane a glide plane of symmetry with translation $c/2$. Laue patterns indicate monoclinic holoedry and no signs of polarity of the crystals could be detected. The space group was consequently assumed to be $P\frac{2_1}{c}$ (C_{2h}^5).

Arrangement of the molecules in the unit cell

The direct analysis of this structure by the application of phase relationships for determination of the signs was not successful, probably because of the rather low values of the unitary structure factors. Actually very few of these approach 0.5 in magnitude. The structure has therefore been derived from Patterson synthesis, *i.e.* a projection of vector-density on (100), and some useful informations were obtained by the consideration of characteristic intensity variations of the reflexions. Thus, for example, 011 is too weak to be observed, 022 is the strongest one of the $0kl$'s, and 033 too weak to be observed. This might possibly indicate that the molecules lie approximately parallel and symmetrically to the plane (011) or (01$\bar{1}$) and at a distance of approximately one quarter of a diagonal, in the (100)-projection, from the centres of symmetry. On the other hand, an atom located in such a way in the unit cell that its projection on (100) lies on or near a line connecting the centres of symmetry will make no, or only a small contribution to reflexions for which $k + l$ is odd. The fact that reflexions with $k + l$ odd are on the average as strong as reflexions with $k + l$ even suggests that the approximate centres of the molecules are translated about $|b/4|$ and $|c/4|$ from the centres of symmetry.

Fig. 1. Projected vector-density on (100) for dimethanesulphonyl disulphide. One quarter of the unit cell is shown. The orientation of one sulphur chain and the intramolecular S—S distances are indicated.

The Patterson synthesis

With this knowledge of the probable orientation and location of the molecules as backing the further attack on the structure was made by examination of the Patterson projection on (100). The assymmetric part of this projection is shown in Fig. 1. The four sulphur atoms were first assumed to lie approximately in a plane and such that they would fall on a line in the (100)-projection. This assumption led, however, either to unreasonable interatomic distances or to serious contradictions between observed and calculated structure factors. If the four sulphur atoms are placed on a somewhat irregular zigzag line in (100)-projection, the interatomic vectors become more compatible with the maxima of the vector-density projection, and leads simultaneously to an appreciable amelioration of the agreement with observed intensities. The orientation and form of the zigzag sulphur chain is indicated in the vector-

density projection in Fig. 1. The projection of the molecule was then moved along the [011]-direction until a rough agreement with observed structure factors was obtained. The maxima along the line 001/2 in the projection (Fig. 1), some of which are clearly resolved, were also of help in finding the right position of the molecule. With the approximate structure, thus obtained, the signs of the $0kl$ reflexions were calculated and then the first Fourier-projection of electron density on (100).

Fourier and least squares refinement

The first Fourier-projection on (100) showed clearly the positions of the sulphur atoms, though the corresponding maxima were of somewhat uneven heights. The two oxygen atoms attached to the one end sulphur atom were also resolved, but the two other oxygens and the two methyl groups did not show up distinctly. Space considerations led, however, to a probable arrangement of these atoms, and successive calculations of structure factors and the electron density projection improved the resolution of the peaks and the agreement between observed and calculated structure factors. The observed intensities were originally converted to an absolute scale by a method suggested by Wilson [10], as the intensities were determined by visual and relative estimates. The scale factor was readjusted before the final calculation of the electron density projection so as to make $\Sigma|\mathrm{F}(0kl)|$ observed closely equal to $\Sigma|\mathrm{F}(0kl)|$ calculated.

The projection on (100) is shown in Fig. 2, where a little more than a quarter of the unit cell is reproduced in order to illustrate the structure of one complete molecule $(CH_3S_2OS)_2$. The final calculation of the $\mathrm{F}(0kl)$'s resulted in an average discrepancy between observed and calculated values of 18 % for about 170 observed $0kl$-reflexions.

Intensity estimates of about 60 $h0l$-reflexions of a Weissenberg photograph around [010] formed the basis for determination of x-coordinates of the atoms. With the knowledge of the y- and the z-parameters and with assumed interatomic distances (from previous determinations of the structures of similar sulphur compounds), the $F(h0l)$'s could be expressed by one parameter for the displacement of the molecule along the a-axis. The value of this parameter was then chosen so as to give the best possible agreement with experimental data. Two possible arrangement had, however, to be considered. Thus, the direction of the longest extension of the molecule could make either a positive or a negative angle with the c-axis direction. These two possibilities are not widely different since the angle β differs only a few degrees from 90°. In effect, the latter alternative gave only a slightly better agreement with experimental

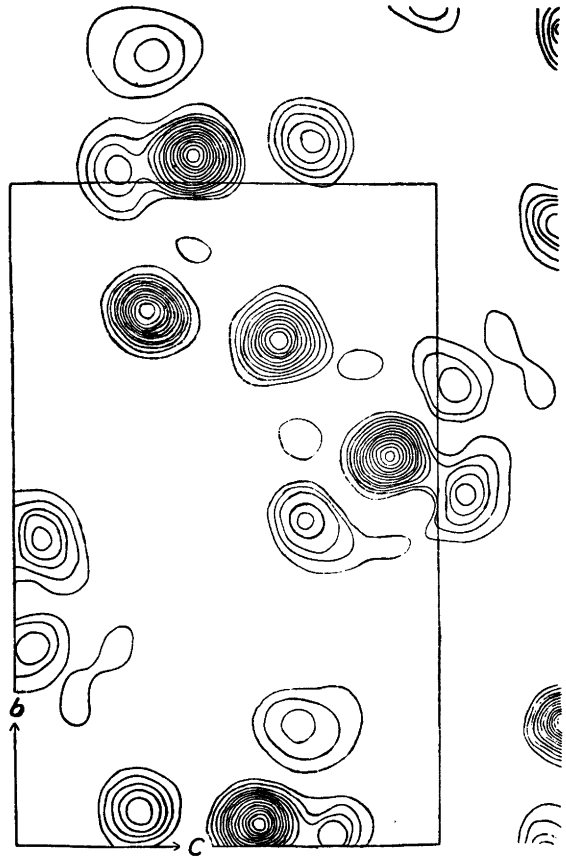

Fig. 2. Projection of electron density on (100) of dimethanesulphonyl disulphide. A little more than one quarter of a unit cell is indicated in order to show the projection of one complete molecule. Contour lines are drawn at intervals of 1 electron per Å^3, beginning with the 3-electron contour.

data than the first alternative in the first calculation of the F($h0l$)'s. The subsequent least squares refinement, by a method including a simplified technique to be described elsewhere [14], led, however, to a substantial improvement in case of the latter alternative, whereas only a slight improvement was obtained in the first case. In addition, some rather unreasonable bond-lengths and bond-angles resulted in the first case, while being within reasonable limits in the latter. Thus, a discrimination between the two alternative structures proved possible and decisive.

Table 1. Atomic coordinates for dimethanesulphonyl disulphide, given in Å. Estimated limits of error are ± 0.02 Å for the S-atoms and ± 0.04 Å for other atoms.

	S_1	S_2	S_3	S_4	O_1	O_2	O_3	O_4	$(CH_3)_1$	$(CH_3)_2$
x	1.95	1.17	−0.34	0.48	3.34	1.78	−0.77	1.27	0.66	1.67
y	0.31	−1.53	−1.89	−3.24	0.13	0.48	−3.73	−4.03	1.36	−2.32
z	2.88	3.42	1.84	0.56	3.69	1.47	−0.34	1.56	3.35	−0.22

The average discrepancy between observed and calculated structure factors, after the least squares refinement, is 19 % for the $h0l$-reflexions alone. For all the reflexions together this error coefficient is 18 %. It seems not unreasonable to claim that this agreement is sufficient to ensure the correctness of the proposed structure. The refinement could possibly be carried a little further, but the intensities are of moderate accuracy, implying that it would not be justified to attempt any great improvement on the ground of these data.

The obtained atomic coordinates are set out in Table 1, and indicated in Fig. 3 for the structure viewed along the a-axis. Observed and calculated structure factors for about 250 reflexions are listed in Table 3. Bond-lengths, bond-angles and interatomic distances are quoted in Table 2.

The structure of the $(CH_3SO_2S)_2$-molecule

It is evident from Fig. 2 that the molecule of $(CH_3SO_2S)_2$ has an unbranched chain of four sulphur atoms, with two oxygens and one methyl group attached to either end sulphur. These four sulphur atoms are, however, not lying in a plane. On the contrary, planes through the two sulphur atoms in the middle of the chain and one or the other of the end sulphurs respectively, would make an angle of approximately 90° with each other. This means that the one CH_3SO_2-group is twisted about 90° out of the plane through the rest of the sulphur chain. The structure of the molecule, with bond-lengths and angles, is indicated in Fig. 4. It is interesting to note the striking similarity between the structure of this molecule, as viewed along the short axis, and the structure of a pentathionate, which has recently been reported in this journal by Foss, Furberg and Hadler [13]. The arrangement of oxygens, methyl and sulphur around the end sulphur atoms is tetrahedral, though somewhat irregular, as it may be seen from the calculated values for the bond-angles in Table 2.

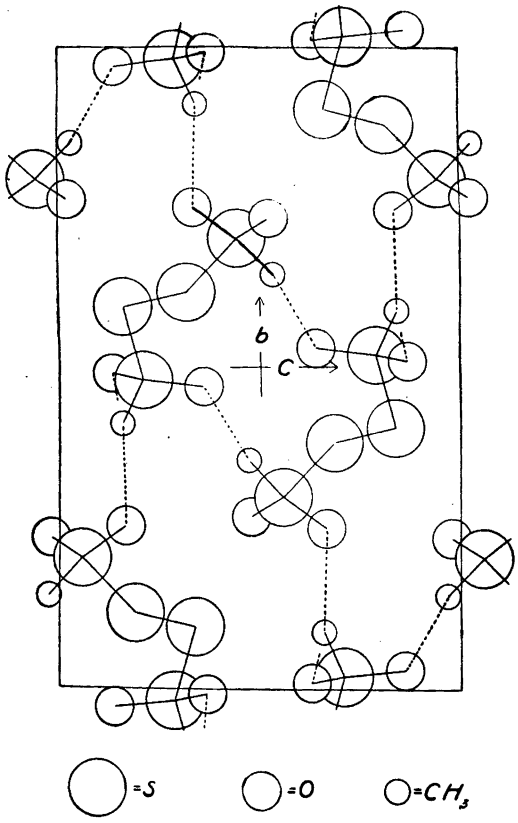

Fig. 3. The structure of dimethanesulphonyl disulphide, viewed in direction of the a*-axis. The centre and the boundaries of the unit cell are indicated. Bonds between the atoms are illustrated by full lines, and dashed lines indicate some short distances, corresponding to weak bonds, between oxygen atoms and methyl groups of different molecules. This weak bond from the O_1 oxygen atom points approximately in the direction of the* a*-axis.*

DISCUSSION OF THE STRUCTURE

The bond-lengths, set out in Table 2, may reasonably be claimed as accurate to within $\pm$ 0.03 Å for the S—S distances and $\pm$ 0.05 Å for the other distances. The diffraction effect of the heavier sulphur atoms may affect the positions of the methyl and oxygen peaks of the projection considerably, especially for the rather close oxygens, and this effect has only been partially removed by the least squares refinement.

The bond-lengths found in this work compare well with those previously reported for sulphur-oxygen compounds. The S—S bonds between the end

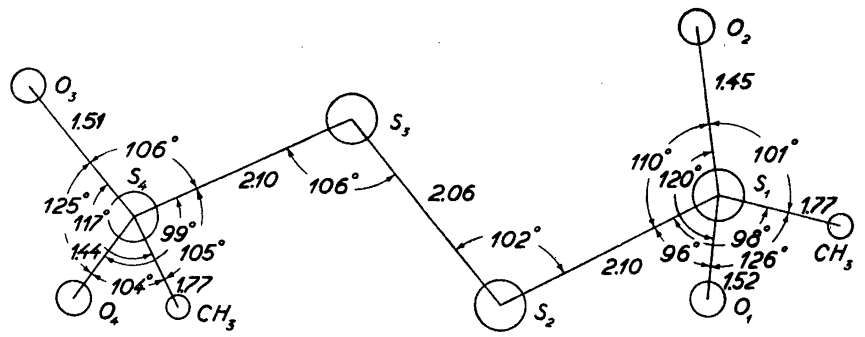

Fig. 4. The structure of the $(CH_3SO_2S)_2$-molecule. Bond-lengths and bond-angles are shown. In this figure the (CH_3SO_2)-group on the left hand side has been turned through an angle of approximately 90° around the line between the two sulphur atoms in the middle of the molecule.

sulphur and the nearest sulphur in the chain are both 2.10 Å, whereas the distance between the two sulphur atoms in the middle of the chain is 2.06 Å. and some significance may be assigned to the difference between these two values. Values ranging from 2.04 Å to 2.16 Å for the S—S distance have been reported in the literature, thus, for example 2.16 Å in trithionate (Zachariassen [3]), 2.07 Å in bisphenylsulphonyl sulphide (Robertson *et al.*[5]), while Warren and Burwell [11], Maxwell and Hendricks [12] determined the S—S distance in elementary sulphur as 2.10 Å. The distance of 2.10 Å, obtained here, may correspond closely to single covalent bond, whereas the slightly shorter S—S distance in the middle of the molecule may indicate some double bond character for that bond.

The S—O distances are on the average 1.48 Å, and thus being close to the value for that bond found in the sulphate ion. The accuracy, attained in this work, does not justify the assignment of any certain significance to the difference of the two S—O bonds, found for both groups, thus, 1.52 Å and 1.45 Å for the first group, 1.51 Å and 1.44 Å for the other group. The sum of the double covalent bond radii for S and O is 1.49 Å and the value of 1.48 Å may, therefore, suggest a considerable double bond character of the S—O bonds. The O—S—O angles are 120° and 125° respectively, thus being close to the expected value for the O—S—O angle of 125.16°, and to the angles found in sulphate and sulphite ions.

The S—CH_3 distances of 1.77 Å are only slightly shorter than the sum of the covalent single bond radii for S and CH_3, thus 1.04 + 0.77 = 1.81 Å.

The distances between non-bonded atoms in the molecule lies within reasonable limits, though the O—O distances of 2.57 Å and 2.62 Å, the O—CH_3

Table 2. Bond lengths, bond angles and some interatomic distances in dimethanesulphonyl disulphide. The limits of error are estimated as ± 0.03 Å for S—S bonds and ± 0.05 Å for other bonds. Errors in the angles are not likely to exceed ± 3°. Values given in brackets refer to distances between atoms of different molecules.

	S_1	S_2	S_3	S_4	O_1	O_2	O_3	O_4	$(CH_3)_1$	$(CH_3)_2$
S_1		2.10	3.24		1.52	1.45			1.77	
S_2	2.10		2.06	3.32	2.72	2.93			2.94	
S_3	3.24	2.06		2.10			2.91	2.72		3.08
S_4		3.32	2.10				1.51	1.44	2.94	1.77
O_1	1.52	2.72				2.57			(3.17)	
O_2	1.45	2.93			2.57					(3.18)
O_3			2.91	1.51				2.62	2.50	2.80
O_4			2.72	1.44			2.62		(3.21)	2.54
$(CH_3)_1$	1.77	2.95			2.94 (3.17)	2.50		(3.21)		
$(CH_3)_2$			3.08	1.77		(3.18)	2.80	2.54		

$\angle S_1-S_2-S_3 = 102°$, $\angle S_2-S_3-S_4 = 106°$, $\angle O_1-S_1-S_2 = 96°$,
$\angle O_2-S_1-S_2 = 110°$ $\angle CH_3-S_1-S_2 = 98°$, $\angle O_1-S_1-O_2 = 120°$,
$\angle CH_3-S_1-O_1 = 126°$, $\angle CH_3-S_1-O_2 = 101°$, $\angle O_3-S_4-S_3 = 106°$,
$\angle O_4-S_4-S_3 = 99°$, $\angle CH_3-S_4-S_3 = 105°$, $\angle O_3-S_4-O_4 = 125°$,
$\angle CH_3-S_4-O_3 = 117°$, $\angle CH_3-S_4-O_4 = 104°$

distances of 2.50 Å and 2.54 Å appear to be rather short. There are significant differences in the distances between $O_1-(CH_3)$ and $O_2-(CH_3)$ for the first group, $O_3-(CH_3)$ and $O_4-(CH_3)$ for the other group. These differences are of the order of 0.3—0.4 Å and may be attributed to steric hindrances.

The molecules are stacked together in pairs around the centres of symmetry, with the direction of their longest extension alternating in the [011] and the [01$\bar{1}$] direction, as may be seen from Fig. 3. The distances between the atoms of different molecules are all above the sum of the van der Waal's radii, except for some distances between oxygen atoms and methyl groups. Such distances are indicated by dashed lines in Fig. 3. The sum of the normal van der Waal's radii for O and CH_3 is 2.0 + 1.4 = 3.40 Å and such distances are usually not found shorter than 3.32 Å. The values of 3.17 Å, 3.18 Å and 3.21 Å found in this structure, are significantly shorter than previous values for this distance, and this observation forces one to conclude that weak bonds, possibly hydrogen bonds, are exerted between oxygen atoms and methyl groups belonging to different molecules. These weak bonds, in addition to normal van der Waal's attraction are holding the molecules together in the crystal.

Table 3. Observed and calculated structure factors for dimethanesulphonyl disulphide. The average discrepancy between observed and calculated values is 18%.

$h\,k\,l$	$F_{obs.}$	$F_{calc.}$	$h\,k\,l$	$F_{obs.}$	$F_{calc.}$
100	52	+50	$\bar{4}04$	—	+18
200	45	−34	$\bar{5}04$	—	−12
300	16	+21	$\bar{6}04$	22	+35
400	39	−36	106	34	+41
500	—	−11	206	73	−76
600	—	−15	306	—	−8
102	35	+27	406	26	+32
202	27	+32	506	13	−2
302	34	−35	$\bar{1}06$	12	−4
402	—	−4	$\bar{2}06$	39	+35
502	12	−18	$\bar{3}06$	—	−12
602	14	+12	$\bar{4}06$	—	−1
$\bar{1}02$	17	+23	$\bar{5}06$	15	−23
$\bar{2}02$	13	−5	$\bar{6}06$	21	−23
$\bar{3}02$	13	−15	108	38	−52
$\bar{4}02$	11	+6	208	—	+5
$\bar{5}02$	23	−30	308	11	−17
$\bar{6}02$	27	−33	$\bar{1}08$	38	−40
104	61	−65	$\bar{2}08$	66	−56
204	28	−31	$\bar{3}08$	29	+23
304	28	−34	$\bar{4}08$	37	+32
404	41	−45	$\bar{5}08$	14	−6
504	17	−24	1010	15	−21
$\bar{1}04$	68	+67	2010	14	−13
$\bar{2}04$	102	+85	3010	22	+13
$\bar{3}04$	78	+80			
$\bar{1}010$	14	−13	0131	25	−18
$\bar{2}010$	48	+47	0151	30	+22
$\bar{3}010$	28	+34	0171	—	−6
1012	16	+25	0191	7	+4
2012	14	+3	021	39	+36
$\bar{1}012$	—	+1	041	18	−21
$\bar{2}012$	19	−20	061	70	−74
$\bar{3}012$	42	−42	081	76	−72
020	31	+26	0101	28	−14
040	26	+40	0121	—	+2
060	22	−20	0141	43	−45
080	74	+67	0161	25	−28
0100	74	+64	0181	24	−24
0120	18	−15	022	100	−95
0140	20	−16	042	12	+17

Table 3 continued.

h k l	$F_{obs.}$	$F_{calc.}$	*h k l*	$F_{obs.}$	$F_{calc.}$
0160	15	−15	062	9	−2
0180	20	−19	082	42	−45
0200	8	+8	0102	16	−17
002	34	−38	0122	14	−13
004	30	−25	0142	29	+31
006	57	+61	0162	—	−1
008	34	−43	012	40	+39
0010	21	−30	032	27	−29
0012	—	−4	052	—	−4
011	—	−1	072	—	+4
031	56	−60	092	15	+17
051	36	+37	0112	34	+30
071	16	−20	0132	18	−19
091	—	+7	0152	—	−2
0111	36	−39	0172	25	+30
0192	13	+20	054	—	+1
013	49	+48	074	9	+16
033	—	+6	094	57	−65
053	23	+27	0114	12	+5
073	22	−29	0134	16	−17
093	33	+27	0154	12	+14
0113	36	+37	0174	16	−28
0133	31	+35	015	18	−15
0153	16	−16	035	35	−38
0173	26	−29	055	35	−33
0193	21	+9	075	—	+13
023	6	+6	095	—	+5
043	14	−13	0115	20	−21
063	40	+43	0135	—	−5
083	—	−6	0155	—	+12
0103	—	+7	0175	19	+26
0123	35	+26	025	21	−24
0143	—	+3	045	33	−33
0163	39	+35	065	41	+56
024	16	−20	085	11	+4
044	16	+16	0105	12	−7
064	27	+28	0125	12	−12
084	—	−4	0145	26	−19
0104	15	−16	0165	16	+23
0124	14	−14	0185	8	−8
0144	16	+18	026	20	+16
0164	11	+3	046	33	−40
0184	8	−4	066	33	−36

Table 3 continued.

h k l	$F_{obs.}$	$F_{calc.}$	*h k l*	$F_{obs.}$	$F_{calc.}$
014	26	−9	086	31	+34
034	16	−19	0106	—	+7
0126	14	+13	0108	22	−26
0146	30	−26	0128	24	+26
016	15	+16	0148	9	+4
036	27	+25	018	14	−8
056	—	+8	038	24	−30
076	27	+18	058	14	−12
096	—	−4	078	—	−6
0116	50	+58	098	15	−16
0136	14	+9	0118	17	−17
0156	15	+12	0138	27	−28
0176	14	+6	0158	19	−20
017	—	−1	019	14	−15
037	48	+42	039	14	−6
057	22	+22	059	37	−47
077	33	+36	079	—	−12
097	19	−21	099	14	−16
0117	14	−15	0119	—	+16
0137	—	+1	0139	17	+17
0157	14	−11	029	19	−15
027	40	+35	049	—	+6
047	—	+1	069	—	+9
067	14	−15	089	9	−5
087	24	−24	0109	16	−18
0107	14	+17	0129	18	+11
0127	25	+30	0210	15	+19
0147	12	−22	0410	10	−9
028	14	+17	0610	17	+23
048	19	+17	0810	18	+13
068	14	+13	01010	15	−14
088	—	−3			
0110	15	−20	0911	15	+12
0310	15	+9	0211	18	−21
0510	10	+12	0411	10	+4
0710	—	+18	0611	10	−8
0910	—	−1	0811	16	+10
01110	10	+8	0212	9	−4
0111	10	+14	0412	19	−32
0311	15	+12	0612	18	−28
0511	10	−11	0312	20	+29
0711	19	+32			

SUMMARY

The structure of dimethanesulphonyl disulphide has been determined in detail from X-ray intensity data by Fourier and least squares methods.

Atomic coordinates, bond-angles and interatomic distances are given, the structure is described and discussed. It is shown that the $(CH_3SO_2S)_2$ molecule has an unbranched zigzag sulphur chain. Weak hydrogen bonds between oxygen atoms and methyl groups appear to be acting as intermolecular forces in this structure.

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α-Aryloxyesters. II. Further Studies on Certain Self-Condensations

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In a previous paper [1] the self-condensations of ethyl and *t*-butyl 2,4,6,-trichlorophenoxyacetates * to the corresponding α, γ-di-TCPO-acetoacetates were described, and it was reported that the yield was considerably lower with the ethyl ester than with the *t*-butyl ester. Furthermore, the condensation product of the ethyl ester had a surprisingly lower melting point (78°) than both that of the *t*-butyl ester (131°) and that of ethyl 2,4-dichlorophenoxyacetate (98°). The purpose of the present investigation has been to elucidate, firstly, whether ortho-substituents in the phenyl groups have any significant influence on the yield of self-condensation products; and, secondly, whether any connection could be found between the melting

Table 1.

Substituents in benzene ring of aryloxyacetic ester	Alkyl in ester group	Yield of α,γ-diaryloxy acetoacetic ester %	m.p. °C	Analyses: Formula	Mol. weight calc.	% Halogen Calc.	Found
2-Chloro	Ethyl	68	72–74	$C_{18}H_{16}O_5Cl_2$	383.22	Cl 18.50	18.54
2,4-Dichloro	Ethyl [1]	60	96–98				
2,4,6-Trichloro	Ethyl	50	77–78 [1]				
2,4,6-Trichloro	Methyl	55	136–37	$C_{17}H_{10}O_5Cl_6$	506.99	Cl 41.96	42.31
2,4,6-Trichloro	*i*-Propyl	70	88–89	$C_{19}H_{14}O_5Cl_6$	535.04	Cl 39.76	39.77
2,4,6-Trichloro	*t*-Butyl [1]	51	129–31				
2,4-Dibromo	Ethyl	62	119–20	$C_{18}H_{14}O_5Br_4$	629.95	Br 50.74	51.09
2,6-Dibromo-4-methyl	Ethyl	59	100–02	$C_{20}H_{18}O_5Br_4$	658.00	Br 48.58	48.73
2,4-Dichloro	Methyl [14]	65	102–04				

* Throughout this paper the 2,4,6-trichlorophenoxy group is abbreviated to TCPO.

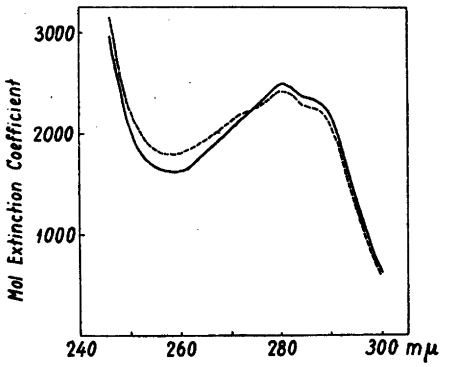

Fig. 1. Ultraviolet absorption spectra of solutions in absolute ethanol of ethyl (– – –) and t-butyl (————) α,γ-di-TCPO-acetoacetate. Concentrations 0.0002 M, cell-thickness 1.000 cm.

point of the acetoacetic ester and the size of the esterifying alcoholic group. To this end, a number of new self-condensations were carried out with suitable aryloxy esters as represented in Table 1. Attempts also have been made to search for structural differences between ethyl and *t*-butyl α,γ-di-TCPO-acetoacetates. The ultraviolet and infrared absorption spectra were determined; in the infrared, a number of structurally related compounds were investigated as well.

Apparently the size of ortho-substituents in the phenoxy groups has no important influence on the yield of self-condensation products. The low yield previously reported for ethyl TCPO-acetate must have been due to the procedure used in working up the reaction mixture. This procedure has been improved and now ethyl TCPO-acetate gives satisfactory yield. Even ethyl 2,6-dibromo-4-methylphenoxyacetate gives more than 50 % yield. Nor do the melting points show any systematic variation: although both ethyl and *i*-propyl α,γ-di-TCPO-acetoacetate have low melting points, the melting point of the methyl ester appears to be "normal". The absorption curves in ultraviolet (2 400—3 000 Å) for ethyl and *t*-butyl α,γ-di-TCPO-acetoacetate in absolute ethanol (Fig. 1) and in *n*-heptane do not give much information about the structures of the compounds. They are very similar and show a maximum at 2 800 Å, probably due to the TCPO-group *.

* The values of the extinction coefficients (ε = 2 415 and 2 490) are much too high for a carbonyl group (ε = 15—20 [2]). Ethyl acetoacetate shows a maximum at 2 430 Å (ε = 1 900), which has been ascribed to its enol content [3]. Any appreciable enolization of the di-TCPO-acetoacetates would be expected to effect a shift to lower wave-lengths. The ultraviolet spectra of ethyl TCPO-acetate, TCPO-acetone, and 2,4,6-trichloroanisole are almost identical and show likewise a band at 2 800 Å (ε = 800); in addition they show another band at 2 880 Å (ε = 800), while the di-TCPO-acetoacetates show only a shoulder at that wave-length.

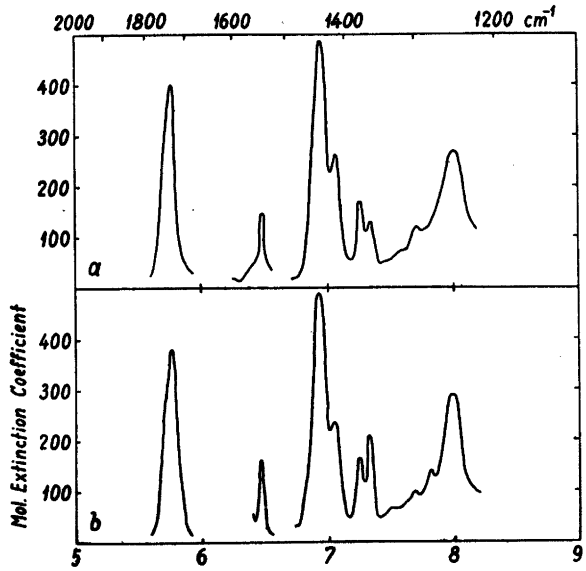

Fig. 2. Infrared absorption spectra of solutions in carbon tetrachloride of (a) ethyl and (b) t-butyl α,γ-di-TCPO-acetoacetate. Concentrations (a) 0.1093 M and (b) 0.0641 M; cell-thickness 0.1 mm.

The infrared absorption curves of the same two substances in carbon tetrachloride solution (Fig. 2) are practically coincident in the 5—7 μ region, showing a single strong absorption at 5.76 μ (1 735 cm^{-1}) presumably due to the carbonyl group. On the other hand, the infrared spectra of both compounds as solids suspended in nujol (Fig. 3 b and d) showed two peaks, at about 5.70 μ (1 755 cm^{-1}) and 5.82 μ (1 720 cm^{-1}). While the latter band was of about the same strength in the two spectra, the first band was considerably weaker in the spectrum of the ethyl ester. The methyl (Fig. 3 a) and the *i*-propyl (c) esters show the same two bands, the 5.70 μ band being of intermediate strengths *. No band was observed either in the 3 μ or the 6 μ region (corresponding to hydroxyl groups and carbon-carbon double-bonds respectively).

* In an attempt to interpret these bands, the spectra of certain related compounds as solids in nujol were measured: while 2,4,6-trichloroanisole (Fig. 3 k) shows no absorption at all in this region, and TCPO-acetone (i), TCPO-acetic acid (g), and TCPO-acetamide (h) all show one broad band, ethyl TCPO-acetate (f) (and ethyl phenoxyacetate), surprisingly, show two bands, at 5.65 μ (1 770 cm^{-1}) and 5.75 μ (1 740 cm^{-1}). Ethyl TCPO-acetate shows these bands also in solution (Fig. 4). α,γ-Diphenoxyacetoacetamide (Fig. 3 e), however, show two bands at 5.74 μ (1 742 cm^{-1}) and 5.88 μ (1 702 cm^{-1}), very near those of the acetoacetic esters investigated.

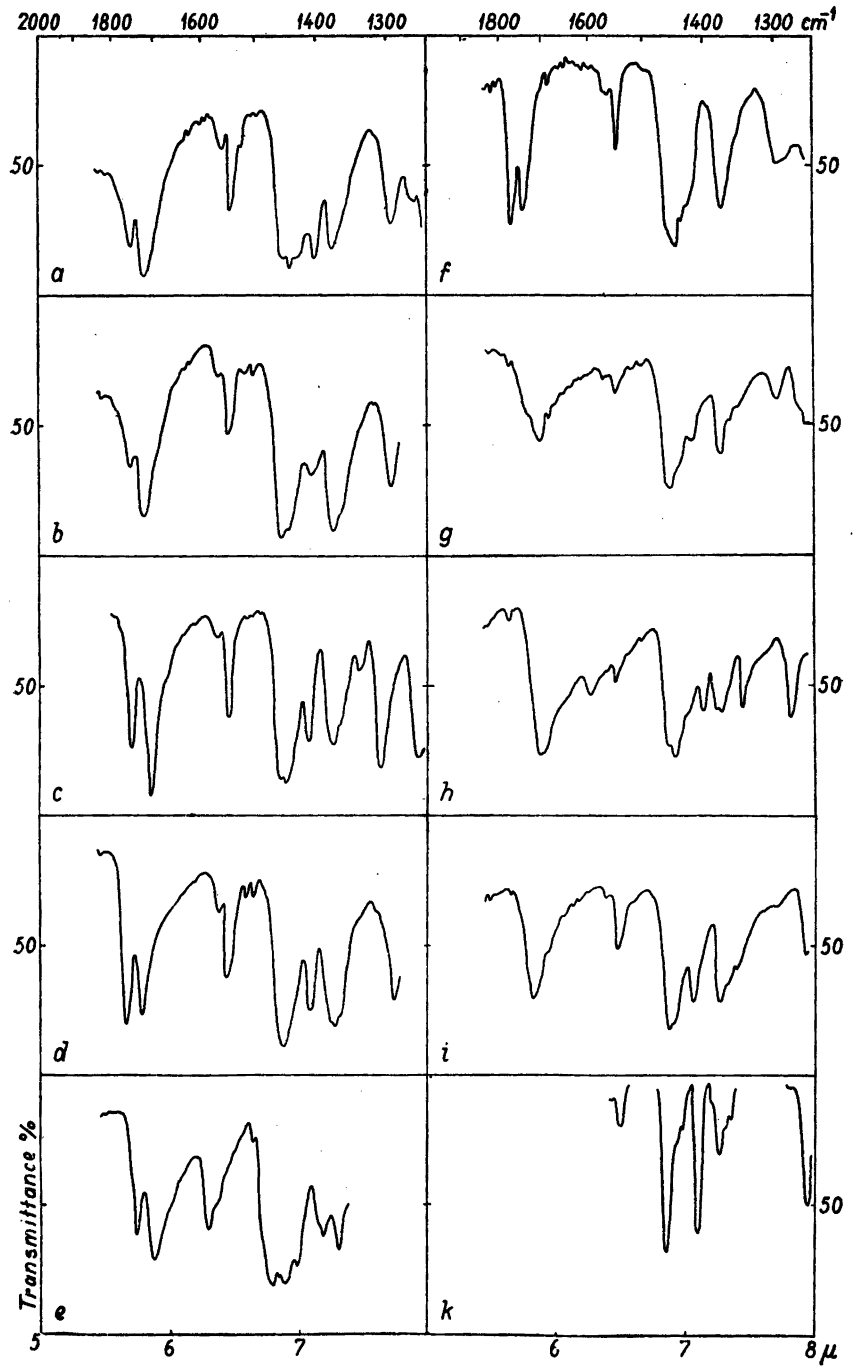

Fig. 3. Infrared absorption spectra of suspensions in nujol of: (a) methyl, (b) ethyl, (c) i-propyl, (d) t-butyl α,γ-di-TCPO-acetoacetate; (e) α,γ-diphenoxyacetoacetamide; (f) ethyl TCPO-acetate; (g) TCPO-acetic acid; (h) TCPO-acetamide; (i) TCPO-acetone; (h) 2,4,6-trichloroanisole. Approximate concentrations 1.3 × 10^{-4} mole in 0.2 ml nujol; cell-thickness approximately 0.01 mm.

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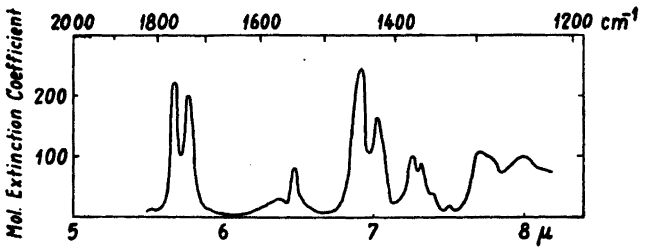

Fig. 4. Infrared absorption spectrum of ethyl TCPO-acetate in carbon tetrachloride solution. Concentration 0.247 M, cell-thickness 0.1 mm.

Thus, no indication of enolization has been obtained, and the compounds appear to be essentially ketonic in structure. According to their spectra in solution, they seem to be chemically almost identical, and the difference between them appears to be connected with the crystalline state. The solubilities (Table 2) show good correspondence with the melting points, as the two low-melting compounds are almost ten times as soluble as the two high-melting ones. A reasonable possibility, therefore, is that the differences observed are due to the degree of intermolecular association caused by steric effects of the alkyl of the ester group and the large TCPO group.

Table 2. Solubilities of α,γ-di-TCPO-acetoacetic esters at 20° C in moles per liter of absolute ethanol.

		m.p. °C
Methyl	0.032	137
Ethyl	0.282	78
i-Propyl	0.252	89
t-Butyl	0.030	131

EXPERIMENTAL

All melting and boiling points are uncorrected.

Starting materials. The following two esters were prepared from the corresponding phenol and ethyl chloroacetate, as previously described [1]: *Ethyl o-chlorophenoxyacetate*, b.p. 151—153° at 15 mm (m.p. 32° [4]), yield 70 %. Alkaline hydrolysis: $C_{10}H_{11}O_3Cl$ (214.65) Equiv.wt. found 214.2. *Ethyl 2,4-dibromophenoxyacetate*, b.p. 162—166° at 1 mm (m.p. 39.5—40.5°), yield 52 %. Alkaline hydrolysis: $C_{10}H_{10}O_3Br_2$ (338.01) Equiv.wt. found 335.0. 2,4-Dibromophenoxyacetic acid isolated: m.p. 150—152° (reported 151.8—153.3° [5]).

i-Propyl TCPO-acetate, b.p. 152—154° at 1 mm, was obtained by esterification of the acid [1], yield 65 %. This ester solidifies partially at room temperature. Alkaline hydrolysis: $C_{11}H_{11}O_3Cl_3$ (297.57) Equiv.wt. found 296.2.

The following two (solid) esters were obtained as previously [1] described for ethyl TCPO-acetate: *Methyl TCPO-acetate*, m.p. 62—63°; alkaline hydrolysis: $C_9H_7O_3Cl_3$ (269.52) Equiv.wt. found 268.3. *Ethyl 2,6-dibromo-4-methylphenoxyacetate*, m.p. 83—85°, yield 72 %; alkaline hydrolysis: $C_{11}H_{12}O_3Br_2$ (352.05) Equiv.wt. found 352.0. 2,6-Dibromo-4-methylphenoxyacetic acid isolated: m. p. 175° (reported 200° [6]); titration with 0.1 *N* sodium hydroxide: $C_9H_8O_3Br_2$ (323.98) Neutr.equiv. found 324.7. The required 2,6-dibromo-4-methylphenol, b.p. 153—156° at 20 mm (reported 139—40° at 11 mm [7]) was obtained in 48 % yield, by a procedure analogous to that given for 2-bromo-4-methylphenol [8], from 3,5-dibromo-4-aminotoluene, m.p. 73°, which was prepared by the bromination of *p*-toluidine in methanol.

2,4,6-Trichloroanisole, m.p. 60° [9], was prepared by shaking the phenol in potassium hydroxide solution with dimethyl sulfate [10].

TCPO-acetone, m.p. 78—79° (reported 75° [11]) was obtained from bromoacetone [12], the phenol, and potassium carbonate in dry acetone [13], yield 72 %. Found Cl 42.10; Calc. for $C_9H_7O_2Cl_3$ (253.52) Cl 41.96.

TCPO-acetic acid, *TCPO-acetamide*, *ethyl TCPO-acetate*, *ethyl α, γ-di-TCPO-acetoacetate*, *t-butyl α, γ-di-TCPO-acetoacetate*, and *α, γ-diphenoxyacetoacetamide* were the products previously described [1].

Self-condensations. The procedure previously described [14,1] was modified. To the ether suspension of di-*i*-propylaminomagnesium bromide * (generally 0.1 or 0.2 moles) was added, during ½—1 hour, one equivalent of the ester in ether solution. An oily precipitate usually formed **. After stirring at room temperature for about ½ hour the mixture was refluxed for two hours. The flask was cooled in an ice-salt bath and crushed ice and concentrated hydrochloric acid added until acidic reaction. Stirring was continued at room temperature until two clear layers were obtained. The ether layer was separated and the water layer extracted with ether. The combined ether solutions were washed with saturated sodium bicarbonate solution and water, dried by shaking with anhydrous sodium sulfate for 10—15 minutes, filtered, and concentrated to a small volume (50—100 ml). After standing over-night in the ice-box, generally most of the acetoacetic ester had crystallized. The products were recrystallized from the corresponding alcohols. The yields, data, and analyses are given in Table 1.

The ultraviolet absorption spectra (Fig. 1) were obtained by means of a Beckman model DU spectrophotometer, using quartz cells and a hydrogen discharge lamp.

The infrared absorption spectra (Figs. 2—4) were measured by using a Beckman model IR 2 spectrophotometer with a rock-salt prism. The slit was automatically adjusted by means of a mechanical slit drive coupled to the wave-length drive; the slit being 0.140 mm at 5.4 μ (1 850 cm^{-1}) and 0.285 mm at 8.5 μ (1 177 cm^{-1}). The substances were examined between rock-salt plates as dilute solutions in carbon tetrachloride (Figs. 2 and 4), or as suspensions in nujol (Fig. 3).

The solubilities in ethanol were determined by recrystallization of the pure compounds from that solvent. After standing over-night at 20°, the mother liquors were regarded as

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* The author is indebted to *A/S Dansk Shell, Copenhagen*, and to *Shell Chemical Corporation, New York*, for generous samples of di-*i*-propylamine.

** In the case of 2,6-dibromo-4-methylphenoxyacetic ester the precipitate formed lumps which made stirring difficult and probably decreased the yield. In the case of *i*-propyl TCPO-acetate the reaction mixture remained a clear solution.

saturated solutions. Chlorine was determined on aliquote samples, according to Stepanow's sodium reduction procedure followed by Volhard titration *, and from the chlorine content the concentration of acetoacetic ester was calculated.

SUMMARY

A number of halogen substituted aryloxyacetic esters have been satisfactorily self-condensed, apparently independently of the size and the position of the substituents in the phenyl ring.

Attempts have been made to explain some irregularities concerning the melting points of the self-condensation products of different 2,4,6-trichlorophenoxyacetic esters. The suggestion is made that the irregularities are due to differences in the crystalline, rather than in the chemical structures.

The ultraviolet and infrared spectra of some of the self-condensation products and certain related compounds are given.

The author wishes to express his gratitude to Mrs. Susanne Refn, M.S., for the infrared spectra and for valuable discussions concerning these spectra.

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* All halogen analyses were carried out using this method.

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An Ion Exchange and Extinctiometric Investigation of the Nickel Thiocyanate System

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The aim of the present investigation is a quantitative study of the complex equilibria between nickel and thiocyanate ions. The investigations of this system reported in the literature, give information only of the composition of the saturated complex, existing at high thiocyanate ion concentrations, but not of the successive complex formation at lower thiocyanate ion concentrations, or of the strengths of the complexes formed. Thus, Brintzinger and Ratanarat [1] consider that dialysis measurements prove that at the concentration 2 C of thiocyanate ions and small nickel concentrations all of the nickel is present as the complex $Ni(SCN)_4^{2-}$. From extinction curves of solutions containing varying amounts of nickel and thiocyanate ions Csokán [2] concludes that, with nickel in excess, $NiSCN^+$ is formed, while at a large excess of thiocyanate the complex $Ni(SCN)_4^{2-}$ predominates. According to Major [3] an uncharged complex exists in saturated solutions of nickel thiocyanate.

THE INVESTIGATION METHODS

The cation exchange method, described in two preceding papers (Fronæus [4,5], referred to below as I and II), was used in the present investigation for the determination of the complexity constants of the mononuclear complexes. In order to check the results an independent extinctiometric method was also applied.

As in previous investigations on other systems, the measurements were carried out at the constant ionic strength $I = 1$ C with sodium perchlorate as a supplementary neutral salt in the solutions. The temperature was kept at 20.0° C.

CHEMICALS USED

Sodium thiocyanate, p.a. The concentration of a stock solution, prepared from a calculated amount of the dried preparation, was checked by Volhard titration.

The *other chemicals* used were prepared as described in II. All stock solutions had the ionic strength 1 C. The cation exchanger (Amberlite IR-105), transformed to the sodium form, and air-dried, had the exchange capacity 2.31 meq. per gram. This was determined by transforming a sample of the exchanger to the hydrogen form, treating it with sodium acetate until equilibrium was established, filtering off and washing the exchanger and titrating with sodium hydroxide the acetic acid set free.

THE ION EXCHANGE INVESTIGATION

Before the treatment with the cation exchanger the complex solutions had the composition:

C'_M mC $Ni(ClO_4)_2 + C'_A$ mC NaSCN + $(1\,000 - 3C'_M - C'_A)$mC $NaClO_4$

with a constant value of C'_M and varying values of C'_A within each measurement series. The ratio m/v between the mass of the dried resin and the initial volume of the solution was kept constant (50.0 g · l^{-1}) at all measurements. After shaking the solution with the resin for twenty-four hours an exchange equilibrium was attained, and the solution was separated from the resin.

The equilibrium nickel concentration C_M was determined extinctiometrically at the wave length 500 mμ by means of potassium dithio-oxalate. The apparatus and the method of measurement were the same as those described in II. As nickel thiocyanate complexes are weak compared with nickel dithio-oxalate complexes, the molar extinction with respect to nickel is independent of the thiocyanate ion concentration, when the dithio-oxalate is added in excess.

The determination of the equilibrium thiocyanate concentration C_A was carried out by Volhard titration after addition of an excess of silver nitrate to a portion of the solution.

For the reasons given in II it is impossible to determine the nickel load C_{MR} on the ion exchanger by direct analysis of the resin phase, whenever one of the complexes formed can be absorbed by the exchanger. Thus C_{MR} must be indirectly calculated from eq. (6) of II. As m/v was kept constant, the quantity δ in this equation, depending upon the swelling of the resin, was the same for all measurements. The concentration of free anions in the resin phase being negligible, the factor δ was obtained as the quotient C'_A/C_A from solutions with $C_M = 0$. At these measurements the value of δ was 0.985.

The transformation of the ion exchanger from the hydrogen to the sodium form had been finished at a rather low $p[H^+]$. For that reason, after equilibrium with the exchanger was attained, the value of $p[H^+]$ in the complex solutions

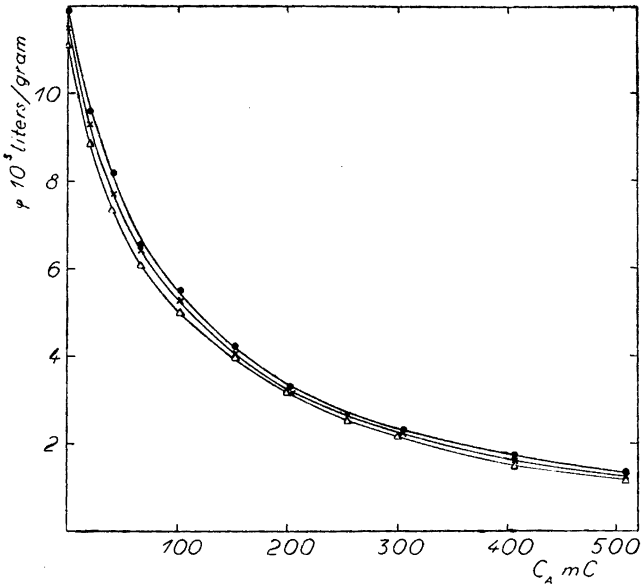

Fig. 1. The function $\varphi(C_A)$ at given values of C'_M.
●: $C'_M = 1.010$ *mC;* ×: $C'_M = 2.52$ *mC;* Δ: $C'_M = 5.05$ *mC.*

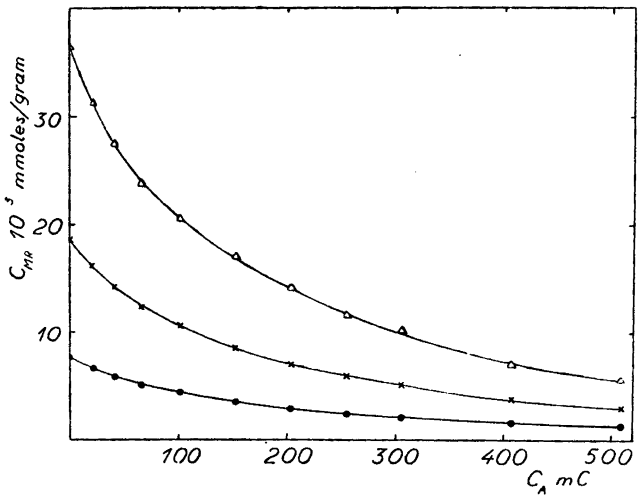

Fig. 2. The function $C_{MR}(C_A)$ at given values of C'_M.
●: $C'_M = 1.010$ *mC;* ×: $C'_M = 2.52$ *mC;* Δ: $C'_M = 5.05$ *mC.*

determined by emf measurements with a glass electrode, was constant and equal to 3.0. As the sorption affinity of Ni^{2+} for the exchanger used is dependent on the $p[H^+]$, it is necessary to work at a constant $p[H^+]$. Thiocyanic acid being a strong acid (*cf.* Gorman and Connell [6]), it is possible to choose a low $p[H^+]$-value, and this is expedient, as will be shown below.

The values obtained from the measurements are collected in Figs. 1 and 2, the same notations being used as in II. The quantities φ and C_{MR} can be considered as functions of the initial nickel concentration C'_M and the equilibrium thiocyanate concentration C_A. Thus, in these figures the functions $\varphi(C_A)$ and $C_{MR}(C_A)$ are represented at three given values of the parameter C'_M. From these curves, pairs of values (C_{MR}, φ), corresponding to some given concentrations C_A, are obtained. They are to be found in Table 1.

Table 1. Determination of corresponding values of C_A, C_M, and φ at $C_{MR} = 6.00 \cdot 10^{-3}$ mmoles $\cdot$ g^{-1}.

C_A mC	C'_M = 1.010 mC		C'_M = 2.52 mC		C'_M = 5.05 mC		$C_{MR} \cdot 10^3 = 6$ mmole $\cdot$ g^{-1}
	$C_{MR} \cdot 10^3$ mmole $\cdot$ g^{-1}	$\varphi \cdot 10^3$ l $\cdot$ g^{-1}	$C_{MR} \cdot 10^3$ mmole $\cdot$ g^{-1}	$\varphi \cdot 10^3$ l $\cdot$ g^{-1}	$C_{MR} \cdot 10^3$ mmole $\cdot$ g^{-1}	$\varphi \cdot 10^3$ l $\cdot$ g^{-1}	$\varphi \cdot 10^3$ l $\cdot$ g^{-1}
0	7.60	11.90	18.6	11.50	36.4	11.10	12.00
20.0	6.70	9.70	16.2	9.30	31.3	8.85	9.75
40.0	6.00	8.20	14.3	7.75	27.5	7.35	8.20
70.0	5.10	6.55	12.3	6.25	23.5	5.95	6.55
100.0	4.45	5.50	10.6	5.25	20.6	5.05	5.45
150.0	3.60	4.25	8.6	4.10	17.0	3.95	4.20
200	2.90	3.35	7.1	3.25	14.2	3.15	3.30
250	2.45	2.75	6.1	2.70	11.9	2.60	2.70
300	2.10	2.35	5.3	2.25	10.1	2.15	2.25
400	1.70	1.75	3.8	1.65	7.3	1.55	1.60
500	1.35	1.35	3.1	1.30	5.7	1.20	1.20

φ is then considered as a function of C_{MR} and C_A. From Table 1 it is evident that the dependence of φ upon C_{MR} at $p[H^+] = 3$ is very slight, so, by graphical interpolation, it is easy to determine the function φ (C_A) at a constant value of C_{MR} with great accuracy. In this case it was possible to choose the value of C_{MR} as low as $6.00 \cdot 10^{-3}$ mmoles $\cdot$ g^{-1}. The corresponding equilibrium nickel concentrations C_M, that can be obtained from the relation $C_M = C_{MR} \cdot \varphi^{-1}$, are so small that the approximation $[A^-] = C_A$ is quite satisfactory at every C_A.

The basic principle of the calculations is that at small C_{MR} the distribution coefficients l_0 and l_1 of Ni^{2+} and $NiSCN^+$ are functions almost solely of C_{MR} and not of the ligand concentration $[A^-]$. This principle has been tested experimentally for the exchanger used on other complex systems in I and II and will be explained theoretically in a future paper.

In the present paper the calculation procedure of II is applied. The φ-value at $[A^-] = 0$ and at the constant C_{MR}-value selected gives the distribution coefficient $l_0 = 12.00 \cdot 10^{-3}$ l · g^{-1} (Table 1, last column). Then in Table 2

Table 2. Determination of corresponding values of φ_1, f and the polynomial X_2 at different values of $[A^-]$ and at $C_{MR} = 6.00 \cdot 10^{-3}$ mmoles · g^{-1}.

$[A^-]$ mC	φ_1 C^{-1}	f C^{-2}	$\frac{\Delta\varphi_1}{[A^-]}$ C^{-2}	$\frac{\Delta f}{[A^-]}$ C^{-3}	X_2 C^{-2}
0	*11.4*	*127*			
20.0	11.5				
40.0	11.6	128			46
70.0	11.9	129			50
100.0	12.0	131			49
150.0	12.4	134			52
200	13.2	141	9.0	70	57
250	13.8	147	9.6	80	60
300	14.4	154	10.0	90	62
400	16.2	173	12.0	115	70
500	18.0	192	13.2	130	78

the function φ_1 has been computed according to eq. (2) of II and from a graphic extrapolation of this function to $[A^-] = 0$, $\beta_1 - l = 11.4$ C^{-1} is obtained. In the next column of Table 2 the function f has been computed from eq. (3) of II and extrapolated to $[A^-] = 0$. The differences $\Delta\varphi_1$ and Δf in eq. (5) of II have the following signification in the present paper:

$$\Delta\varphi_1 = \varphi_1 - 11.4 \text{ C}^{-1}, \quad \Delta f = f - 127 \text{ C}^{-2}$$

They can be calculated accurately enough only at ligand concentrations $\geqq 200$ mC. If $\Delta f/[A^-]$ is plotted against $\Delta\varphi_1/[A^-]$ the relation can be represented by a straight line, the slope of which gives the complexity constant of the first mononuclear complex:

$$\beta_1 = 15.0 \pm 0.5 \text{ C}^{-1}$$

Table 3. The ligand numbers $\bar{n}$ and $\bar{n}_R$ and the distribution of C_M between the nickel ion and the different complexes at given values of $[A^-]$.

$[A^-]$ mC	$\bar{n}$	$\bar{n}_R$	$100\alpha_0$	$100\alpha_1$	$100\alpha_2$	$100\alpha_3$
10	0.136	0.035	86.7	13.0	0.3	0
50	0.532	0.155	53.6	40.1	5.9	0.4
100	0.857	0.265	33.3	49.9	14.6	2.2
200	1.29	0.42	15.9	47.8	28.0	8.3
300	1.58	0.52	8.9	40.1	35.3	15.7
400	1.79	0.59	5.5	33.0	38.7	22.8
500	1.95	0.64	3.6	27.2	39.8	29.4

Combining this value with that obtained for $\beta_1 - l$ the value $l = 3.6 \pm 0.5$ C^{-1} is found. In the last column of Table 2 the polynomial X_2 has been calculated from eq. (4) of II. The connection between X_2 and $[A^-]$ can be graphically represented by a straight line. The intercept on the X_2-axis gives: $\beta_2 = 44 \pm 4$ C^{-2} and from the slope: $\beta_3 = 65 \pm 10$ C^{-3} is obtained. The linear relation shows that no higher complexes than $Ni(SCN)_3^-$ are formed in measurable amounts within the concentration range of A^- used.

With the constants β_1, β_2, β_3, and l, the ligand numbers $\bar{n}$ and $\bar{n}_R$ (*cf.* I p. 860) and the composition (*cf.* II p. 1207) of the complex system have been calculated in Table 3.

The mass law products k_0 and k_1

As stated above $(\partial\varphi/\partial\bar{C}_{MR})_{[A^-]}$ has a very small value at all concentrations of A^- and low concentrations C_{MR}. Thus, under the conditions mentioned the distribution coefficients l_0 and l_1 are but little dependent upon C_{MR}. The same must hold for the mass law products k_0 and k_1 calculated from eq. (2) of I, as the sodium ion concentrations are nearly constant in both phases. Corresponding values of C_{MR} and l_0 at $C_A = 0$ are to be found in the first line of Table 1 and for $[Na^+]$ and $[Na^+]_R$ the approximate values 1 000 mC and 2.31 mmoles $\cdot$ g^{-1} can be used with sufficient accuracy. The corrections caused by diffusible non-exchange electrolyte absorbed have been neglected. The values of k_0 are collected in Table 4. Thus, at $p[H^+] = 3$ the dependence of k_0 upon C_{MR} is as slight as we should expect, if the nickel ions are either free within the resin phase or partly coordinated to groups that are fixed at the exchanger, and in *great excess*, for instance sulphonate groups.

Table 4. The mass law product k_0 as a function of C_{MR} at p $[H^+] = 3.0$ and $C_A = 0$.

$C_{MR} \cdot 10^3$ mmole · g^{-1}	$k_0 \cdot 10^{-3}$ $l^{-1} \cdot g$
7.6	2.23
18.6	2.15
36.4	2.08

The value of k_1 at $C_{MR} = 6 \cdot 10^{-3}$ mmoles · g^{-1} can be calculated from eq. (2) of II, using the relation $l_1 = l_0 \cdot l \cdot \beta_1^{-1}$; the value $k_1 = 1.3$ is obtained. Thus at the p$[H^+]$ used the affinity of the complex $NiSCN^+$ for the exchanger is of about the same magnitude as that of the sodium ion, indicating that the complex is a free ion within the resin phase.

The almost constant value found for k_0 shows that a small exchange of sodium ions for nickel ions does not appreciably influence the activity coefficients in the resin phase. We should expect that for the exchange $Na^+ - NiSCN^+$, where both ions have equal valency, the influence is even less marked. Then the condition that l_0 and l_1 are independent of $[A^-]$ and can be treated as constants in the calculation of the complexity constants, must be very well fulfilled in the present case.

THE EXTINCTIOMETRIC INVESTIGATION

As shown previously (I p. 862) the cation exchange method can be used for the determination of the complexity constants of the mononuclear complexes at an arbitrary composition of the complex system. However, such measurements cannot give any information whether polynuclear complexes are also formed or not. The extinctiometric determination of the ligand number $\bar{n}$ has, therefore, been chosen as a check, as this method presupposes that mononuclear complexes are formed exclusively. The strength of the nickel thiocyanate system has moreover an appropriate magnitude for an accurate extinctiometric determination. The notations, the method of calculation, and the apparatus used are the same as described in a previous paper [7].

The measurements

The extinction curves of the nickel and thiocyanate ions and of a solution with $C_M = 25.0$ mC and $C_A = 925$ mC were determined in order to select the

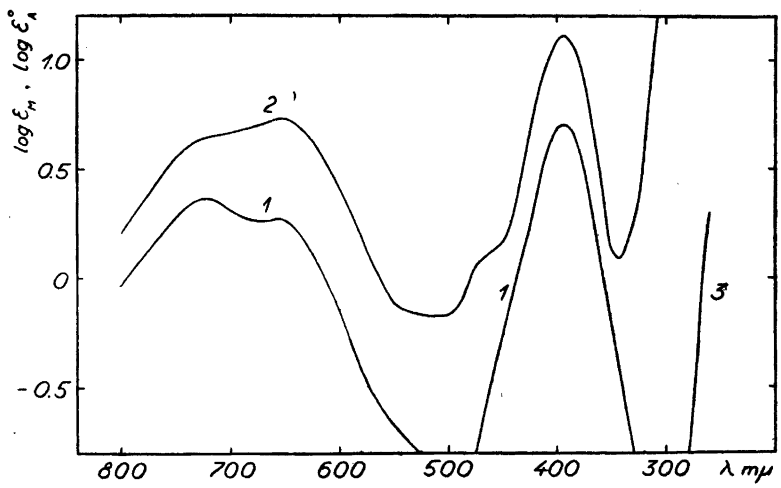

Fig. 3. Extinction curves of: 1. the nickel ion; 2. a complex solution with C_M = 25.0 mC and C_A = 925 mC; 3. the thiocyanate ion.

most suitable wave-length λ for the measurements, and are represented in Fig. 3. The two first-mentioned curves are in agreement in the main with those obtained by v. Kiss and Csokán [8].

On selecting a suitable wave-length the following two conditions should be considered. With the cells available the values of the total extinction must lie within an appropriate variation range. The C_M-values must not be so great that the ionic medium may be changed appreciably, and not so small that the difference $C_A - [A^-]$ cannot be determined with sufficient accuracy in any measurement series. In view of these conditions it can be seen that the low ε_M-values at $\lambda > 310$ mμ and the high ε_M-values at $\lambda < 290$ mμ make measurements impossible at such wave-lengths. Thus a narrow range remains, and 295 mμ was chosen as a suitable value of λ. At 295 mμ $\varepsilon_M^0 \approx 0$ and ε_A^0 is very small.

In one and the same measurement series, C_M and the layer thickness d were kept constant, and in the different series the product $d \cdot C_M$ was constant and equal to 25.0 cm · mC. Thus solutions with the same value of ε_M are measured at the same total extinction E, which is necessary to avoid certain systematic errors in the determination of $\bar{n}$ [7, p. 151].

The values measured are collected in Fig. 4.

The function $\varepsilon_M(C_A)$ is represented at three given values of the parameter C_M. From these curves pairs of values (C_M, C_A), corresponding to nine selected ε_M, are obtained (Table 5).

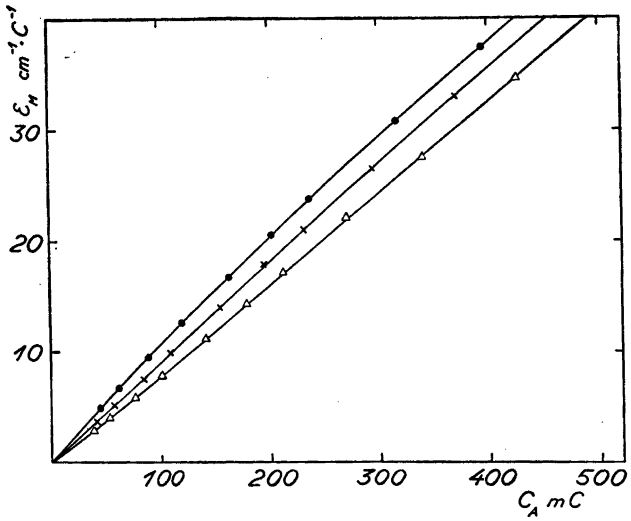

Fig. 4. The function $\varepsilon_M(C_A)$ at different values of C_M.
●: $C_M = 8.33$ mC; ×: $C_M = 25.0$ mC; Δ: $C_M = 50.0$ mC.

If C_A is plotted against C_M at a constant value of ε_M, practically a straight line is obtained, indicating that only mononuclear complexes are formed[7, p. 150]. Then the slope gives $\bar{n}$, and from the intercept on the C_A-axis we get the corresponding $[A^-]$. Owing to the almost rectilinear course of the curves in Fig. 4 all the values of $\bar{n}$ in Table 5 are obtained with about the same accuracy.

Table 5. Determination of the complex formation function from the extinction measurements.

ε_M cm^{-1} · C^{-1}	C_M = 50 mC	C_M = 25 mC	C_M = 8.33 mC	C_M = 0	$\bar{n}$	$\frac{\bar{n}}{[A^-]}$ C^{-1}
	C_A mC	C_A mC	C_A mC	$[A^-]$ mC		
5.00	67.5	56.5	46.0	42.0	0.53	12.6
7.00	92.0	79.0	65.0	61.0	0.65	10.7
10.00	129.0	111.0	93.5	87.5	0.86	9.85
15.0	189.0	165.5	144.5	137	1.06	7.75
20.0	250	221	197.0	187	1.28	6.85
25.0	310	278	251	240	1.42	5.90
30.0	371	335	308	297	1.48	5.00
35.0	431	394	367	355	1.54	4.35
40.0	492	454	426	414	1.56	3.75

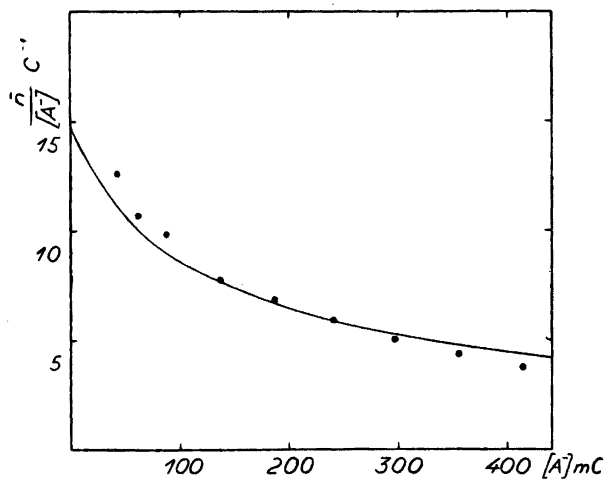

Fig. 5. $\bar{n}/[A^-]$ *as a function of* $[A^-]$. *Full circles relate to the extinction measurements, and the fulldrawn curve has been calculated from the ion exchange measurements.*

In Fig. 5, representing the function $\bar{n}/[A^-]$, the results from the two investigations can be compared. It will be seen that within the limits of experimental random errors the values determined extinctiometrically fall very well along the fulldrawn curve calculated from the values of $\bar{n}$ and $[A^-]$ in Table 3. This means that the extinction measurements give the same set of complexity constants as the ion exchange investigation.

Thus we are justified in concluding that the two methods have functioned well and that the constants obtained are real equilibrium constants. Furthermore it has been proved that mononuclear complexes are formed exclusively.

SUMMARY

The equilibria between nickel and thiocyanate ions are investigated at 20° C and at a constant ionic strength $I = 1$ C. Sodium perchlorate is used as a supplementary salt in the solution.

The investigation is performed in two ways, by an ion exchange method [4,5] and extinctiometrically [7]. The results obtained by the different measurements are consistent, proving the applicability of the methods to the present system. At thiocyanate ion concentrations $\leqq 0.5$ C the following three mononuclear complexes are formed: $NiSCN^+$, $Ni(SCN)_2$, and $Ni(SCN)_3^-$. In the conditions stated the values found for the complexity constants are:

$$\beta_1 = 15.0 \pm 0.5 \text{ C}^{-1}, \quad \beta_2 = 44 \pm 4 \text{ C}^{-2}, \quad \beta_3 = 65 \pm 10 \text{ C}^{-3}$$

From the extinction investigation we arrive at the conclusion that practically no polynuclear complexes are formed.

The absorption of the complex $NiSCN^+$ in the ion exchanger is studied quantitatively. The measurements indicate that at $p[H^+] = 3$ this complex is free within the resin phase and not coordinated to structurally bound groups of the ion exchanger.

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On the Substrate Specificity of Rhodanese

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Lang [1] showed in 1932 that cyanide in the presence of thiosulfate and liver tissue was converted to thiocyanate. An enzyme present in the liver and named rhodanese was shown to be responsible for the reaction. Lang also found that liver tissue catalyzed the formation of thiocyanate from colloidal sulfur and cyanide and assumed that rhodanese was responsible also for the latter reaction. Rhodanese was later studied by Saunders and Himwich [2,3] and Bénard *et al.*[4], using tissue homogenates as a source of the enzyme. Different sulfur containing compounds were investigated with respect of their ability to replace thiosulfate as sulfur donor in the enzyme system. The French workers claimed that sodium hydrosulfite $Na_2S_2O_4$ was active in this respect, other compounds were found to be practically inactive. The present study was undertaken in order to reinvestigate the results reported for colloidal sulfur and hydrosulfite with the use of a purified enzyme preparation. In connection with the work the efficiency of some other compounds as sulfur donors in the rhodanese system was investigated.

METHODS

Materials. Colloidal sulfur solution was prepared as follows: 40 mg sulfur was dissolved in 100 ml hot 96 % ethanol, the solution was rapidly cooled to room temperature, diluted to 500 ml with distilled water and then evaporated *in vacuo* to a volume of 200 ml. The solution had to be prepared fresh each day because sulfur gradually precipitated out of the solution.

Sodium ethyl thiosulfate (Bunte's salt) was prepared according to Otto and Troeger [5] and recrystallized from ethanol.

Sodium *p*-toluene thiosulfonate was prepared from *p*-toluene sulfonchloride and sodium sulfide according to the directions of Troeger and Linde [6]. It was recrystallized from ethanol.

Sodium hydrosulfite, potassium ethyl xanthate and sodium diethyldithiocarbamate were commercial products and used without further purification.

Rhodanese was purified from beef liver using an improvement of the method described in a preceding paper [7]. The liver was extracted, treated with lead acetate and fractionated with ammonium sulfate as described before. It was then dialyzed against 0.02 *M* sodium acetate, the pH adjusted to 5.0 and ethanol added to 10 % by volume. The solution was shaken in the cold room for two minutes with chloroform and the precipitated proteins and excess chloroform was centrifuged off. The supernatant was then fractionated with acetone at −5° C and the precipitate appearing between 25 and 45 % acetone was collected and dissolved in *M*/15 disodium phosphate. The pH was adjusted to 8.0 and the enzyme precipitated by raising the acetone concentration to 50 %. The enzyme was dissolved in *M*/150 phosphate buffer pH 7.4 and again precipitated by raising the acetone concentration to 50 %. The last two steps gave only an insignificant purification and can advantageously be omitted. The acetone remaining in the precipitate was now removed by evaporation at −18° C and the precipitate was then dissolved in distilled water. The pH was adjusted to 4.6 and the solution fractionated with ammonium sulfate. The precipitate appearing between 7.5 and 46 % saturation was collected and dissolved in *M*/15 disodium phosphate. The preparation thus obtained represented a 400-fold purification from the starting material and had a specific activity over twice that of the preparation obtained by the original method. It was stored for 3 months at −18° C without any loss of activity. When a 0.5 % solution of this preparation was examined in the "Spinco ultracentrifuge" a homogenous boundary was observed with $s_{20} = 2.76$ *S*. A similar preparation was then studied with respect to solubility in ammonium sulfate solutions of different concentrations and appeared homogenous. When the concentration of ammonium sulfate was brought to incipient precipitation of the enzyme, the latter crystallized in small plates. Further details concerning the new method of purification will be published in the near future.

Liver homogenates were prepared by disintegrating frozen, thawed beef liver with 3 volumes distilled water in an "Atomix" blender and filtering the obtained suspension through glass wool in order to remove coarse particles.

Determinations. The formation of thiocyanate from an equimolar mixture of the sulfur donor and cyanide was with two exceptions studied in the following test system. To 1.0 ml of a 0.125 *M* solution of the sulfur donor studied (in the case of hydrosulfite this solution was 0.08 *M* with respect to NaOH in order to obtain the desired pH in the test) was added 0.5 ml of a 0.40 *M* phosphate buffer (equimolar mixture of disodium and monopotassium phosphate) and 0.5 ml of the properly diluted enzyme. The reaction was started by adding 0.5 ml of a 0.25 *M* KCN solution and stopped after 5 minutes at 20° C by adding 2.5 ml of a ferric nitrate-nitric acid reagent. The latter contained 100 g $Fe(NO_3)_3$ 9 H_2O and 200 ml 65 % HNO_3 per 1 000 ml. The test solution was diluted to 30 ml and in the case of ethyl xanthate and diethyldithiocarbamate centrifuged, as the latter compounds gave precipitates with the ferric nitrate reagent. 30 minutes after the addition of ferric nitrate the optical density at 4 600 Å was determined in the Beckman spectrophotometer. The values obtained were compared with those obtained using a standard solution of thiocyanate in the test system. A blank determination was always carried out, using 0.5 ml distilled water instead of enzyme. The pH in this test system was 8.75 which was found to be the pH-optimum for the enzyme when thiosulfate was the substrate. In the case of *p*-toluene thiosulfonate preliminary experiments showed, however, that the spontaneous reaction between this compound and cyanide was so rapid at pH 8.75, that any effect of the enzyme would be imperceptible. By lowering the pH to 7.4 better conditions were obtained and the test

was in this case carried out as follows. At the start of reaction 1.0 ml of a solution, which was 0.125 *M* with respect to cyanide and 0.20 *M* with respect to phosphate and adjusted to pH 7.4 with HCl was added to 1.0 ml of a 0.125 *M* solution of *p*-toluenethiosulfonate and 0.5 ml enzyme. After 5 minutes at 20° C 2.5 ml ferric nitrate reagent and 0.5 ml 38 % formaldehyde were added and the test solution diluted to 30 ml. A precipitate was then centrifuged off, and the optical density at 4 600 Å was determined 30 minutes after the addition of ferric nitrate. Formaldehyde was added in this test in order to remove the interfering colour, which *p*-toluene thiosulfonate and *p*-toluene sulfinate (formed during the enzyme reaction) give with ferric ions. The activity of rhodanese in the *p*-toluene thiosulfonate test was compared with the activity obtained in a similar test system, which contained thiosulfate instead of *p*-toluene thiosulfonate. In the case of colloidal sulfur it was for practical reasons not possible to use a higher concentration of this compound than equivalent to 0.005 *M*. The same concentration of of cyanide and pH 8.75 were used. The components in the test were the following: 2.0 ml colloidal sulfur solution, 0.4 ml enzyme and 0.1 ml of a solution of pH 8.75, containing 0.125 *M* cyanide and 0.20 M phosphate. After 5 minutes incubation at 20° C, 2.5 ml ferric nitrate reagent was added and the test solution diluted to 30 ml. 30 minutes later the optical density at 4600 Å was determined. Turbidity was removed by a preceding centrifugation.

In these investigations the effect of dilution on the enzyme was considered. During the purification experiments it was found that the purified enzyme was considerably inactivated when it was diluted with distilled water or buffer before the assay. We never observed this inactivation with crude enzyme preparations in contrast to Saunders and Himwich [3] and Rosenthal *et al.*[8], who observed a rapid inactivation of rhodanese when tissue homogenates were diluted over a certain range. These authors also reported that the inactivation could be prevented by diluting the enzyme with a solution of thiosulfate. Thiosulfate could in fact prevent the inactivation observed with the purified beef liver enzyme. In consequence of this the assay method as described by Cosby and Sumner [9] was abandoned and the purification of rhodanese was followed with a new method of assay. The cyanide concentration was here 0.05 *M*, the thiosulfate concentration 0.10 *M*, the pH was 8.75 and the enzyme was always diluted with thiosulfate before assay. One μq protein of our purified enzyme as described above was in this test able to form 1.8 μ equivalents CNS^- during 5 minutes incubation at 20° C. In the experiments with the different sulfur donors the enzyme was diluted before incubation with a 0.01 *M* solution of the sulfur donor (in the case of hydrosulfite this solution was neutralized with NaOH). The only exception was colloidal sulfur in which case the enzyme was diluted with distilled water. The decrease in activity was here compensated for by comparing the obtained results with those obtained using the diluted enzyme in a simultaneous test in which colloidal sulfur was replaced by thiosulfate in the same molar concentration.

RESULTS

The results obtained with the purified enzyme are summarized in Table 1.

Because no activity was obtained with hydrosulfite and colloidal sulfur in contrast to previous reports, the effect of a crude liver homogenate on these systems was investigated. In the hydrosulfite system 0.5 ml homogenate di-

luted 1—20 gave 0.17 μeq. CNS^-, in the corresponding thiosulfate system the same amount of homogenate gave 7.02 μ eq. CNS^-. The activity obtained with hydrosulfite was thus 2.5 % of that obtained with thiosulfate. The colloidal sulfur and corresponding thiosulfate system contained 0.4 ml undiluted homogenate, which was adjusted with NaOH to pH 8.75 due to the weak buffer capacity of the system. With thiosulfate 4.35 μ eq. and with colloidal sulfur 1.96 μ eq. CNS^- were obtained. As the kinetics of the thiocyanate formation in these systems does not follow the zero order curve, the fractional activity can in this case not be expressed in %, but the activity of the colloidal sulfur is evident.

Table 1. Specificity of purified rhodanese

The test systems as described in the text contained 7.5 μg enzyme protein with the exception of *p*-toluene thiosulfonate and corresponding thiosulfate system, which contained 3 μg protein, and colloidal sulfur and corresponding thiosulfate system, which contained 60 μg protein.

Sulfur donor	Concentration of sulfur donor *M*	pH	CNS^- formed μeq.	Activity of thiosulfate system %
Thiosulfate	0.05	8.75	10.9	100
Ethyl thiosulfate	0.05	8.75	0	0
Ethyl xanthate	0.05	8.75	0.18	1.7
Diethyldithiocarbamate	0.05	8.75	0	0
Hydrosulfite	0.05	8.75	0.51	4.7
Thiosulfate	0.05	7.40	2.54	100
p-Toluene thiosulfonate	0.05	7.40	11.6	457
Thiosulfate	0.005	8.75	6.55	100
Colloidal sulfur	0.005	8.75	0	0

DISCUSSION

The results obtained with purified rhodanese showed that thiosulfate and *p*-toluene thiosulfonate but not ethyl thiosulfate could function as sulfur donor in the enzyme system. Considering the structure of these compounds [10a,11] it is seen that thiosulfate and *p*-toluene thiosulfonate contain a free thiol group. In ethyl thiosulfate this is blocked and the activity seems thus to be connected with the presence of this thiol group in the substrate molecule.

$^-S-SO_2-O^-$	$^-S-SO_2-C_6H_4CH_3$	$C_2H_5-S-SO_2-O^-$
Thiosulfate	*p*-Toluene thiosulfonate	Ethyl thiosulfate

Gutman [12] also found that no thiocyanate was formed in the spontaneous reaction between ethyl thiosulfate and cyanide under conditions when thiosulfate or aryl thiosulfonates gave a high yield of thiocyanate. In a recent communication it was concluded from inhibition data that the active group in rhodanese was a disulfide bond and a possible reaction mechanism was proposed [13]. Thiosulfate was assumed to form an intermediate enzyme-substrate compound, which decomposes in the presence of cyanide giving thiocyanate, sulfite and the free enzyme. This enzyme-substrate compound would, in its molecule, contain a chain of three sulfur atoms. The lability of such sulfur chains in the presence of cyanide is well known, for instance in the case of polythionates. In consequence of this all compounds containing a thiol group linked to sulfur would be expected to function as substrate for rhodanese. Sodium disulfide was then studied as a possible substrate. It was found, however, that the rate of the spontaneous reaction between the disulfide and cyanide was so high even at pH 7, that it was impossible to investigate the effect of the enzyme. But as a solution of sulfur in sodium sulfide with a composition corresponding to Na_2S_2 contains an equilibrium mixture [14] of Na_2S and Na_2S_4 these results are easily explained. Salts of dithioacids, with the group $-CSS^-$, in which, however, the two sulfur atoms are separated by a carbon atom, were also of interest as possible substrates. Ethyl xanthate and diethyldithiocarbamate were studied but found to be inactive. The latter compound was of special interest as its disulfide, tetraethylthiuram-disulfide is known to react spontaneously with cyanide, giving thiocyanate [15]. Previous workers have established [2,4] that compounds, containing a thiol group not linked to sulfur, are devoid of activity as substrates for rhodanese. Their stabilizing and protecting effect on the enzyme [2,7,13] must, however, be interpreted as a reaction between them and the enzyme.

These investigations do not confirm the claim of Bénard *et al.*[4] that hydrosulfite can function as a sulfur donor in the rhodanese system. Only insignificant amounts of thiocyanate was formed in the presence of the purified enzyme or a liver homogenate. As solutions of hydrosulfite rapidly decompose with the formation of thiosulfate [10b], the results obtained here are attributable to impurities of thiosulfate. This decomposition is retarded in alkali, and the high pH used in the present investigation was thus an advantage.

In the case of colloidal sulfur no formation of thiocyanate was observed with the purified enzyme, but with a liver homogenate a strong reaction was observed. Further experiments showed that this reaction obtained in the presence of liver homogenates was caused by a heat labile factor, also present in blood serum and muscle homogenates, which are entirely devoid of the catalytic activity on cyanide and thiosulfate. The enzyme catalyzing the

reaction between colloidal sulfur and cyanide must thus be different from that catalyzing the reaction between thiosulfate and cyanide. We suggest that the first enzyme is called rhodanese S, while the old name rhodanese is kept for the latter enzyme. The properties of rhodanese S will be reported in a following paper.

SUMMARY

1. Different sulfur containing compounds were investigated with respect to their efficiency as sulfur donors in the rhodanese system.

2. *p*-Toluene thiosulfonate was more active than thiosulfate. Ethyl thiosulfate, ethyl xanthate, diethyldithiocarbamate, hydrosulfite and colloidal sulfur were inactive.

3. The catalytic effect of liver homogenates on the formation of thiocyanate from colloidal sulfur and cyanide is due to an enzyme different from rhodanese.

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α-Keto-Acids in Green Plants

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The occurrence of α-keto-acids in plants, with particular reference to the biological N_2-fixation, was studied in this laboratory in the 1930's. Virtanen and Laine [1] believed that they had found oxaloacetic acid in pea plants by the hydrazine method of Szent-Györgyi and Straub [2]. Since this method gives unreliable results with green parts of plants it was modified later [3]. Virtanen *et al.*[4] were also able to detect oxaloacetic acid in young pea plants by determining the CO_2 produced by the action of aniline. Since oxaloacetic acid seemed to disappear rapidly from green plants on crushing them in dilute sulphuric acid, thus giving variable results, Virtanen and Arhimo [3] modified the method by adding sodium hydroxide to the plants on crushing in order to raise the pH to about 12—13. The addition of alkali stops respiration immediately and consequently, the decomposition of oxaloacetic acid is prevented more effectively than by the use of acid. Oxaloacetic acid could regularly be found by this method both in pea and clover leaves. Virtanen *et al.*[5, 6] studied in detail this method and the effect of light and other factors on the oxaloacetic acid content of different plants (*Pisum*, *Faba*, *Trifolium pratense*, *Phleum*, *Hordeum* and *Avena*). It was noticed that oxaloacetic acid disappeared in the dark, and it was not detected in maturing plants after flowering. In young pea plants 30—50 μg oxaloacetic acid were found per 1 g fresh weight, in red clover about double this amount.

Other methods were also used for estimation of keto-acids in green plants. Virtanen *et al.*[7] determined these acids in crushed plant material by forming from them 2,4-dinitro phenylhydrazones, which were then reduced with sodium amalgam to the respective amino acids. In this manner pyruvic, oxaloacetic, and α-keto glutaric acids were demonstrated in growing pea and red clover (*e.g.* in red clover 12 μg pyruvic acid, 18 μg oxaloacetic acid and 42 μg α-keto glutaric acid per 1 g fresh material). The method was, however, laborious and

unreliable, the results varying in the control experiments from 40 to 80 % of the theoretical values. Moreover, ascorbic acid and dehydroascorbic acid interfered with the determination of oxaloacetic acid [6].

Ketoglutaric acid has also been determined [6] in plants by oxidizing the 2,4-dinitro phenylhydrazones with potassium permanganate according to Krebs [8] and by determining the succinic acid formed from ketoglutaric acid.

Thus we considered that by using different methods we had been able to prove the occurrence of α-keto-acids, essential to the carbohydrate metabolism, in green plants.

Later, James and James [9] have observed that barley roots poisoned with formaldehyde or certain aromatic sulphonic acids give reactions for pyruvic acid not shown by normal plants. Bennett [10] observed that nitroprusside and other tests indicate that pyruvic acid may accumulate in the Ebenezer onion. Morgan [11] estimated pyruvic acid in the juice of onion as 2,4-dinitro phenylhydrazone. He found 0.1034 g pyruvic acid per 100 ml juice. Since no acid was found in the intact onion, he concluded that pyruvic acid was formed from a precursor or precursors while the onion was crushed. Wilson *et al.*[12] were unable to find oxaloacetic acid in pea plants and thus could not confirm the observations made in this laboratory. It is, however, true that these determinations were made on the expressed plant sap from which the proteins were precipitated with tungstate in a sulphuric acid solution, this treatment, according to the above, preventing ineffectively the decomposition of oxaloacetic acid.

In order to check our previous findings of the occurrence of keto-acids in green plants we carried out a number of determinations with pea using a paper chromatographic method for separation and identification of 2,4-dinitro phenylhydrazones of keto-acids.

EXPERIMENTAL

Being labile compounds α-keto-acids are not suitable to the paper chromatographic technique. Their phenylhydrazones are more stable and therefore more suitable for the purpose. The first to determine keto-acids in preparations of animal origin by paper chromatography as 2,4-dinitro phenylhydrazones were Cavallini *et al.*[13]. Since the detection of keto-acids in plants requires pretreatment of the material for stabilization of these acids, particularly oxaloacetic acid, and special measures for liberation of the 2,4-dinitro phenylhydrazone fraction from impurities disturbing the chromatography, a suitable method had to be devised. Development of the chromatograms also required more elucidation, in order to enable separation of the three α-keto-acids in question. After the affecting factors had been proved experimentally the following method was worked out and used in the determination of keto-acids in plant material.

Treatment of the plant material: 10 g of plant material were crushed in a mortar with 10 ml of 2 *N* NaOH which brought the pH to about 13. The crushed material was squeezed

through linen, the mortar and the precipitate were washed with 200 ml of 8 % trichloroacetic acid and the washings added to the extract whereby its pH fell to about 1. The solution was centrifuged and the supernatant liquid added to 10 ml of 1 % 2,4-dinitro phenylhydrazine reagent, mixed well and allowed to stand at room temperature for about 1 h. Crushing and centrifugation were regularly performed within 15 min. Since the plants taken from nature could not be treated as rapidly as the plants taken from the greenhouse of the laboratory, attempts were made to cool the plants rapidly by placing them in a glass bowl in a mixture of CO_2-ice and alcohol. The keto-acids then remained undecomposed for a longer time.

Reagents: 8 % solution of trichloroacetic acid was regularly made just before use from a 50 % basal solution which was kept in an icebox and renewed weekly. 1 % solution of 2,4-dinitro phenylhydrazine was prepared by dissolving 1 g in 5 *N* H_2SO_4. This, also, was kept in an icebox and renewed weekly.

Extraction: The solution containing 2,4-dinitro phenylhydrazones was shaken with ethylacetate until no further colour could be extracted. The combined extracts were then shaken with small amounts of 10 % Na_2CO_3 until the new soda phases remained colourless. 2,4-dinitro phenylhydrazones of keto-acids were brought to the soda solution. To this was added 2 *N* H_2SO_4 until the reaction was acid. The yellow colour was again extracted into ethylacetate and dried with anhydrous Na_2SO_4 overnight, after which the salt was separated by filtering and washing with a small amount of ehylacetate. The combined ethylacetate solutions were then evaporated *in vacuo* to about 1 ml.

Removal of fatty acids and other impurities: The paper chromatographic analysis of the ethylacetate solutions proved to be impossible because the spots of the dinitro phenylhydrazine derivatives were badly deformed due to impurities, principally fatty acids, present in the solution. These were removed as follows: Equal amounts of "bentonite"* and "Celite 545"** were mixed and of this mixture an adsorption column of 1 cm diam. and approximately 3 cm length was formed. Ether was allowed to pass through the column at the rate of 4 drops per minute, after which the ethylacetate extract was added to the column. Fatty acids *etc.* were then eluted with approximately 30 ml of ether. The adsorbent, which contained the adsorbed 2,4-dinitro phenylhydrazones of keto-acids, was then pushed out from the chromatographic tube into a centrifuge tube, where the dinitro phenylhydrazone derivatives were displaced from the adsorbent by three extractions with 10 ml of 10 % soda solution, the extract being clarified each time by centrifugation.

The combined extracts were made acid with 2 *N* H_2SO_4 and the 2,4-dinitro phenylhydrazones were extracted into ethylacetate which was then dried and concentrated *in vacuo* to 1 ml. 0.1 ml of this solution, added in small fractions to form a sharp concentrated starting spot, usually contained enough material for one chromatogram.

Paper chromatographic analysis: Both partition chromatographic separations with many organic solvents and adsorption chromatographic separations with different buffers in water solution were studied. Although separation by both principles was possible the latter method proved to be superior in our work. All buffers above pH 7.0 gave a distinct separation, the glycine-NaOH buffer of Sörensen, with pH 8.4 being the most suitable. This buffer was, therefore, used as a 0.1 molar solution whereby the three keto-acids in question were already well separated in one-dimensional runs (Fig. 1). Best results

* "Wyoming's" bentonite.

** Johns Manville Co., Box 60, New York 16, N.Y., U.S.A.

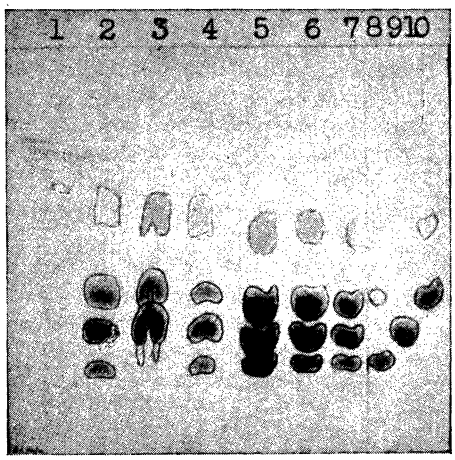

Fig. 1. Paper chromatographic analysis of an experiment with pea 20—21.VI.1952. Paper adsorption chromatogram by 0.1 M *Sörensen glycine-NaOH-buffer, pH 8.4. Whatman No. 1 paper, at 22° C for 8 h.*

1. *Plants harvested in the night, no sun for 9 h, NaOH added.*
2. *Plants harvested in the afternoon, kept at −10° C for 1 ½ h after which NaOH added.*
3. *Plants harvested in the afternoon, H_2SO_4 added.*
4. *Plants harvested in the afternoon, NaOH added.*
5. *Control, pyruvic, α-ketoglutaric, and oxaloacetic acids, 80 μg of each.*
6. *Control, as 5 but 50 μg of each acid.*
7. *Control, as 5 but 30 μg of each acid.*
8. *Control, oxaloacetic acid, 30 μg.*
9. *Control, α-ketoglutaric acid, 30 μg.*
10. *Control, pyruvic acid, 30 μg.*

were obtained in an atmosphere containing phenol vapours and traces of HCN. R_F-values: oxaloacetic acid 0.78, ketoglutaric acid 0.69, and pyruvic acid 0.45 (the faster and more distinct of the two spots produced by pyruvic acid). The "satellite" spots of the dinitro phenylhydrazone derivatives, probably due to isomeric forms, were all more strongly adsorbed having R_F-values below 0.45.

In the semiquantitative determinations the procedure was as follows: The 2,4-dinitro phenylhydrazone spots were cut off and the paper strips extracted in a beaker with 3 × 1 ml of 10 % Na_2CO_3. 3 ml of 2 *N* NaOH were added to the filtered solution. The red colour formed was measured photometrically by using the 525 mμ filter and the solution obtained by the same treatment from a strip of paper cut from the same sheet outside the spots was used as a standard. The "satellite" spots were thus left out from the determinations but usually they did not represent more than about 10 % of the total material.

As an example of the determination of keto-acids in plants by the above method we present the following:

20.6.1952. Samples were taken in the afternoon from pea plants growing in the greenhouse of the laboratory. The plants had been sown on May 21 and were not yet in flower. The weather was sunny on the day of harvest and had been so on the day before. The aim of the experiment was to elucidate not only the presence and quantities of ketoacids in plants but also the effect of alkali and acid treatment on the preservation of keto-acids. Further, the effect of low temperature on the preservation of keto-acids was examined.

Next morning before sunrise new samples were taken from the same lot of plants and crushed with alkali. This experiment was performed to control the earlier findings about the rapid disappearance of oxaloacetic acid in the dark when assimilation does not take place.

The experiments thus included the following:

1. 10 pea plants, fresh weight 12 g. The plants were crushed with alkali. (About 20 μg oxaloacetic acid, 40 μg α-ketoglutaric acid, and 20 μg pyruvic acid per 1 g fresh weight were found).
2. 10 pea plants, fresh weight 18 g. The plants were crushed with diluted sulphuric acid. (Oxaloacetic acid was not found.)
3. 10 pea plants, fresh weight 18 g. The plants were cooled to below $-$ 10° C by placing them in a glass bowl and keeping this in a mixture of CO_2-ice and alcohol for about 1 $\frac{1}{2}$ h, fresh CO_2-ice being added to the alcohol solution as required. The plants were then crushed with alkali. The results were practically the same as in experiment 1.
4. 10 pea plants, fresh weight 12 g. The plants which had received no sun for 9 h, were crushed with alkali. No oxaloacetic acid, and very small amounts of α-ketoglutaric acid and pyruvic acid were found.

(In experiments 1,2, and 3 the plants were removed in the afternoon at 16.30—17.00, in experiment 4 in the morning at 3.00.)

DISCUSSION

Fig. 1 illustrates the results of the experiments described above (20.6—21.6). The results show that all the three keto-acids, oxaloacetic acid, α-ketoglutaric acid and pyruvic acid are found in pea plants grown in the light, thus confirming the earlier results from this laboratory. Oxaloacetic acid evidently produces some pyruvic acid in the analysis but, judging from the chromatograms, pyruvic acid is present to some extent in the intact plants and is not formed only from oxaloacetic acid during the treatment and analysis of the plant material. The values found are obviously too low for oxaloacetic acid and too high for pyruvic acid. The two spots given by 2,4-dinitro phenyl-hydrazones of pyruvic acid and oxaloacetic acid, the slower ones being much weaker than the faster, are probably caused by cis-trans-isomeric forms. For this further reason the values recorded in the experimental part are only semiquantitative.

Oxaloacetic acid disappears rapidly from the plants in the dark in accord with the previous results from this laboratory. Under this condition both ketoglutaric acid and pyruvic acid are also decreased in quantity. The accumulation of oxaloacetic acid in the light and rapid disappearance in the dark suggest that the formation of this keto-acid is connected with the CO_2-assimilation.

On crushing the pea plants oxaloacetic acid disappears very rapidly, an indication of great activity of oxaloacetic acid decarboxylase and possibly even of some other oxaloacetic acid-consuming system in the cells. Since oxaloacetic acid is stabilized both in acid and alkaline reaction, it can be expected that its disappearance is prevented when the plant is crushed either with acid or alkali. The results obtained in this work as well as in the previous work of this laboratory indicate that the effect of alkali is far superior to that of acid, the chief reason obviously being that acid does not intrude into the cell so rapidly as does alkali.

When the plants are crushed with dilute sulphuric acid, oxaloacetic acid often disappears entirely as happened in the experiment described above. In two out of three samples of young pea plants, crushed with sulphuric acid, no oxaloacetic acid was found; in the third was found a little, which shows that in the treated plant material oxaloacetic acid may appear sometimes, but not regularly. In all these samples oxaloacetic acid was found when the pea plants were crushed with alkali. Oxaloacetic, α-ketoglutaric, and pyruvic acids were found both in the plants grown in the greenhouse of the laboratory and in nature providing that they were harvested in light. Rapid cooling of plants to below $-10°$ C prevents the disappearance of oxaloacetic acid for at least 2 hours.

Pea seeds germinated in the dark for 7 days contained no oxaloacetic acid but only α-ketoglutaric and pyruvic acids.

SUMMARY

A paper chromatographic method has been worked out for determination of α-keto-acids in green plants in the form of 2,4-dinitro phenylhydrazones. This has corroborated the results obtained in this laboratory in the 1930's by different methods on the presence of oxaloacetic acid, α-ketoglutaric acid and pyruvic acid in green plants. Other keto-acids were not detected in our chromatograms. In accord with the earlier observations it has been possible to confirm the rapid disappearance of oxaloacetic acid in the dark. On crushing the plant material oxaloacetic acid disappears, more readily in acid than in alkaline solution.

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Isotope Distribution in Amino Acids from Regenerating Rat Liver after Administration of Labelled Acetate

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In a series of investigations by Ehrensvärd *et al.* various microorganisms have been grown on $C^{13}H_3C^{14}OONa$[1–5] or $C^{13}H_3C^{14}OONa + CO_2$[6–8] as the only sources of carbon and the protein-bound amino acids investigated for their isotope content. In these cases the organisms had to synthesize all their amino acids from acetate or acetate plus carbon dioxide and consequently they were all found to be labelled. It might, therefore, be of interest to make a similar investigation on an organism, which by nutrition experiments has been found unable to synthesize some amino acids — the essential ones for the organism in question. In view of the extensive work carried out on regenerating rat liver, it was thought that this system would be suitable for carrying out an experiment with labelled acetate administred to rats after partial hepatectomy. Such an experiment has been made in which 14 amino acids have been isolated from the liver TCA-proteins after an 11 hour period of regenerative growth.

EXPERIMENTAL

Ten rats were subjected to partial hepatectomy and injected intraperitoneally with 2.5 *mM* of labelled acetate (C^{13} of methyl group = 35.25 % excess, C^{14} of carboxyl group = 142 500 counts/min per 15 mg barium carbonate) per 100 g body weight. This was divided into four doses given at three hours intervals; the rats were allowed to feed *ad libitum* during the experiment. The animals were sacrificed two hours after the last injection and the livers dried immediately with alcohol and ether. The proteins were precipitated with 10 per cent TCA, washed and dried with alcohol and ether; weight 8.2 g. *

* This part of the work was kindly performed by the staff of Professor Hammarstens laboratory, Karolinska Institutet, Stockholm.

The TCA-protein was hydrolyzed with 20 per cent HCl during 24 hours, whereafter the amino acids were isolated on a Dowex column, according to Ehrensvärd *et al.*[4,5]. For some reason phenylalanine and cysteine failed to emerge from the column; the amount of methionine was too small to be purified from contaminating isoleucine. A sample of each amino acid was combusted to CO_2, trapped as barium carbonate in order to determine the mean isotope content of all the carbon atoms. The amounts of amino acids isolated and the results of the total combustions are shown in Table 1.

Table 1. Amount and isotope content of amino acids from regenerating rat liver. C^{13} is given in atom per cent excess, C^{14} in counts per minute per 15 mg barium carbonate.

Amino acid	Amount mg	C^{13}	C^{14}	Amino acid	Amount mg	C^{13}	C^{14}
Glutamic acid (as hydrochloride)	575	0.192	340	Valine	315	0.016	5
Aspartic acid	230	0.114	215	Leucine	200	0.009	5
Alanine	220	0.136	124	Isoleucine	100	0.018	7
Serine (as alanine)	215	0.067	56	Threonine	90	0.016	5
Glycine	200	0.059	62	Tyrosine	150	0.013	0
Proline	115	0.058	75	Lysine (as hydrochloride)	290	0.019	2
Arginine (as hydrochloride)	340	0.053	104	Histidine (as hydrochloride)	80	0.022	2

It is easily seen that the amino acids with respect to isotope content are rather sharply divided into two groups, those in the left column with a fair amount of isotope, and those in the right column with a very low labelling. In the last mentioned amino acids no further attempts were made to localize in detail the isotope; the others were degraded as far as possible (for the ring of proline and the four middle atoms of arginine there are as yet no methods available).

Glutamic acid was degraded according to Mosbach, Phares and Carson [9].

Aspartic acid was decarboxylated with ninhydrin, yielding both the carboxyl groups.

Alanine was degraded by decarboxylating with ninhydrin about 45 mg (0.5 mmole) in a small vessel provided with an inlet tube; the mixture of CO_2 and CH_3CHO was flushed with nitrogen through a scrubber according to Phares [10], containing 10 ml of 6 *N* H_2SO_4, saturated with $K_2Cr_2O_7$. The CO_2 passed through and was trapped as usual in $Ba(OH)_2$, whereas the acetaldehyde was oxidized to acetic acid; after dilution with water the acetic acid was recovered by steam distillation and purified by chromatography on silica with 0.5 *N* H_2SO_4 as the stationary phase and 5 % butanol in chloroform as the mobile phase [11]. The acetic acid was then degraded according to Phares [10].

Serine was converted to alanine during the isolation and was degraded as above.

Glycine and *proline* were decarboxylated with ninhydrin.

Arginine was decarboxylated with ninhydrin and hydrolyzed with saturated $Ba(OH)_2$ during 24 hours to yield the carbon atom of the amidine group as $BaCO_3$. The barium carbonate, obtained in this way, was impure and was therefore dissolved in dilute HCl and the CO_2 reprecipitated. The ornithine moiety of arginine was isolated as the hydrochloride and subjected to total combustion.

The results of the degradations are shown in Table 2.

Table 2. Isotope content in different carbon atoms of some amino acids. C^{13} and C^{14} as in Table 1. $'C^{13}$ and $'C^{14}$ denote isotope content in per cent of the corresponding content of the labelled acetate, taken as 100.

	C^{13}	C^{14}	$'C^{13}$	$'C^{14}$	$'C^{14}/'C^{13}$
Acetic acid carboxyl group	—	142 500	—	100	
Methyl »	35.25	—	100	—	
Glutamic acid					
α-carboxyl	0.168	773	0.476	0.542	1.14
γ-carboxyl	0.117	1 230	0.332	0.864	0.26
α-atom	0.165	21	0.469	0.015	
β-atom	0.267	18	0.758	0.013	
γ-atom	0.327	18	0.928	0.013	
Aspartic acid					
α- and β-carboxyl mean value	0.108	443	0.306	0.311	1.03
α- and β-atoms mean value, by diff.	0.120	0	0.340		
Alanine					
carboxyl	0.100	424	0.284	0.298	1.05
α-atom	0.160	10	0.454		
β-atom	0.138	5	0.392		
Serine					
carboxyl	0.051	167	0.145	0.117	0.805
α-atom	0.068	6	0.193		
β-atom	0.067	7	0.193		
Glycine					
carboxyl	0.045	116	0.128	0.082	0.640
α-atom, by diff.	0.073	8	0.207		
Proline					
carboxyl	0.047	112	0.134	0.079	0.590
ring atoms, mean value, by diff.	0.061	66	0.173	0.046	0.266
Arginine					
carboxyl	0.046	120	0.131	0.084	0.641
amidine carbon	0.140	673	0.398	0.472	0.840
ornithine, mean value, by diff.	0.066	98	0.187	0.069	0.370

DISCUSSION

As mentioned before, a significant amount of isotope appeared only in the following amino acids: glutamic acid, aspartic acid, alanine, serine, glycine, proline and arginine. This is to be expected, since these amino acids have been found by nutrition experiments to be non-essential for the rat, whereas valine, leucine, isoleucine, threonine, tyrosine, lysine and histidine must be supplied in the diet. An important question, raised among others by Rose, is whether the essential amino acids cannot be synthesized at all, or whether the rate of synhesis is too low to supply the organism with an adequate amount of them. In the first case the essential amino acids in this experiment would, theoretically, be entirely non-labelled. However, in spite of repeated recrystallizations they retained (with the exception of tyrosine) a small amount of C^{14} (the C^{13}-content will be commented upon later). To infer from these findings, that *e.g.* leucine can be synthesized to a very small extent by the organism, whereas tyrosine cannot be synthesized at all, seems however, quite premature; probably the results only reflect the ease, with which tyrosine, because of its low solubility in water, can be purified from labelled contaminants.

If the labelling of the non-essential amino acids is regarded in more detail, it is seen that the distribution of the isotopes is in full accordance with the supposition of the Krebs' cycle in rat liver. The central position of glutamic acid, the labelling of which is at least twice as high as that of the other amino acids, is apparent; this has also been found by Åqvist [2]. As is now known with certainty [13], the product of the condensation of acetate with oxalacetate is citric acid — a chemically symmetrical compound. Nevertheless, glutamic acid, which reasonably might be supposed to represent α-keto-glutaric acid with respect to labelling, is unevenly labelled; the γ-atom has a higher content of C^{13} than the α-atom and the γ-carboxyl a higher content of C^{14} than the α-carboxyl, indicating that citric acid does not indergo any randomization on its pathway to α-keto-glutaric acid, as was also found by Potter and Heidelberger [14]. In the Krebs' cycle citrid acid apparently behaves like an asymmetrical compound. The labelling of the β-atom is somewhat obscure; it ought to have the same amount of C^{13} as the α-atom but has definitely more.

The isotope values of serine might indicate a connection with alanine (or pyruvate, assumed to be in equilibrium vith alanine). Its connection with glycine has been established by many investigators and is not contradicted by this experiment.

Because of the incomplete degradation of proline, little can be said about its origin, except that the data do not contradict the assumed connection with ornithine (the carboxyl groups of these two compounds have almost identical isotope values).

In connection with this work, the author would like to point to a technical question of some importance. It has been found in this laboratory[15], that combustion of amino acids, *commercially* obtained, yields a CO_2, which contains a small amount of C^{13} in excess of the standard (ethyl alcohol) employed here; it is generally between 0.010 and 0.020. Ninhydrin decarboxylation of these amino acids yields an even higher C^{13}-excess, in some instances rising to 0.035. Whatever the cause of this anomality, it is obviously necessary to take the figures for the C^{13}-content (especially the low ones) with some reservation.

SUMMARY

Partially hepatectomized rats have been fed $C^{13}H_3C^{14}OONa$ and 14 amino acids from the regenerating liver isolated. Glutamic acid, aspartic acid, alanine, serine, glycine, proline and arginine were labelled; valine, leucine, isoleucine, threonine, tyrosine, lysine and histidine contained only insignificant amounts of isotope. The distribution of C^{13} and C^{14} in the labelled amino acids was in accordance with the supposition of a Krebs' cycle in rat liver.

I wish to express my gratitude to Dr. Ehrensvärd, who suggested the work, kindly supplied the labelled acetate and was of great help throughout.

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Intermolecular Free Lengths in the Liquid State
II. Surface Tension

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Owing to the great biological and chemical importance of surface phenomena, the surface tension of liquids has been closely studied. A large number of relations has been demonstrated between surface tension and other properties (see Partington [1]). However no attention appears to have been devoted to the intermolecular free length. As this constitutes a simple and characteristic property of the liquid state, we shall in this work study the surface tension as a function of it.

The free length between the surfaces of the molecules in the bulk of the liquid, hereafter designated the free length (L), may be defined as

$$L = 2(V_T - V_0)/Y \qquad (1)$$

where V_T and V_0 are the molar volumes at T°K and O°K respectively, and Y is the molecular surface of one mole. This definition, which involves an approximation, has been thoroughly discussed in a previous work [2].

PURE LIQUIDS

In Fig. 1 the surface tension (σ) for 53 non-associated organic liquids has been plotted as a function of the free length (L) at 20° C. All liquids have been included for which it was possible to obtain accurate values for surface tension and zero volume (V_0). The surface tension values have been taken from Timmermann *et al.*[3] Harkins *et al.*[4] and Landolt-Börnstein Tabellen. Where several divergent values for the same liquid were found, the average value has been used. The free lengths have been computed according to equation (1)

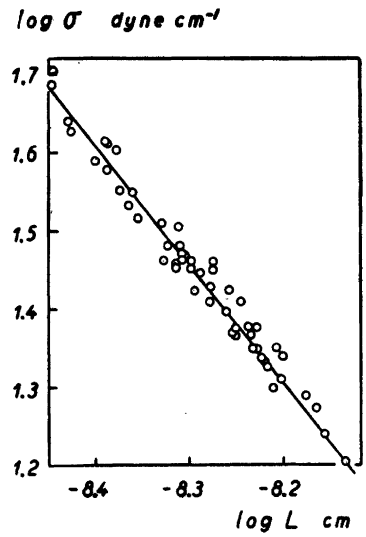

Fig. 1. Surface tensions (σ) plotted as a function of intermolecular free lengths (L) in 53 non-associated liquids at 20° C. L has been calculated from the definition (1).

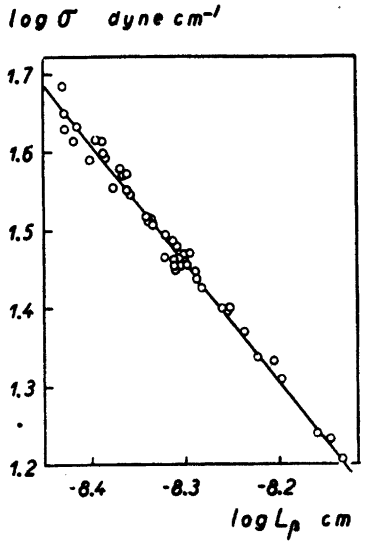

Fig. 2. Surface tensions (σ) plotted as a function of intermolecular free lengths (Lβ) in 46 nonassociated liquids at 20° C. Lβ has been calculated from the adiabatic compressibilities.

from molar volumes, and these have been given for most of the liquids in Table I : 1 (Jacobson [2]). Fig. 1 suggests a relation of the type

$$\sigma = kL^{-\frac{3}{2}} \tag{2}$$

where $\log k = -10.986$ forcgs units at 20° C.

In order to test the validity of equation (2) free lengths have been computed from surface tension values according to this equation and compared with those computed from the definition (1). The average deviation in the values thus computed for L for 53 liquids amounts to 3.6 %. This deviation is due partly to errors in the values used for σ and V_0 and partly to equation (2) being approximate. The errors in V_0 can be surmounted, as the free lengths may be computed from the adiabatic compressibility (β) according to Jacobson [2]. In Fig. 2 the surface tension for 46 liquids has been plotted as a function of the free length (L_β) computed from compressibility values. As may be seen from the figure, there is better agreement between σ and L_β than between σ and L. It is probable, though not proved, that this better agreement is due to the error in V_0, and thus in free length, being eliminated when L_β is employed. The mean divergence between L_β and the free length computed

Table 1. Examples of intermolecular free lengths calculated from surface tension (σ), adiabatic compressibility (β) and molar volumes (V_O and V_T) at 20° C.

Substance	σ dyne/cm	Intermolecular free length, 10^{-8} cm		
		Calc. from σ Eq. (2)	Calc. from β	Calc. from V_O and V_T Eq. (1)
Pentane	16.0	0.75	0.74	0.74
Hexane	17.3	0.71	0.70	0.70
Heptane	20.4	0.64	0.64	0.64
Octane	21.5	0.61	0.61	0.60
Benzene	28.9	0.50	0.50	0.50
Toluene	28.9	0.50	0.50	0.49
Cyclohexane	26.5	0.53	0.55	0.55
Ethylbenzene	28.0	0.50	0.50	0.49
Ethyl ether	17.0	0.72	0.72	0.72
Ethyl acetate	23.8	0.57	0.55	0.59
Butyl acetate	24.8	0.56	0.56	0.60
Carbon tetrachloride	26.5	0.53	0.52	0.51
Chloroform	26.8	0.53	0.50	0.52
Chlorobenzene	32.7	0.46	0.46	0.44
Bromobenzene	37.2	0.43	0.44	0.41
Iodobenzene	39.2	0.41	0.41	0.41
Fluorobenzene	27.8	0.52	0.51	0.51
Aniline	43.4	0.38	0.37	0.37

according to (2) is 2.2 %. This divergence may be due in part to experimental errors, since the determinations of surface tension and compressibility were done on different samples of the liquids, so that different degrees of purity probably had an effect. Some examples of surface tension values employed are given in Table 1, as are also free lengths computed from molar volumes, from surface tension and from adiabatic compressibility.

A close study of equation (2) at different temperatures shows that k and the exponent vary somewhat between 0 and 50° C. The variation in the exponent, however, is small and for practical purposes it may be put equal to $-3/2$ within the temperature range stated. The constant k then assumes the values given in Table 2. *If definitions other than (1) for the free length are used, then*

Table 2. Values for the constant k at different temperatures.

Temp °C	log k
0	−11.032
10	−11.008
20	−10.986
25	−10.976
30	−10.966
40	−10.948
50	−10.932

the constant k and possibly also the exponent must be recalculated if equation (2) is to hold good.

Equation (2) also holds good for associated liquids, if account is taken of the degrees of association. This means that association factors can be calculated from surface tension values by means of the relation demonstrated.

SOLUTIONS AND GIBBS' ADSORPTION EQUATION

Equation (2) should also be valid for solutions and liquid mixtures. This is analogous to the situation for compressibility in which the corresponding equation derived from data for pure liquids also holds good for mixtures, with an accuracy of around one per cent [2]. In actual fact the relation demonstrated makes it possible in certain cases to compute surface concentrations from the surface tensions of the solutions *via* free lengths. For this, however, the dependence of the free length on the concentration must be known. For non-associated solutions this can be obtained from equation I : (6) [2]. For practical purposes it is then advisable to plot a curve for this, which can subsequently be employed for locating concentrations that correspond to free lengths, which are computed by means of (2) from the surface tensions of the solutions.

It is of interest to compare surface concentrations thus computed with the results obtained with Gibbs' adsorption equation. For dilute solutions this may be written

$$\Gamma = -\frac{c}{RT}\frac{\mathrm{d}\sigma}{\mathrm{d}c} \qquad (3)$$

where Γ is the concentration of the solute at the surface, in moles per square centimetre, in excess of that in the bulk, c is the bulk molar concentration of the solute, R the gas constant and T the absolute temperature. Many attempts

have been made to verify Gibbs' equation. Most of these, however, have failed, and the reason for this has been disscused by Michaud [5] and others. However excellent agreement was obtained by McBain and Humphrey [6] with their microtome technique, and their experiments constitute strong evidence for the validity of Gibbs' equation.

In our case, the comparison is rendered more difficult by the fact that Gibbs' equation gives the adsorption in number of moles per square centimetre of liquid surface, whereas the concentrations computed *via* free length are expressed as moles per litre or the like. In order to make the results comparable, the thickness of the surface layer in which the adsorption takes place must be estimated. The adsorption is greatest in the top monomolecular layer, rapidly approaching zero in the layers immediately below. In view of this, we have assumed a mean thickness equivalent to about two monomolecular layers. In this way the adsorption according to Gibbs' equation may be expressed in moles per litre.

Surface concentrations have been computed for a number of solutions of non-associated liquids, both from Gibbs' equation and from surface tensions *via* free lengths. The requisite surface tension values have been taken from smoothed curves plotted from values according to Taubmann [7]. Solutions of

Table 3. Surface concentrations of heptane and cyclohexane in nitrobenzene solutions calculated from surface tensions over the free lengths (c_L) and from Gibb's adsorption equation (c_G).

Bulc conc. mole/lit	Surface tension σ dyne/cm	Surface conc. c_L mole/lit	$-\frac{d\sigma}{dc}$	Surface conc. c_G mole/lit	$\frac{c_G}{c_L}$
Heptane					
0.1	41.7	0.62	19.4	0.77	1.24
0.2	39.7	1.12	16.8	1.35	1.21
0.3	38.0	1.59	14.8	1.83	1.15
0.5	35.2	2.32	12.3	2.61	1.13
0.7	32.9	2.95	10.1	3.61	1.22
1.0	30.3	3.60	7.4	3.54	0.98
Cyclohexane					
0.1	42.9	0.35	9.4	0.43	1.23
0.2	42.0	0.75	9.0	0.82	1.09
0.3	41.1	1.05	8.3	1.16	1.10
0.5	39.5	1.64	7.1	1.72	1.05
0.7	38.1	2.18	6.7	2.31	1.06
1.0	36.3	2.94	5.3	2.82	0.96

heptane, octane, cyclohexane and benzene, each in nitrobenzene, have been studied at bulk concentrations between 0.05 and 1.00 mole per litre. In all cases the thickness of the adsorption layer has been assumed to be 12 Å. To enable computation of surface concentrations from the equations (2) and I(6), the necessary density values for the solutions were determined with a pycnometer. Examples of the values employed and the results obtained for solutions of heptane and cyclohexane can be seen in Table 3. The last column shows the ratios between surface concentrations computed via Gibbs' equation (c_G) and via free lengths (c_L). The ratio has a value close to one, and similar values were obtained for other solutions studied. At low concentrations the computations are made uncertain by experimental errors and at high concentrations equation (3) does not hold good.

The fact that the values for c_G/c_L is close to one for different solutions and different concentrations supports the assumption that equation (2) is also applicable to solutions. Indirectly, the conclusion may also be drawn that the thickness of the adsorption layer was correctly estimated. Solutions of associated substances were not investigated, as the conditions with these become unsurveyable if their degree of association is not known.

DISCUSSION

It should be noted that the free length L in the empirical relation demonstrated refers to conditions inside the bulk of the liquid. Whether this bulk length in pure liquids is equal to the free length in the surface layer cannot be decided. Nevertheless, there must be a simple relation between these two entities, a relation which is included in equation (2). Therefore computation of surface concentrations in the study of solutions presented above was possible.

The compressibility (β) for a liquid is approximately proportional to the square of the free length [2]. If this is combined with the relation (2) given, we get

$$\beta\sigma^{\frac{4}{3}} = constant \qquad (4)$$

which is a well known relation (*cf.* Partington [1], p. 139). It holds good, approximately, for both adiabatic and isothermal compressibility, but the constant assumes different values in the two cases.

SUMMARY

The empirical relation $\sigma = kL^{-\frac{3}{2}}$ is shown between the surface tension σ in normal and associated liquids and the free intermolecular length L in the bulk of the liquids. The constant k varies to some extent with the temperature

and $\log k = -10.986$ holds good at 20° C for cgs units. The relation has been tested on 53 organic liquids and it has been found that L can be computed from the surface tension with an accuracy of a few per cent. The relation is also valid for solutions and in certain cases makes possible a computation of surface concentrations. Comparison has been made with Gibbs' adsorption equation and good agreement was obtained, on the assumption that the thickness of the layer within which adsorption takes place is equivalent to about two monomolecular layers.

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Intermolecular Free Lengths in the Liquid State
III. Viscosity

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Many empirical and theoretical relations have been given for the viscosity of liquid systems (Partington,[1] Doolittle[2]). Much attention has thus been paid to the free volume, which is an essential feature in studying viscosity. However, we have found it better to relate viscosity to another similar property, namely the intermolecular free length. By employing this concept it is possible to formulate a simple equation that describes the viscosity of liquid systems as a function of this free length and of the molecular weight.

ABSOLUTE FREE LENGTHS AND FREE FLUIDITY LENGTHS

In studying compressibility[3] and surface tension[4] it was found suitable to use the concept of free length between the surfaces of the molecules $L = 2(V_T - V_0)/Y_0$. V_T and V_0 are the molar volumes at T°K and O°K. Y_0 is the molar surface of one mole. L is called the absolute free length or often only the free length.

In studying physical properties that depend on molecular motions in a direction tangential to the surfaces of the molecules, *e.g.* viscosity and diffusion, the free length should be defined as

$$L_\Phi = 2(V_T - V_{\Phi_\infty})/Y \qquad (1)$$

where L_Φ is called the free fluidity length as distinguished from the absolute free length. The molecular surface of one mole is $Y = (36\pi N V_{\Phi_\infty}^2)^{\frac{1}{3}}$. V_{Φ_∞} is the molar zero fluidity volume; it is a fundamental property which will therefore be discussed further.

The molar zero fluidity volume can be obtained from Friend's equation, which gives the change of fluidity Φ with density[5]

$$\Phi = \frac{1}{\eta} = k_1(V_T^{\frac{8}{3}} - V_{\Phi_\infty}^{\frac{8}{3}}) \qquad (2)$$

where k_1 is a constant characteristic of each substance. In order to find the molar zero fluidity volume of a substance from this equation it is necessary to know the fluidities and densities at two different temperatures. Then the constant k_1 can be eliminated. To

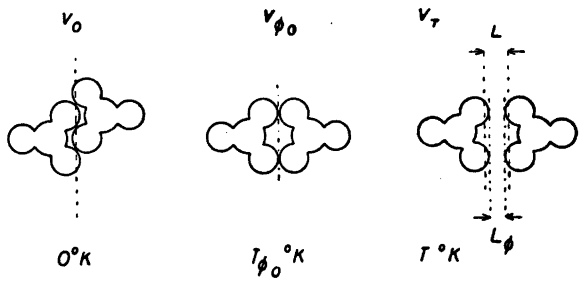

Fig. 1. The difference between the absolute free length L *and the free fluidity length* L_Φ *for polyatomic molecules. At O°K the molecules have their closest packing possible and the molar volume is* V_0. V_{Φ_0} *is a theoretical molar volume at a temperature* T_{Φ_0} *when the molecules have just sufficient space to move tangentially against each others.* V_T *is the molar volume at* T°K.

obtain sufficient accuracy in V_{Φ_0} it is desirable to have fluidities and densities determined on the same sample and at exactly the same temperature.

The molar zero fluidity volume is a characteristic property of each substance. It is the molar volume of the supercooled liquid at the temperature of zero fluidity based on the assumption that the liquid does not solidify but continues to conform to equation (2). It should be observed that the zero fluidity volume is always smaller than the volume at the melting point, which is evident from the fact that it is possible to supercool a liquid.

The difference between the absolute free length and the free fluidity length is illustrated in Fig. 1. Some examples of values are given in Table 1. It should be observed that the definition of the free fluidity length involves an approximation in the same manner as the definition of the absolute free length. The free lengths will therefore be somewhat larger than the shortest possible perpendicular distance between the surfaces of the molecules. Both the absolute free length and the free fluidity length most closely resemble the free path in the liquid. Their error varies with the temperature and thus the definition according to (1) should be used only within limited temperature ranges. For a further discussion of the limitations see Jacobson [3].

FLUIDITIES OF LIQUID SYSTEMS

In a preliminary report [6] the following relation was given between the fluidity Φ and the free fluidity length L_Φ in a liquid

$$\Phi = KL_\Phi^p \tag{3}$$

At 20° C the values for the constants are $p = 1.27$ and $\log K = 13.104$ for cgs units. Equation (3) holds with rather good accuracy for most organic liquids. However, in some cases errors of 30 to 40 per cent for L_Φ are obtained, especially for certain halides. Systematic attempts have therefore been made to find a more universal relation.

Through empirical studies we have obtained the following equation

$$\Phi + c = kL_\Phi M^{-\frac{1}{2}} \tag{4}$$

Table 1. Examples of values for molar volumes at different states of some liquids and their free lengths.

Substance	Molar volume at absolut zero V_0	Molar zero fluidity volume V_{Φ_0}	Molar volume at melting point V_m	Molar volume at 20° C V_T	Fluidity Φ rhe	Absolute free length at 20° C L, 10^{-8}cm	Free fluidity length at 20°C, L_Φ 10^{-8} cm		Per cent deviation
							Calc. from definition Eq. (1)	Calc. from Φ Eq. (4)	
Pentane	86.7	92.1	96.6	115.2	417.5	0.713	0.555	0.537	− 3.3
Hexane	100.8	110.2	114.9	130.7	306.9	0.676	0.437	0.437	0
Cyclohexane	86.2	100.5	106.5	108.1	102.4	0.550	0.172	0.158	− 8.1
Benzene	71.3	79.6	87.5	88.9	153.0	0.501	0.246	0.218	−11.4
Aniline	77.4	89.0	89.4	91.1	21.8	0.370	0.052	0.053	+ 2.7
Pyridine	66.4	74.8	76.4	80.5	103.3	0.422	0.158	0.155	− 1.9
Iodobenzene	94.1	104.4	106.3	111.4	61.3	0.410	0.155	0.161	+ 3.9
Carbon tetrachloride	77.4	87.6	92.5	96.5	103.2	0.516	0.221	0.215	− 2.7
Ethyl acetate	76.4	85.0	87.4	97.8	220.0	0.583	0.325	0.323	− 0.6
Ethylether	77.2	82.9	87.0	103.9	408.5	0.722	0.541	0.533	− 1.5

where M is the molecular weight, $c = 15.8$ and $k = 6.85 \cdot 10^{-11}$ for cgs units. Equation (4) has been tested for 81 organic non-associated liquids of different types. In Fig. 2 the fluidities have been plotted as a function of the term $L_\Phi M^{-\frac{1}{3}}$. The necessary data have been taken from Timmermann [7] and Landolt Börnstein Tabellen. The equation holds with an average accuracy of about 5 per cent for L_Φ. For various reasons it is difficult to determine the degree of accuracy exactly. Thus, in several cases it has been impossible to obtain values for density and viscosity that have been measured on the same sample and at exactly the same temperature, as is desireable in calculating the zero fluidity volume. Furthermore we have abandoned the procedure of taking into account the form factors of the molecules. Since the molecules are not spherical the actual surface of the molecules is somewhat larger than that calculated and used in the definition (1). The actual free length is then smaller than that calculated from (1). This is apparent in the fact that the error is greater for compounds with asymmetric molecules *e.g.* benzene (see Table 1).

We have tried to use the factor $M^{-\frac{1}{2}}$ instead of $M^{-\frac{1}{3}}$ in equation (4). The results, however, are slightly in favour of the equation with $M^{-\frac{1}{3}}$.

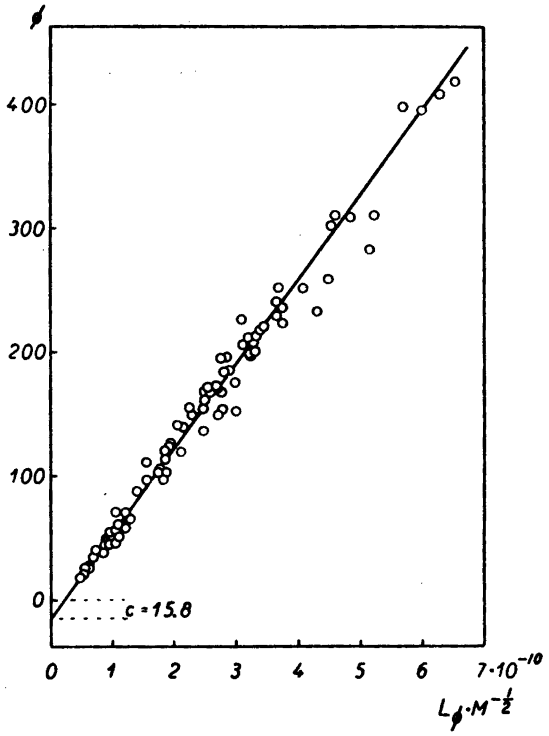

Fig. 2. Fluidities (Φ) for 81 organic compounds plotted as a function of the factor $L_\Phi M^{-\frac{1}{2}}$ *at 20° C.*

The fluidity of associated liquids has also been investigated. However, equation (4) is not suitable for calculating association factors. This depends on the fact that with increasing association both the free length and the molecular weight increase and therefore the fluidity changes only slightly despite variation in the association factor. Also, Friend's equation (2) is not always valid for associated compounds and, it is difficult, therefore, to obtain accurate values for zero fluidity volumes.

We have also tested the given relation (4) on 20 liquid mixtures of the type benzene — chlorobenzene, benzene — cyclohexane, heptane — bromobenzene, heptane — α-bromonaphthalene and 2,2,4-trimethylpentane — ethyl acetate. The viscosities were determined with an Ostwald viscosimeter giving an accuracy of better than 1 per cent. The free lengths for the mixtures are obtained from a definition I(6)[3] corresponding to (1). In this it is assumed that the zero fluidity volumes are additive which, however, should not always be exactly the case. The molecular weight for a mixture is $M_{12} = m_1M_1 + m_2M_2$ where m_1 and m_2 are the molar fractions of the two liquids with the molecular weights M_1 and M_2 respectively.

For all series of liquid mixtures the fluidities have been a linear function of $L_\Phi M^{-\frac{1}{2}}$. The average error in L_Φ has been 2.4 per cent for the 20 mixtures. In Fig. 3 the results are given for mixtures of heptane-α-bromonaphthalene. Good results are also obtained in

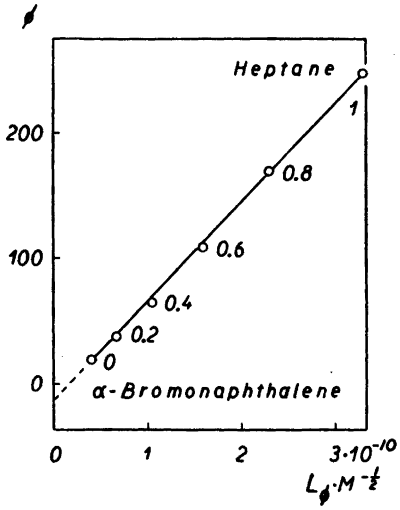

Fig. 3. Fluidities (Φ) at 20° C as a function of the factor $L_\Phi M^{-\frac{1}{2}}$ *for mixtures of heptane and α-bromonaphthalene at various volume fractions of heptane.*

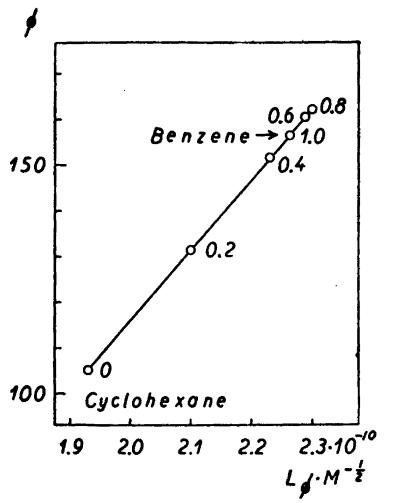

Fig. 4. Fluidities (Φ) at 20° C as a function of the factor $L_\Phi M^{-\frac{1}{2}}$ *for mixtures of benzene and cyclohexane at various volume fractions of benzene.*

cases in which the fluidities for some concentrations of the mixture are higher than the fluidities for the two pure liquids. Such systems are for instance 2,2,4-trimethylpentane-ethyl acetate and benzene-cyclohexane. For the latter system the results are given in Fig. 4.

Equation (4) is also approximately valid for the dependence of fluidity on temperature within moderate limits *e.g.* 0 to 60° C. Errors amounting to 3 per cent arise from the fact that L_Φ has such a large systematic error within the limits mentioned. This can be surmounted by selecting new values for the constants k and c for each compound. In this manner errors that depend on inaccurate values for the zero fluidity volumes are also eliminated.

DISCUSSION

The general applicability of the given relation as demonstrated here, has been achieved through the introduction of the concept of free fluidity length. This concept should also be of value in studying various other physical properties of liquids. Thus, for instance, preliminary investigations have shown that the diffusion constant of an organic compound is a linear function of the free fluidity lengths in the different diffusion media.

One disadvantage of equation (4) is the necessity of the constant c, due to inaccuracies in Friend's equation (2). To overcome this disadvantage a new method must be sought for obtaining the correct zero fluidity point

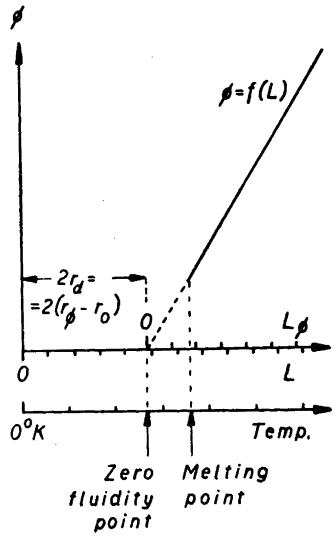

Fig. 5. The the difference between the apparent molecular radius at zero fluidity (r_Φ) and the radius at absolute zero (r_0) can be obtained by extrapolating the absolute free length (L) to zero fluidity

values. We are of the opinion that these could most conveniently be attained by extrapolating the absolute free length L to zero fluidity as indicated in Fig. 5. The intersection of the curve with the absolute free length axis gives the difference r_d between the molecular radius at absolute zero r_0 and the apparent molecular radius at zero fluidity r_Φ. Thus

$$2r_d = 2(r_\Phi - r_0) = L - L_\Phi \tag{5}$$

The apparent molecular radius at zero fluidity r_Φ should be a characteristic property of each compound, and is always larger than the molecular radius at absolute zero for polyatomic molecules. The difference r_d depends on the "roughness" of the molecular surface. The advantage in this procedure of using r_Φ instead of V_{Φ_0} lies in the fact that fluidity is an intermolecular phenomenon and should be related in a simpler way to the free lengths than to any function of the molar volumes. However, before this suggested method could be used to treat the fluidity problem the exact dependence of the free length on temperature must be known; this problem is not as yet satisfactorily solved.

SUMMARY

An empirical relation, $\Phi + c = kL_\Phi M^{-\frac{1}{2}}$ has been shown between the fluidity Φ, the intermolecular free fluidity lenght L_Φ and the molecular weight M of liquids. The relation has been tested on 81 non-associated organic liquids and it holds with an average accuracy of about 5 per cent for L_Φ, using the values

for the constants $c = 15.8$ and $k = 6.85 \cdot 10^{-11}$. The corresponding accuracy for 20 mixtures of organic liquids is 2.4 per cent. The relation is also approximately valid for the dependence of fluidity upon temperature. The concept of free fluidity length should also be of value in studying other physical properties of liquids.

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Heats of Combustion of Organic Chloro Compounds Determined by the "Quartz Wool" Method

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A determination of the heats of combustion in the calorimetric bomb of compounds containing chlorine is difficult, owing to the fact that a mixture of free chlorine and hydrogen chloride is formed during the combustion. Thus the final state is not defined. Berthelot and Matignon [1] proposed to place a solution of arsenious acid in the bomb (that is to say on its bottom), in order to reduce the formed chlorine to hydrogen chloride. This method, however, has proved to be unsatisfactory [2,3]. The surface of the arsenious solution has to be enlarged. For this purpose the inside of the bomb must be covered with some material [4] moistened with the arsenious solution.

Quartz fibers proved to be satisfactory. A string of loosely joined fibers is wound in a spiral on the upper part of the inside of the bomb. The weight of the spiral is 4 g and it retains about 4 ml of the arsenious solution, the total quantity of which is 20 ml. The total surface, covered by the string of quartz threads and its interspaces, amounts to 50—60 % of the interior surface of the bomb. The spiral can easily be put in its place. The unpractised investigator might perhaps at first moisten the fibers with water, which is afterwards evaporated in a heating box.**

The effect of the quartz spiral moistened with arsenious solution is twofold. On one hand the process of reduction is promoted. At the end of the main period — in this paper always 5 minutes after the ignition — no traces of

* Further particulars about the collaboration on p. 67 and p. 78. D. Folkesson was my calculating assistant (p. 82).

** The name "quartz wool method" is misleading, for it is quite impossible to wind *wool* of quartz to a durable spiral.

chlorine can be detected in the gases from the combustion. On the other hand the total amount of free chlorine formed is also diminished, which is certainly due to the fact, that the moistened spiral delivers water vapor to the combustion zone, thus affecting the equilibrium [3]:

$$2\ H_2O + 2\ Cl_2 \rightleftharpoons 4\ HCl + O_2$$

By the aid of the quartz spiral method the heats of combustion of more than 130 organic chloro compounds were determined in the Department of Organic Chemistry of the University of Lund *. Most of these values were published in 3 dissertations for the degree of doctor of philosophy, namely by E. Schjånberg [4] 1934, E. Eftring [5] 1938 and K. J. Karlsson [6] 1941. Some determinations were made by others, especially by Smith and collaborators and by G. Sjöström [7] 1936.

The principal features of the quartz wool method have been described by Schjånberg [12]. Here I have reason to remind of the following details. The heat equivalent was determined in the presence of the spiral, moistened with arsenious solution (0.35 *N*, 20.0 ml). Mean error of the mean values of the heat equivalent ± 0.01 %. The bomb was lined with platinum; its volume was 280 ml. Between crucible and shield about 3 cm. Oxygen pressure 25 atm. Total heat evolved 5 000 cal. Average total rise of temperature 1.7°. Room at constant temperature, generally 20.0°, in a few cases 18.3° or 18.5°.

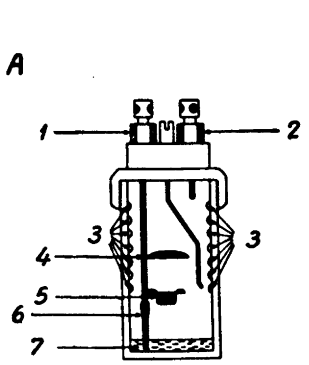
Schjånberg and Karlsson burned liquid compounds of relatively low volatility in open crucible with a cellophane cover, a method recommended by W. A. Roth, while Eftring — in a few cases also Schjånberg and Karlsson — for this purpose used glass ampoules, whose necks were sealed with a drop of paraffin oil.

The authors in question applied the following corrections: for paraffin oil, used as auxiliary substance, for cellophane, for the oxidation of arsenious acid (10—14 cal), for dissolved platinum and gold (1—3 cal).

The accuracy arrived at in the combustion of chloro compounds is dependent on their content of chlorine, since on one hand the necessary percentage quantity of paraffin oil rises with the chlorine content, on the other hand an increased chlorine content in a compound diminishes its heat of combustion. In the combustion of carbontetrachloride *e.g.* this compound contributes about 4 % to the total evolution of heat, in the combustion of amylchloride more than 50 %. Of course the carbontetrachloride represents an extreme case. From some figures of Schjånberg I have calculated that the mean error of a combustion rises from ± 0.01 % at 20 % of chlorine to 0.1 % at 65 %. As for the accuracy of the determination of the heat equivalent see above.

The heats of combustion of chloro compounds can also be determined by another method. The final communication on the quartz wool method was published in 1931. Two years later Popoff and Shirokich [9] described such combustions in a swinging bomb, driven by a wire; the construction of the driving device was not given. Later on we

* (19 iodo compounds were also burned in Lund; Karlsson in his dissertation.)



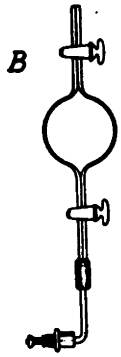


Fig. 1. A. The bomb adapted for the taking of a sample. 1) outlet valve for sample, 2) inlet valve for oxygen, 3) quartz wool coil, 4) shield, 5) platinum crucible, 6) tube extension, 7) arsenious oxide solution. B. the bulb with two stopcocks.

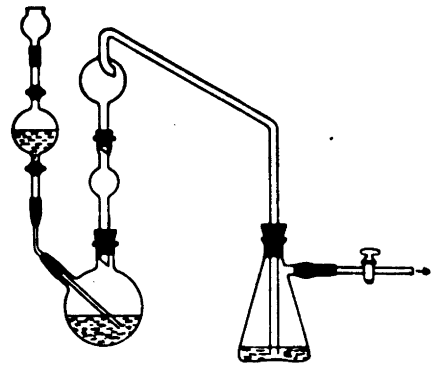


Fig. 2. The apparatus for determination of carbon dioxide.

started experiments with swinging as well as with rotating bombs [10,11] and attained by that means about the same reproducibility and the same values of combustion as with the stationary bomb.

I. FURTHER CORRECTIONS ON THE HEATS OF COMBUSTION *

As reported in a previous paper [8], the values of the above mentioned authors are not entirely correct, inasmuch as Washburn corrections have not been applied, among which the heat of solution of dissolved carbon dioxide in this case is the most important. Furthermore a correction must be applied for the heat, accompanying the dilution of the different solutions of hydrochloric acid, formed during the combustion, to a uniform final concentration.

These two corrections can not be *calculated* directly from the values communicated by the authors. Special experimental investigations were necessary for this purpose. After this the principal part of the remaining Washburn corrections may be calculated from his formulas by anyone, who has available the dissertations referred ta above **. Concerning the magnitude of these Washburn corrections see p. 80.

The determinations of the two first mentioned corrections are described below.

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* In collaboration with Sven Krook, and Henrik Westermark.

** Data necessary for these calculations are also given in the Tables 6—9 of this paper.

1. Determination of dissolved carbon dioxide. This determination must be made in the bomb solutions at the termination of the main period (5 min.) without diminishing the oxygen pressure, *i.e.* without opening the bomb previously. An approximative knowledge of the concentration of carbon dioxide after the final period (10 min.) is likewise necessary.

For this purpose we made combustions with varying quantities of carbon (paraffin oil) and with varying concentrations of hydrochloric acid (0; 0.2; 0.4 *N*) in the 0.35 *N* arsenious acid. During the combustion the bomb was immersed in a thermostat, kept at 19.0 °. As described below a sample was taken of the solution on the bottom, where about 80 % of the whole bomb liquid is to be found, and analysed. Samples were taken after 5 as well as after 10 min.

Fig. 1 A represents a schematic drawing of the bomb, adapted for our analyses. As the figure shows, the inlet tube is extended to the bottom of the bomb for this purpose. A sample of the solution on the bottom is forced by the interior pressure through a silver tube, serving as connecting joint (see Fig. 1 B), into an evacuated bulb, fitted with two gastight stopcocks. The bulb is then transferred to and connected with the distillation apparatus, shown in Fig. 2. The determination of the carbon dioxide dissolved in the sample was carried out according to Vesterberg's vacuum method [13]. The correctness of our analyses were checked by special tests. The total quantity of carbon dioxide, dissolved in other parts of the solution, was calculated by an indirect method.

After a combustion in the presence of the moistened quartz string the bomb is brought into horizontal position and slowly turned around (two rotations in one minute was deemed adequate) while swinging the bomb slightly. Hereby the differences in concentrations are equalized, and an analysis of a sample from the layer on the bottom, after the bomb is brought into vertical position, will give an average concentration of the total amount of carbon dioxide *now* dissolved.

The motion of the solution in contact with the gaseous phase brings about an increase of the concentration in the water layer, which is not yet saturated with carbon dioxide. This increase was approximately determined in the following way. We analysed the bottom layer after two combustions with *dry* quartz string (all the solution on the bottom), the first time without turning the bomb, the second turning it. — These two combustions were identical in other respects. — The difference between these two determinations will give the "rotation-increase" per g substance in the content of carbon dioxide in the solution. The subtraction of this quantity from the above mentioned total amount of carbon dioxide per g, and the multiplication by the weight

of the bomb solution give the *total quantity of dissolved carbon dioxide in an ordinary combustion — in a first approximation.* For a more accurate calculation of this quantity we had also to apply two further small corrections, the determinations of which, however, may be omitted here.

Concerning our determinations of dissolved carbon dioxide I will also mention that every rotation experiment was made twice, and that it was possible to attain an accuracy of 1—3 % in the average value of parallel determinations. It will be seen from what precedes that every value of a total concentration of carbon dioxide is the result of 8 combustions, all of them with the same quantity of paraffin oil: dry quartz string, bomb turned and not turned respectively, moistened quartz with the same two alternatives and then parallel experiments. One of these last ones ("bomb not turned") served as a control.

All the concentrations *after 10 minutes* were determined in this way (Table 1).

Table 1.

Milliatoms of carbon burned	Dissolved carbon dioxide in mg. 20 ml of arsenious solutions with hydrochloric acid of the following concentrations		
	I 0.0 *N*	II 0.2 *N*	III 0.4 *N*
24.8	—	34.5	33.9
31.1	41.1	39.6	39.0
41.4	52.3	50.6	48.3
50.0	58.7		

The dissolved quantity diminishes with increasing acidity of the solutions. The decrease, however, is very small and of no importance: 1 mg carbon dioxide corresponds to only 0.1 cal. In Fig. 3 the curves for the relation between the values of the table and milliatoms of carbon burned are given. As a result of a miscalculation, discovered too late, the point for 50 milliatoms was determined only for solution I. The approximate positions for solutions II and III were found by extrapolation, as it appears from the diagram. Nevertheless this manner of proceeding may be considered satisfactory, since these "10-minutes" values are not used for the corrections of the heats of combustion, but only for the estimation of the heats of solution during the final period. It is desirable to know these values approximately because these heats may represent a systematic, though small error.

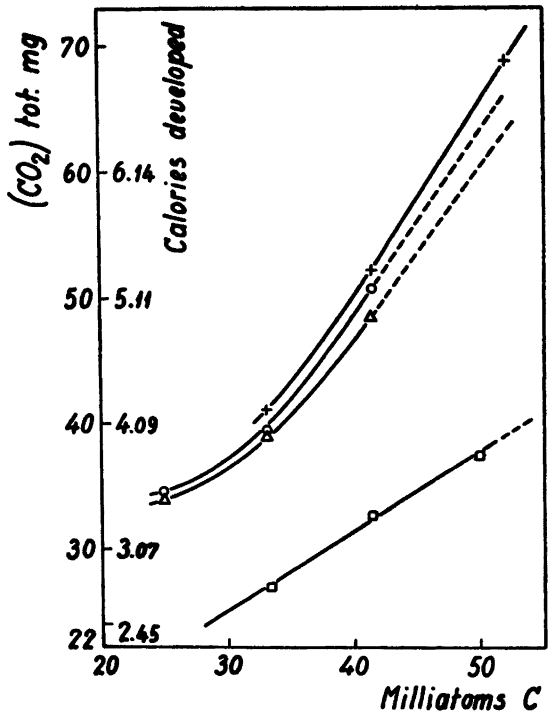

Fig. 3. Correction for dissolved carbon dioxide. CO_2 dissolved after 10 min. in solution I × ×, II ○—○, III △—△. CO_2 dissolved after 5 min., mean values □—□.

The procedure just described is circumstantial and suffers besides from an uncertainty. Since the turning of the bomb must be started one minute before the sample is to be taken, the state in the interior is not the same at this last moment as at the beginning of the rotation. At the end of the final period this uncertainty in the definition of the time must be of less importance than after the end of the main period, when the convective movements of the gases have just ceased. Hence we chose another way of proceeding for the end of the main period. For its description we need the following notations:

$[CO_2]_{tot}$ = total quantity (mg) of carbon dioxide dissolved

$[CO_2]_b$ = concentration (mg/g) of the carbon dioxide in the bottom layer (moistened spiral, without rotation)

$[CO_2]_{sp}$ = average concentration (mg/g) of carbon dioxide in the spiral and other places in the bomb than the bottom

g_{tot} = weight of the total solution, including water formed by the combustion

g_b = weight of the solution on the bottom.

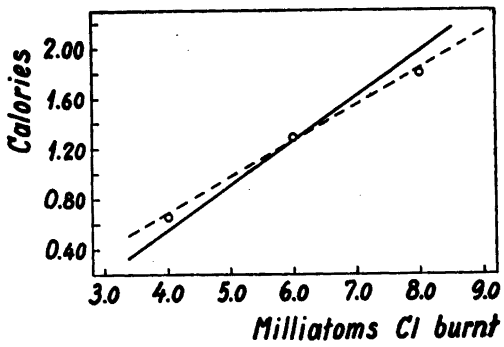

Fig. 4. Correction for HCl-dilution. ($Corr_{HCl}$). – – – 5 minutes, ——— 10 minutes.

Then we have:

$$[CO_2]_{tot} = g_b \cdot [CO_2]_b + (g_{tot} - g_b) \cdot [CO_2]_{sp} \quad (1)$$

Knowing $[CO_2]_{tot}$ one can calculate $[CO_2]_{sp}$ from equation (1). We determined in the manner above described $[CO_2]_{tot}$ at 10 min and found for $[CO_2]_{sp}$ an average value of 2.2, nearly independent of the amount of carbon dioxide (we used solution II). At 6 min the value 2.0 ± 0.2 was found. The uncertainty in these determinations is of less importance, however, owing to the fact that the quantity g_b is about 4 times greater than $(g_{tot} - g_b)$. Hence we considered the value 2.2 valid also for 5-minute analyses, determined $[CO_2]_b$ (moistened spiral, without rotation) at this moment and then used equation (1). The results are found in Table 2.

Contrary to Table 1 this table shows no decided diminishing of the amount of dissolved carbon dioxide with increasing acidity of the solutions. Hence it is justified to take mean values. The connexion between these and the quantities of burned carbon is found in Fig. 3 (the lower curve). Evidently

Table 2.

Milliatoms of carbon burned	Dissolved carbon dioxide in mg. 20 ml of arsenious solutions with hydrochloric acid of the following concentrations			
	I 0.0 *N*	II 0.2 *N*	III 0.4 *N*	Mean values
33.2	26.5	28.8	25.6	27.0
41.4	32.3	33.3	32.0	32.5
49.7	38.6	37.6	35.9	37.4

the best representation is a straight line. The total amount of dissolved carbon dioxide (in mg) can be calculated by aid of the equation:

$$[CO_2]_{tot} = 0{,}632 \cdot n_C + 0{,}620 \qquad (2)$$

n_C here denotes milliatoms of carbon burned. The dissolution of 1 millimole of carbon dioxide gives a heat of 4.5 cal. In Fig. 3 the corresponding heats are also indicated *.

2. *Determination of the different concentrations of hydrogen chloride.* The hydrogen chloride formed by the combustion is mainly deposited in the quartz spiral and its interspaces, partly in other places in the bomb: in the bottom solution, on walls and cover. On the shield only very small quantities can be found. Eftring [5] examined for the first time the concentrations in these places, and calculated that a dilution of them to a common concentration would cause an appreciable production of heat: up to 3 cal for one combustion. Eftrings examination was nevertheless a little superficial and limited to two combustions.

We burned chloro compounds in quantities containing 4, 6, and 8 milliatoms of chlorine, using monochloroacetic acid, *p*-dichlorobenzene and chloral hydrate, and used paraffin oil as the auxiliary substance. The bomb was opened after 5, and 10 min. respectively as before. Even in the first case we found only small traces of hydrogen chloride in the gasphase, although the smell of this compound was easily detected.

After the opening of the bomb the quartz string was removed and washed with hot water on a suction filter. In the filtrate the chlorine was determined. The condensed liquids on cover, the lower part of the wall and under the quartz spiral (after its removing) were absorbed separately by filter paper, immediately weighed on a torsion balance and analysed. The solution on the bottom was also weighed and analysed, but we calculated the amount of water in the quartz spiral as a difference, taking into consideration also the water formed by the combustion. The concentrations of the acid under the spiral and in it are almost the same. Accordingly they are dealt with together.

The hydrogen chloride, formed by the combustion of the above mentioned quantities of chloro compounds, forms in a 20.0 ml solution as final state 0.2 *N*; 0.3 *N* and 0.4 *N* hydrochloric acid, having the molar ratios $HCl : H_2O = 1 : 282$; 1 : 188 and 1 : 141. The corrections in the Tables 3 and 4 are calculated on the assumption that the different solutions of hydrogen chloride found after the combustion are diluted to these molar proportions.

* As in all papers from Lund dealing with heats of combustion of chloro compounds, developed heat is considered positive; also here.

Table 3. Correction for HCl-dilutions.

Figures for *p*-dichlorobenzene; parallel experiments nos. 1 and 2. The figures refer to the end of the main period. The heats of dilution were taken from "selected values" of Bureau of Standards and are calculated for dilution to the molar proportion 1 : 188, see above.

Exp. no. 1: $C_6H_4Cl_2$, 441.2 mg, paraffin oil 305.4 mg
» » 2: » 442.0 » » » 431.0 »

No.	Cover		Wall		Quartz-spiral		Bottom layer		HCl total [2]	
	Sol. mg	HCl mmoles	Sol. mg	HCl mmoles	Sol. g [1]	HCl mmoles	Sol. g	HCl mmoles	mmoles found	mmoles calc.
1	135	0.50	102	0.25		4.79	15.36	0.39	5.94	6.00
2	116	0.41	211	0.33		4.73	15.71	0.40	5.89	6.00
	Water mg	Corr cal	Water mg	Corr cal	Water g [1]	Corr cal	Water g	Corr cal	Total cal	mean
1	117	0.43	93	0.14	5.2	0.81	15.06	−0.06	1.32	1.28
2	101	0.34	199	0.11	4.9	0.85	15.40	−0.06	1.24	

[1] Calculated (see above)

[2] The correction for the solution in crucible and on shield is very small and for that reason neglected.

In Table 4 there is given the thermal effect (in cal) accompanying the change in concentrations of the hydrochloric acid, as it is formed in the bomb, to a uniform concentration in 20.0 ml solution.

Table 4.

milliatoms Cl in comp.		4	6	8
corr. after	5 min	0.67	1.28	1.81
	10 »	0.58	1.26	1.98
for dilution to		0.2—	0.3—	0.4—N

All the figures are mean values of two experiments. The deviation from the mean in the 5 min. experiments is ± 0.03—0.07 and in the 10 min. series is somewhat more. The differences between calculated milliatoms chlorine 4, 6, and 8 in the compounds and amount of hydrochloric acid found (see

Table 3) in the bomb solutions are very small: the largest difference observed is the one in Table 3.

Table 4 shows that the total corrections after the lapse of 5 and 10 min. are almost equal, which evidently is a result of the rate at which gaseous HCl goes into solution. It also follows from the table that the total correction is nearly proportional to the chlorine content of the compound burned. The very good agreement between the parallel experiments is perhaps somewhat astonishing in consideration of the fact that the concentrations of the solutions of hydrochloric acid in a definite part of the bomb (cover, wall, quartz spiral etc.) are not always easy to reproduce, *i.e.* often differ more in parallel experiments than the sum of all the corrections.

The relation between correction (corr_{HCl}) and milliatoms of chlorine in the compound (n_{Cl}) is given, as Fig. 4 shows, by straight lines represented by the following equations:

$$\text{at } 5 \text{ min: } \text{corr}_{\text{HCl}} = 0.29\ n_{\text{Cl}} - 0.46 \text{ (cal)} \tag{3}$$

$$\text{at } 10 \text{ min: } \text{corr}_{\text{HCl}} = 0.36\ n_{\text{Cl}} - 0.88 \text{ (cal)} \tag{4}$$

3. *Formulas for the calculation of the corrections for the dissolved carbon dioxide.* The calculation is of an elementary sort, but I think that the somewhat complicated formulas may be convenient for readers, who wish to check the following corrections. It should be observed that heat evolved is always considered positive, (see note on page 72).

The following notations are used. The unit for heat is calory.

E and E_{corr}	heat equivalent of the calorimeter, uncorr. and corr. respectively.
Q, Q_{b}, Q_{p}, Q_{cell}	heat of combustion of substance, benzoic acid, paraffin oil and cellophane.
Q_{corr}, $Q_{\text{b corr}}$, $Q_{\text{p corr}}$, $Q_{\text{cell corr}}$	the same heats corr.
g, g_{b}, g_{p}, g_{cell}	weights of substance, benzoic acid etc.
Δt, Δt_{b}, Δt_{p}, Δt_{cell}	rise of temperature in combustion of substance, benzoic acid etc.
n, n_{b}, n_{p}, n_{cell}	millimoles of dissolved carbon dioxide in the combustion of substance etc.
w_{sol}	heat of solution of carbon dioxide (per millimole).
q	sum of other small amounts of heat evolved during a combustion.
corr, corr_E, corr_{p}, $\text{corr}_{\text{cell}}$	these quantities are defined in equations 7, 10, 11, and 13.

a. Correction for the heat equivalent.

$$E\Delta t_{\text{b}} = g_{\text{b}}Q_{\text{b}} + q \tag{5}$$

$$E_{\text{corr}}\Delta t_{\text{b}} = g_{\text{b}}Q_{\text{b}} + n_{\text{b}}w_{\text{sol}} + q \tag{6}$$

$$E_{\text{corr}} - E = \frac{n_{\text{b}}w_{\text{sol}}}{t\Delta_{\text{b}}} = \text{corr}_E \tag{7}$$

b. Correction for the heat of combustion of paraffin oil.

$$E\Delta t_p = g_p Q_p + q \tag{8}$$

$$E_{corr}\Delta t_p = g_p Q_{p\,corr} + n_p w_{sol} + q \tag{9}$$

Then with the aid of (7) we get

$$Q_{p\,corr} - Q_p = \frac{corr_E \Delta t_p - n_p w_{sol}}{g_p} = corr_p \tag{10}$$

c. In the same way we get for cellophane:

$$corr_{cell} = \frac{corr_E \Delta t_{cell} - n_{cell} w_{sol}}{g_{cell}} \tag{11}$$

d. Correction for the chloro compound, burned in the presence of paraffin oil and cellophane.

$$E_{corr}\Delta t = gQ_{corr} + g_p Q_{p\,corr} + g_c Q_{c\,corr} + q + nw_{so} \tag{12}$$

Finally with the aid of equations (7), (10) and (11) we obtain:

$$Q_{corr} - Q = \frac{corr_E \Delta t - g_p corr_p - g_{cell} corr_{cell} - nw_{sol}}{g} = corr \tag{13}$$

This last correction thus represents the total "carbon dioxide correction" for the chloro compound. Owing to the fact that the corrections for benzoic acid, paraffin oil and cellophane, *i.e.* $corr_E$, $corr_p$, and $corr_{cell}$, are practically constant, the use of this somewhat complicated formula is considerably simplified. The correction term nw_{sol} may be taken from Fig. 3 or be calculated with the help of equation (2).

To the correction of equation (13) we then have to add the correction for the dilution of the formed hydrochloric acid ($corr_{HCl}$) according to Fig. 4 or equation (3).

II. COMMENTS ON THE "QUARTZ WOOL" METHOD.

1. The main feature of the "quartz wool" method is the *spiral* made of a string of fine quartzthreads. One may imagine that in laying the windings on the wall of the bomb there may arise small discrepancies between different experimenters, possibly giving *accidental* deviations in the results. Still such possible deviations are certainly less than those caused by other factors. This is shown by some combustions of paraffin oil by Schjånberg [4] and Bjellerup.

Schjånberg burned paraffin oil a) with b) without quartz spiral and found in series a) 11001 ± 3, in series b) 11001 ± 4 cal/g. Bjellerup burned paraffin oil using in series a) the rotating bomb [11], in series b) the quartz spiral method. Results in a) 10975.1 ± 0.7, in b) 10975.7 ± 1.0 cal/g. The errors in temperature readings or other factors in Bjellerups combustions [14] are very much diminished compared with those of Schjånberg but in spite of this the accuracy

found by Bjellerup is about the same in series b) as in series a), which must show that neither *accidental* nor *systematic* errors are of importance even with more exact methods than those used by the first workers in this field.

2. The corrections made here for the heat of dilution of hydrochloric acid have been calculated on the assumption that the values found in the literature are valid also in the presence of some amount af arsenious acid. We made a few investigations concerning the correctness of this assumption. Mixing first water and pure hydrochloric acid solution and second water and hydrochloric acid containing arsenious oxide, we determined the rise of temperature in both cases. The final concentrations were the same as in the combustion experiments. We used a glass calorimeter in a laboratory with constant temperature ($\pm$ 0.1°).

For quantities 20 times greater than the ones used in the combustions a small difference between the two types of experiments were found. This difference (0.5 cal), however, has no noticeable effect on the heat of dilution in the case in question here.

3. The heat evolved during the final period by the dissolution of carbon dioxide can be calculated with the aid of the curves in Fig. 3. We find as average values for 30 milliatoms burned carbon about 1.5 and for 50 milliatoms nearly 3 cal evolved. When determining the heat equivalent we use about 50 milliatoms carbon, when determining the heats of combustion of the chloro compounds not quite so many milliatoms. The average of the amounts found in Karlssons paper (aromatic compounds) is 42, in Schjånbergs and in Eftrings 35 milliatoms. Yet these smaller quantities of carbon cancel most of the corresponding error in the calibration experiments. The remaining part of this error we need not take into consideration.

4. It is well known that a diluted solution of arsenious acid slowly diminishes its concentration in contact with the air. The most important presumption for the use of arsenious acid for the reduction of chlorine according to Berthelot-Matignon is of course that the bomb oxygen itself does not oxidize the arsenious acid during the combustion experiment. If such an oxidation takes place, in any case it must be very slight and the error caused may be neglected *e.g.* on account of its compensation by the same error, when the heat equivalent is determined.

Karlsson observed [6, p. 3] that the concentration of the solution of the arsenious acid in the bomb does not remain quite unchanged even by combustions of compounds, containing no chlorine. The magnitude of the change observed was "very much varying" and impossible to reproduce.

On my request Sunner a few years ago carried out some experiments concerning this question. He used the above mentioned quantity of arsenious

acid (20 ml 0.35 *N* solution) and oxygen of 25 and 35 atm. pressure and found that in the first case the arsenious acid lost about 0.1 % of its normality in half an hour (the usual time in our experiments for combustion with a main period of 5 min.). This gives a heat effect of 0.2 cal.*

Sunner, however, did not use a quartz spiral in his experiments. On that account we now moistened the quartz string with arsenious solution in the ordinary way. Then we immediately made a combustion (using paraffin oil) with oxygen of 25 atm. We found no difference between the blank and the combustion experiment as regards the consumption of permanganate, that is no change in the concentration of the arsenious solution present in the bomb during the combustion. This was the case, whether the solution contained hydrochloric acid or not. We conclude from these experiments that even in the presence of the moistened quartz string, the errors arising from oxidation by the bomb oxygen are negligible. Nor did Bjellerup, in his experiments with bromo compounds, find any oxidation of this kind (private communication).

Karlsson's results may be explained by insufficient washing of the quartz spiral after the combustion. Some arsenious acid may thus have been left behind in it.

5. In the present investigation we have for the first time taken an interest in the quantity of arsenious solution, retained in the quartz string. The authors of previous papers in this field — even Smith — had an exaggerated idea of this quantity; see *e.g.* Eftring, who writes that about half of the volume of the solution (20 ml) runs down to the bottom. As has previously been stated, in reality about 4/5 of the solution runs down and only 4 g remain in the string.

But even this small quantity is obviously sufficient to accelerate the reduction process, which may be considered to take place mainly in the quartz string. It is interesting to compare the number of milliequivalents contained in these 4 g of the solution (about 1.4) with the number totally reduced during a combustion.

Burning in a crucible brings about a greater reduction than burning in ampoules, probably owing to a faster combustion and a higher temperature. Thus Eftring's values, found by the use of ampoules, are less than 1.4 meq. with the exception of the one for tetrachloro ethylene (1.6). The highest figure for the rest is 1.25 for CCl_4. For the solid compound C_2Cl_6 (crucible) the value is 1.75. Schjånbergs crucible values for compounds with similar constitution

* On the same occasion Sunner investigated the oxidation of hydrazine chloride and found it about 3 times as oxidable as arsenious acid.

are about twice as great as Eftring's. But only for the chloroacetic acids and some simple derivatives of them, generally containing more than 50 % of chlorine, the reduced quantity is equal to or exceeds 1.5 meq. Karlsson's crucible values are low, as may be expected with aromatic compounds. The highest one is 1.3 for the dichlorobenzenes (48 % chlorine).

From the figures quoted we may conclude that theoretically 4 g of 0.35 *N* solution would be sufficient for the reduction in most cases.

No experiments have been made by myself or the authors referred to in order to investigate the minimum of volume, necessary for a quick reduction *in the presence of a moistened quartz string*. Only a few experiments, by no means systematic, have been made *without a quartz string*.

It is a matter of course that every considerable reduction of the volume of the arsenious solution *may* be advantageous in many respects for the accuracy of the combustion, but for answering this question we must first know the corrections for the heat of solution of the carbon dioxide and the heat of dilution of the hydrogen chloride under the new conditions. For their determination special experiments are required.

III. ON THE MAGNITUDE AND RELIABILITY OF THE CORRECTIONS DETERMINED *.

The magnitude of the sum of the corrections for dissolved carbon dioxide and hydrogen chloride will, above all, depend on the content of chlorine in the burned compound. A few examples will be given to show this: the sum of corrections is given in cal/g of substance burned:

Carbon tetrachloride 6.0; chloroform 5.8; methylene chloride 5.7; 1,2,3-trichloropropane 5.0; *o*-dichlorobenzene 3.3; chlorobenzene 1.9; *n*-amyl chloride 2.7; *p*-chlorobenzoic acid 1.1.

A comparison between values, obtained by the quartz spiral and the moving bomb methods, must be of importance for the estimation of the reliability of the quartz spiral method, as it is probable that the moving bomb gives heats of combustion free from systematic errors. For such a comparison we chose some compounds in the dissertations above referred to[4-6] and calculated the corrections for "carbon dioxide" and "hydrogen chloride" and also the other corrections applied in the tables given in the following chapter (p. 79). Then Bjellerup prepared and purified respectively the compounds and determined their heats of combustion in a moving bomb, using closed ampoules for liquids.

* In collaboration with Lars Bjellerup.

It may be observed, however, that a comparison between the methods is unreliable regarding the volatile compounds, that are burned with cellophane cover (number 1, 2 and 6 a in Table 5), because their heats of combustion often tend to be too low. This uncertainty in the cellophane method was not known to us beforehand, otherwise we should have chosen other compounds than these ones. The compounds 3 and 4 (Eftring) were burned in a glass ampoule with paraffin prop, which is a more reliable method. Number 6 b Bjellerup burned in a closed ampoule. Other details about these and other recently burned chloro compounds will be published subsequently.

Table 5.

No	Substance	Quartz spiral	Moving bomb
1	*n*-Propyl chloroacetate	4820.8 ± 2 [1]	4828.7 ± 2.6
2	*n*-Butyl chloroacetate	5406.7 ± 0.5 [1]	5419.5 ± 2.2
3	*n*-Amyl chloride	7499.3 ± 2	7499 ± 5
4	1,2,3-Trichloro propane	2800.5 ± 3	2808.4 ± 1.3
5	*p*-Chlorobenzoic acid	4681.2 ± 2	4681.2 ± 2.3
6	*o*-Dichloro benzene a)	4808.3 ± 2 [1]	4817.7 ± 2.9
	b)	4814.5 ± 3.5 [2]	

[1] with cellophane.

[2] in ampoule (Bjellerup).

The table gives evidence of the agreement of both methods for the same substance up to 50 % content of chlorine with the exception of Schjånberg's cellophane values (1 and 2). Concerning compounds with more chlorine further investigations are necessary. It should be mentioned, however, that a small correction — about 0.5 cal — is to be added to numbers 1, 2, 5 and 6 a (see below).

IV. THE CORRECTED HEATS OF COMBUSTION OF SOME ORGANIC CHLORO COMPOUNDS

The original values by Schjånberg [4], Eftring [5], Karlsson [6] and Sjöström [7]* are corrected here in the following way.

1. Corrections to weight in vacuum. This correction was not possible to carry out with Sjöström's compounds, whose densities are not known.

2. Corrections for dissolved carbon dioxide and dilution of hydrogen chloride, according to Figs. 3 and 4; both after 5 min. The sum of the corrections is given in the tables.

* For the chlorhydrins mentioned by Smith [3] new determinations are beeing made and will be published later on.

The *heat equivalents* were also corrected for *dissolved carbon dioxide.* I consider it unnecessary to reproduce these corrections here. It appears from the original papers that no corrections on the heat equivalents to weight in vacuum need to be applied.

Concerning the reliability of the values of the standards used by the authors for determination of the heat equivalents it may be sufficient to refer to Table 5 above: Karlsson and Bjellerup have burned the *same sample of p-chlorobenzoic acid* and found the same heat of combustion for this compound. For the determination of the heat equivalent, however, Karlsson used a benzoic acid sample from Schering-Kahlbaum (standardized by Prof. W. A. Roth), while Bjellerup had a benzoic acid from Bureau of Standards. From this we may conclude that the heats of combustion of both samples are identical within the limits of errors. The standard benzoic acid samples of the other authors had likewise the signature of Roth.

3. The four original authors have used wrong values when correcting for the heat accompanying the solution of platinum and gold *. For the latter metal this error is insignificant, but for the platinum Eftring's and Sjöström's figures are altered (Tables 6 and 9). From Karlsson and Schjånberg the original metal corrections were not available any more. For Schjånberg's part this error may be estimated to be 0.5—1.0 cal/g, for Karlsson's, 0.3—0.5 cal/g. These quantities are to be added to the heats of combustion, *which has not been done in the Tables 7 and 8.*

4. The correction for the oxidation of arsenious acid by the chlorine has been calculated using the value 19.6 cal per meq. by the authors in question. In the tables recalculation is made with the new value 18.6.

5. Finally we have considered it more convenient to refer all the heats of dilution of the hydrogen chloride to a common value: the molar proportion $HCl : H_2O = 1 : 600$ (using "selected values" of Bureau of Standards).

By a mistake the corrections for carbondioxide and hydrogen chloride at 10 min. were calculated at first for *all values* in the tables of the authors. — Sjöströms measurements form an exception. — For that reason it was not necessary to compute the corrections at 5 min. for all values. Guided by the corrections at 10 min. we could get the mean correction at 5 min. by the aid of two or three values in a series of combustions.

In the tables below there are also given — for an average value of the corrections at 5 min. — the quantities of compound, paraffin oil and cellophane (with 3 figures).

The quantities just mentioned may be used for the calculation of the Washburn corrections. For this purpose the approximate initial and final temperatures are given in connexion with the tables. Concerning oxygen

* This error was originally observed by Sunner.

Table 6. Eftring's heats of combustion. Compounds burned in glass ampoules, see p. 77. Initial temperature in nos. 47—59 18.7°, in the rest 17.2°. Rise of temperature about 1.7°.

No.	Compound	g sub-stance	g par. oil	Heat of comb. uncorr. cal/g	Vac corr	HCl + CO_2 corr cal/g	Pt corr cal/g	As_2O_3 corr cal/g	1 : 600 corr cal/g	Heat of comb. corr. cal/g
47	CCl_4	0.321	0.442	552.1±3	0.3	6.0	1.6	4.5	3.6	567.5
48	$CHCl_3$	0.320	0.417	934.3±2	0.6	5.8	1.2	3.9	3.1	947.7
49	CH_2Cl_2	0.364	0.384	1689.4±1	1.3	5.7	1.3	3.2	3.1	1701.4
51	C_2Cl_6	0.362	0.426	718.7±2	0.3	5.9	1.3	5.1	3.7	734.4
52	C_2HCl_5	0.310	0.427	1004.7±3	0.6	5.7	1.1	3.9	2.9	1017.7
53	$C_2H_2Cl_4$ (*symm.*)	0.319	0.422	1370.1±2	0.8	5.6	1.3	3.6	3.1	1382.9
54	$C_2H_4Cl_2$—1,1	0.367	0.377	2997.0±2	2.6	5.1	0.9	2.9	2.6	3005.9
55	$C_2H_4Cl_2$—1,2	0.348	0.373	2995.9±2	2.4	4.7	0.6	3.0	2.5	3004.3
56	C_2Cl_4	0.342	0.452	1183.5±3	0.7	5.7	0.9	4.7	3.5	1197.6
57	C_2HCl_3	0.306	0.424	1738.3±2	1.2	5.4	1.0	4.0	3.1	1750.6
58	$C_2H_2Cl_2$-*trans*	0.397	0.372	2689.6±3	2.2	4.7	0.5	3.4	2.8	2698.8
59	$C_2H_2Cl_2$-*cis*	0.336	0.400	2684.3±2	2.1	4.5	0.7	3.1	2.4	2692.9
60	C_3H_7Cl-*n*	0.369	0.283	6162.7±2	7.5	3.6	0.5	1.3	0.9	6161.5
61	C_3H_7Cl-*i*	0.308	0.313	6162.4 *	7.7	3.3	0.7	1.6	0.8	6161.1
62	$C_3H_6Cl_2$—2,2	0.470	0.289	3961.0±2	3.8	4.6	1.0	2.5	2.4	3967.7
63	$C_3H_6Cl_2$—1,2	0.497	0.291	3969.2±2	3.6	4.7	1.2	2.3	2.2	3976.0
64	$C_3H_6Cl_2$-1,3	0.478	0.302	3972.5±2	3.5	4.8	1.0	2.6	2.5	3979.9
65	$C_3H_5Cl_3$-1,2,3	0.380	0.370	2790.7±3	2.0	5.0	0.8	3.1	2.9	2800.5
66	C_4H_9Cl-*n*	0.439	0.204	6971.8±2	8.4	3.3	0.7	1.1	0.7	6969.2
67	C_4H_9Cl-*i*	0.441	0.202	6943.2±2	8.5	3.3	0.5	1.2	0.8	6940.5
68	C_4H_9Cl-*sec.*	0.373	0.263	6938.9±2	8.5	3.2	0.7	1.4	0.8	6936.5
69	C_4H_9Cl-*tert.*	0.365	0.228	6898.1±2	8.8	3.0	0.5	1.3	0.6	6894.7
70	$C_5H_{11}Cl$-*n*	0.365	0.227	7503.9±2	9.1	2.7	0.3	1.1	0.4	7499.3
71	$C_5H_{11}Cl$-*i*	0.350	0.243	7496.5±2	9.1	2.7	0.4	1.1	0.4	7491.9
72	$C_5H_{11}Cl$-*tert.*	0.376	0.202	7452.4±2	9.2	2.7	0.3	1.1	0.4	7447.7

* 3 combustions; deviations from the arithmetical mean = + 3.8; — 2.6; — 1.1.

pressure, volume of the bomb and of the solution of the arsenious acid, see above. The temperature-rise was approximately 1.7° (in Table 7 generally 1.8°), the quantities of benzoic acid burned were 0.80—0.85 g.

It may be suitable here to give an idea of the approximate magnitude of the rest of the Washburn corrections. It is to assume that these corrections do not differ very much from the ones for the bromo compounds, the final state in the bomb being very similar: hydrogen chloride instead of hydrogen bromide. Bjellerup has calculated the corrections for a series of alkyl bromides and told me that the *sum* of the two gas corrections (Washburn's ΔU_{gas} and $\Delta U''_{corr}$) amounts to + 0.4 cal/g for each of these compounds and to + 0.5 for paraffin oil. Certainly these corrections are next in importance to the corrections, applied in this paper.

Table 7. Karlsson's heats of combustion. Some compounds are burned with cellophane cover. Initial temperature about 18.8°. Temperature rise usually 1.8°. Karlsson states that his correction for dissolved gold and platinum amounts to 0.6—1 cal per combustion, original values not available now. Since this correction is wrongly calculated, his statement means that 0.3—0.5 cal are to be added to the corrected values in Table 7.*

No.	Compound	g substance	g par. oil	g cellophane	Heat of comb. uncorr. cal/g	Vac. corr cal/g	HCl + CO_2 corr. cal/g	As_2O_3 corr cal/g	1 : 600 corr. cal/g	Heat of comb. corr cal/g
9	$C_6H_4Cl \cdot CO_2H$-*o*	0.693	0.210		4722.9±2	3.0	1.1	1.2	0.4	4722.6
10	$C_6H_4Cl \cdot CO_2H$-*m*	0.704	0.188		4685.0±1	3.0	1.1	1.2	0.4	4684.7
11	$C_6H_4Cl \cdot CO_2H$-*p*	0.802	0.148		4681.4±2	3.0	1.1	1.2	0.5	4681.2
12	$CH_3 \cdot C_6H_4 \cdot Cl$-*o*	0.524	0.161	0.025	7081.4±3	6.9	2.0	1.2	0.4	7078.1
13	$CH_3 \cdot C_6H_4 \cdot Cl$-*m*	0.459	0.190	0.023	7085.4±2	6.9	1.9	1.3	0.4	7082.1
14	$CH_3 \cdot C_6H_4 \cdot Cl$-*p*	0.513	0.167	0.028	7094.0±3	6.9	2.0	1.3	0.4	7090.8
15	$C_6H_4Cl_2$-*o*	0.554	0.220	0.024	4804.7±2	3.7	3.3	2.5	1.6	4808.4
16	$C_6H_4Cl_2$-*m*	0.527	0.234	0.025	4796.6±2	3.7	3.2	2.4	1.6	4800.1
17	$C_6H_4Cl_2$-*p*	0.512	0.232	0.023	4773.5±1	3.2	3.2	2.5	1.6	4777.6
18	$C_6H_4Cl \cdot CHO$-*o*	0.515	0.208	0.025	5735.8±1	4.7	1.3	1.3	0.4	5734.1
19	$C_6H_4Cl \cdot CHO$-*m***	0.598	0.184		5722.8±2.5	4.7	1.5	1.4	0.4	5721.4
20	$C_6H_4Cl \cdot CHO$-*p*	0.527	0.214		5688.3±1.5	4.7	1.3	1.3	0.4	5686.6
21	$C_6H_4Cl \cdot OH$-*o*	0.605	0.168	0.024	5417.9±2	4.4	1.6	1.4	0.5	5417.0
22	$C_6H_4Cl \cdot OH$-*m* pulverous	0.500	0.243		5375.8±3	4.4	1.5	1.4	0.4	5374.7
23	$CH_4Cl \cdot OH$-*m* in pieces	0.617	0.174		5376.4±2	4.4	1.6	1.2	0.5	5375.3
24	$C_6H_4Cl \cdot OH$-*m* supercooled	0.579	0.197		5407.2±0.7	4.4	1.6	1.6	0.5	5406.5
25	$C_6H_4Cl \cdot OH$-*p* in pieces	0.633	0.168		5392.2±1.5	4.4	1.6	1.4	0.5	5391.3
26	$C_6H_4Cl \cdot OH$-*p* supercooled	0.617	0.212		5422.1±2	4.4	1.7	1.1	0.6	5421.1
27	C_6H_5Cl ***	0.410	0.239		6603.6±3	6.2	1.9	1.5	0.5	6601.3
28	$C_{10}H_7Cl$-α	0.534	0.136		7366.8±1.8	6.4	1.2	0.8	0.2	7362.6
29	$C_{10}H_7Cl$-β	0.699	0.071		7366.8±4.5	5.9	1.5	0.9	0.4	7363.7

* see p. 80.

** some combustions of this substance were carried out with cellophane cover, the weight of which is not given.

*** This compound was burned in a glass ampoule.

In the Tables 6—9 there are given: in the first column the table number in the authors paper (in Table 9 only the number of the compound in this table), in the second the formula of the compound, in the following the weight of the substance, paraffin oil and in certain cases the cellophane. It may be observed that the corrections "corr Pt" and "corr As_2O_3" do not denote the total correction but supplementary corrections (see above). The final values are given without the "mean error", inasmuch as this is to be found in the column of the "orig. values" *.

With the exception of the vacuum correction all corrections are positive.

* Almost all these numerical calculations are carried out by Mr. *D. Folkesson.*

Table 8. Schjånberg's heats of combustion. Most compounds are burned with cellophane cover. Rise of temperature about 1.7°, exceptions are no. 74 (1.2°) and no. 83 (1.4°). Initial temperature usually 17.2°, in nos. 142—147 18.7°, finally in the just mentioned nos. 74 and 83 probably 17.5°. For erroneous correction for dissolving of metal 0.5—1.0 cal/g are to be added to the final values of the table [1].

No.	Compound	g sub-stance	g par. oil	g cello-phane	Heat of comb. uncorr. cal./g	Vac. corr. cal/g	HCl + CO_2 corr. cal/g	As_2O_3 corr cal/g	1 : 600 corr cal/g	Heat of comb. corr cal/g
64	$CH_2Cl \cdot CO_2H$	0.761	0.363		1838.7±1	1.4	2.2	2.0	1.5	1843.2
65	$CH_2Cl \cdot CO_2CH_3$	0.861	0.215	0.024	3198.4±2	2.7	2.2	1.8	1.2	3200.9
66	$CH_2Cl \cdot CO_2C_2H_5$	0.777	0.178	0.022	4080.2±2	3.7	2.0	1.3	0.9	4080.7
67	$CH_2Cl \cdot CO_2C_3H_7$-*n*	0.797	0.136	0.022	4821.6±2	4.6	2.0	1.1	0.7	4820.8
68	$CH_2Cl \cdot CO_2C_4H_9$-*n*	0.711	0.140		5408.8±0.5	5.3	1.9	0.8	0.5	5406.7
69	$CH_2Cl \cdot CO_2C_3H_7$-*i*	0.792	0.126	0.022	4786.8±2	4.6	2.0	1.0	0.7	4785.9
70	$CH_2Cl \cdot CO_2C_4H_9$-*i*	0.689	0.133	0.020	5399.6±1	5.3	1.8	0.8	0.4	5397.3
71	$CH_2Cl \cdot CO_2C_5H_{11}$-*i*	0.691	0.111		5896.7±0.7	6.0	1.8	0.6	0.4	5893.5
72	$CH_2Cl \cdot CO_2C_3H_5$	0.789	0.144	0.024	4628.4±0.7	4.2	1.9	1.2	0.7	4628.0
73	$CH_2Cl \cdot CONH_2$	0.750	0.317		2663.4±1	—	2.7	2.1	1.4	2669.6*
74	$CHCl_2 \cdot CO_2H$	0.546	0.285		1149.0±1	1.7	3.1	4.1	2.1	1156.6
75	$CHCl_2 \cdot CO_2CH_3$	0.722	0.308	0.024	2194.5±1	1.6	3.3	1.7	2.3	2200.2
76	$CHCl_2 \cdot CO_2C_2H_5$	0.582	0.329	0.020	3010.1±2	2.4	3.1	2.4	1.5	3014.7
77	$CHCl_2 \cdot CO_2C_3H_7$-*n*	0.661	0.236	0.020	3680.5±0.9	3.1	3.0	2.2	1.5	3684.1
78	$CHCl_2 \cdot CO_2C_4H_9$-*n*	0.685	0.208		4238.2±1	3.7	2.9	1.9	1.3	4240.6
79	$CHCl_2 \cdot CO_2C_3H_7$-*i*	0.688	0.240	0.022	3655.4±1	3.0	3.0	2.0	1.6	3659.0
80	$CHCl_2 \cdot CO_2C_4H_9$-*i*	0.712	0.202		4233.6±1	3.7	2.9	1.6	1.3	4235.7
81	$CHCl_2 \cdot CO_2C_5H_{11}$-*i*	0.795	0.141		4725.2±2	4.3	2.8	0.7	1.3	4725.7
82	$CHCl_2 \cdot CO_2C_3H_5$	0.657	0.256	0.022	3508.1±0.8	2.8	2.9	2.5	1.5	3512.2
83	$CCl_3 \cdot CO_2H$	0.535	0.370		717.8±1		4.0	4.1	2.9	728.8*
84	$CCl_3 \cdot CO_2CH_3$	0.527	0.390	0.024	1631.9±2	1.1	3.9	3.7	2.4	1640.8
85	$CCl_3 \cdot CO_2C_2H_5$	0.480	0.334	0.024	2338.3±2	1.7	3.6	3.2	1.9	2345.3
86	$CCl_3 \cdot CO_2C_3H_7$-*n*	0.494	0.362	0.021	2928.8±2	2.2	3.6	2.8	1.8	2934.8
87	$CCl_3 \cdot CO_2C_4H_9$-*n*	0.564	0.310		3444.7±1	2.7	3.5	2.6	1.7	3449.8
88	$CCl_3 \cdot CO_2C_3H_7$-*i*	0.530	0.343	0.021	2901.1±1		3.6	2.5	1.9	2909.1*
89	$CCl_3 \cdot CO_2C_4H_9$-*i*	0.565	0.298		3437.2±2	2.8	3.4	2.2	1.7	3441.7
90	$CCl_3 \cdot CO_2C_5H_{11}$-*i*	0.628	0.261		3897.0±1	3.2	3.4	2.3	1.7	3901.2
91	$CCl_3 \cdot CO_2C_3H_5$	0.507	0.352	0.022	2761.3±0.9	2.0	3.5	3.1	1.9	2767.8
92	$CCl_3 \cdot CONH_2$	0.488	0.416		1147.3±1		4.3	4.8	2.8	1159.2*
101	$CH_3CHCl \cdot CO_2H$	0.841	0.262		3069.9±0.8	2.5	2.2	1.7	1.2	3072.5
102	$CH_3 \cdot CHCl \cdot CO_2CH_3$	0.850	0.160	0.024	4108.2±1	3.8	2.1	1.3	0.9	4108.7
103	$CH_3 \cdot CHCl \cdot CO_2C_2H_5$	0.822	0.112	0.023	4795.3±1	4.6	2.0	1.1	0.7	4794.5
104	$CH_3 \cdot CHCl \cdot CO_2C_3H_7$-*n*	0.699	0.119	0.022	5402.1±2	5.4	1.8	0.8	0.5	5399.8
105	$CH_3CHCl \cdot CO_2C_4H_9$-*n*	0.689	0.109	**	5896.3±0.6	6.1	1.8	0.7	0.4	5893.1
106	$CH_3 \cdot CHCl \cdot CO_2C_3H_7$-*i*	0.708	0.116	0.023	5345.1±0.8	5.5	1.8	0.8	0.5	5342.7
107	$CH_3 \cdot CHCl \cdot CO_2C_4H_9$-*i*	0.685	0.102	0.022	5851.0±2	6.1	1.7	0.7	0.4	5847.7
108	$CH_3 \cdot CHCl \cdot CO_2C_5H_{11}$-*i*	0.662	0.101	0.022	6267.7±0.8	6.6	1.7	0.6	0.3	6263.7
109	$CH_2Cl \cdot CH_2 \cdot CO_2H$	0.861	0.256		3011.3±1		2.1	1.5	1.2	3016.1*
110	$CH_2Cl \cdot CH_2 \cdot CO_2CH_3$	0.828	0.164	0.023	4095.4±2	3.7	2.1	1.4	0.9	4096.1
111	$CH_2Cl \cdot CH_2 \cdot CO_2C_2H_5$	0.814	0.116	0.025	4816.1±1	4.6	2.0	1.0	0.7	4815.2

[1] Schjånberg has also burned 21 chlorine free compounds [9]. The heats may be corrected "for CO_2" according to Fig. 3 in this paper.

* no corr. to vac.

** In some combustions the substance has been ignited by a small strip of cellophane (about 0.002 g), in some without this one. A strip has also been used in nos. 140 and 143—147.

Table 8 (continued).

112	$CH_2Cl \cdot CH_2 \cdot CO_2C_3H_7$-*n*	0.728	0.117	**	5422.8±1	5.3	1.9	0.8	0.5	5520.7
113	$CH_2Cl \cdot CH_2 \cdot CO_2C_4H_9$-*n*	0.710	0.097	**	5916.4±1	6.0	1.8	0.6	0.4	5913.2
114	$CH_2Cl \cdot CH_2 \cdot CO_2C_3H_7$-*i*	0.702	0.125	0.022	5394.6±2	5.4	1.8	0.8	0.5	5392.3
115	$CH_2Cl \cdot CH_2 \cdot CO_2C_4H_9$-*i*	0.687	0.109	0.020	5895.1±2	6.0	1.8	0.7	0.3	5891.9
116	$CH_2Cl \cdot CH_2 \cdot CO_2C_5H_{11}$-*i*	0.665	0.097	0.024	6312.6±0.8	6.5	1.7	0.6	0.3	6308.7
124	$C_2H_5 \cdot CHCl \cdot CO_2H$	0.821	0.182		3939.8±1	3.5	2.0	1.4	0.9	3940.6
125	$C_2H_5 \cdot CHCl \cdot CO_2CH_3$	0.828	0.119	0.021	4736.5±1	4.5	1.9	1.1	0.7	4735.7
126	$C_2H_5 \cdot CHCl \cdot CO_2C_2H_5$	0.682	0.128	0.023	5303.3±2	5.3	1.8	1.0	0.5	5301.3
127	$C_2H_5 \cdot CHCl \cdot CO_2C_3H_7$-*n*	0.701	0.098	0.023	5810.3±1	6.0	1.8	0.9	0.4	5807.4
128	$C_2H_5 \cdot CHCl \cdot CO_2C_4H_9$-*n*	0.658	0.099	0.022	6230.1±2	6.5	1.7	0.8	0.3	6226.4
129	$C_2H_5 \cdot CHCl \cdot CO_2C_3H_7$-*i*	0.716	0.105	0.022	5787.4±1	6.0	1.8	0.9	0.4	5784.5
130	$C_2H_5 \cdot CHCl \cdot CO_2C_4H_9$-*i*	0.657	0.102	0.011	6221.3±1	6.6	1.7	0.7	0.3	6217.4
131	$C_2H_5 \cdot CHCl \cdot CO_2C_5H_{11}$-*i*	0.596	0.113	0.023	6583.0±1	7.0	1.6	0.7	0.2	6578.5
132	$CH_3CHCl \cdot CH_2 \cdot CO_2H$	0.812	0.171		3977.2±1	3.4	2.0	1.4	0.9	3978.1
133	$CH_3 \cdot CHCl \cdot CH_2 \cdot CO_2CH_3$	0.797	0.117	0.020	4791.3±2	4.5	2.0	1.1	0.7	4790.6
134	$CH_3 \cdot CHCl \cdot CH_2 \cdot CO_2C_2H_5$	0.722	0.118	0.022	5376.1±1	5.4	1.9	1.0	0.5	5374.1
135	$CH_3 \cdot CHCl \cdot CH_2 \cdot CO_2C_3H_7$-*n*	0.708	0.094	0.019	5869.5±1	6.0	1.8	0.8	0.4	5866.5
136	$CH_3 \cdot CHCl \cdot CH_2 \cdot CO_2C_4H_9$-*n*	0.668	0.094	0.019	6289.3±1	6.6	1.7	0.8	0.3	6285.5
137	$CH_3 \cdot CHCl \cdot CH_2 \cdot CO_2C_3H_7$-*i*	0.702	0.090	0.024	5844.4±0.6	6.1	1.8	0.8	0.4	5841.3
138	$CH_3 \cdot CHCl \cdot CH_2 \cdot CO_2C_4H_9$-*i*	0.660	0.095	0.019	6272.3±1	6.6	1.7	0.6	0.3	6268.3
139	$CH_3 \cdot CHCl \cdot CH_2 \cdot CO_2C_5H_{11}$-*i*	0.602	0.103	0.021	6632.4±0.7	7.1 ***	1.6	0.6	0.2	6627.7
140	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2H$	0.763	0.201	0.002	3957.5±1	3.3	2.0	1.5	0.8	3958.5
141	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2CH_3$	0.799	0.122	0.022	4788.2±1	4.4	2.0	1.2	0.7	4787.7
142	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2C_2H_5$	0.715	0.120	0.023	5375.1±1	5.3	1.8	1.0	0.5	5373.1
143	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2C_3H_7$-*n*	0.712	0.110	0.002	5867.6±2	5.9	1.8	0.9	0.4	5864.8
144	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2C_4H_9$-*n*	0.670	0.099	0.002	6279.5±1	6.5	1.7	0.8	0.3	6275.8
145	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2C_3H_7$-*i*	0.676	0.112	0.002	5845.1±1	6.0	1.8	1.2	0.4	5842.5
146	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2C_4H_9$-*i*	0.658	0.096	0.002	6262.2±1	6.5	1.7	0.8	0.3	6258.5
147	$ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CO_2C_5H_{11}$-*i*	0.619	0.104	0.002	6617.0±1	6.9	1.6	0.7	0.2	6612.6

** In some combustions the substance has been ignited by a small strip of cellophane (about 0.002 g), in some without this one. A strip has also been used in nos. 140 and 143—147.

*** In the thesis there is a misprint in the density figure. This correction is estimated.

The comparison made in Table 5 certainly shows that many of Schjånberg's values must be too low owing to his use of the cellophane method. This error diminishes with rising boiling points of the compounds burned. Table 8 also shows that in a homologous series the mean errors are as a rule greater for the lower members than for the higher ones. This is more pronounced in the derivatives of the monochloro acetic acid than in those of the chloro butyric acids. Certainly the experimental skill of Schjånberg and his great capacity for uniform working is the cause of the fact that the effect of the volatility is not more noticeable. For that reason it is also probable that these "cellophane errors" disappear when calculating differences in the heats of combustions between consecutive members of a series.

Table 9. Sjöströms heats of combustion [7]. The initial temperature is in the combustions of the compounds 1—3 in Table 9 17.0°, in the rest 18.7°. The rise of temperature is everywhere about 1.7°. The final values are corrected for erroneous heat of dissolving of metal, but not to weight in vacuum, the densities of the compounds being unknown.

No.	Compound	g substance	g par. oil	Heat of comb. uncorr. cal/g	HCl + CO_2 corr cal/g	As_2O_3 corr cal/g	Pt corr cal/g	1 : 600 corr cal/g	Heat of comb. corr cal/g
1	monochlorobenzoquinone	0.889	0.105	4347.3±1.2	1.4	1.4	0.3	0.7	4351.1
2	trichlorobenzoquinone	0.486	0.343	2600.0±1.5	3.0	2.7	0.6	1.6	2607.9
3	trichlorohydroquinone	0.496	0.336	2702.7±0.8	3.0	2.6	0.6	1.6	2710.5
4	monochlorobenzoquinone	0.869	0.102	4346.5±1.2	1.4	1.4	0.1	0.7	4350.1
5	monochlorohydroquinone	0.895	0.101	4488.9±1.1	1.5	1.2	0.4	0.7	4492.7
6	2,6-dichlorohydroquinone	0.577	0.289	3408.9±1.3	2.4	2.1	0.3	1.2	3414.9
7	2,6-dichlorobenzoquinone	0.571	0.292	3305.0±1.2	2.3	2.0	0.3	1.2	3310.8
8	2,5-dichlorohydroquinone	0.561	0.284	3404.4±1.1	2.4	2.1	0.3	1.1	3410.3
9	2,5-dichlorobenzoquinone	0.556	0.291	3304.1±1.4	2.3	2.1	0.3	1.2	3310.0
10	2,3-dichlorohydroquinone	0.564	0.287	3418.5±1.2	2.4	2.1	0.3	1.1	3424.4
11	2,3-dichlorobenzoquinone	0.558	0.289	3299.8±1.4	2.3	2.1	0.3	1.2	3305.7
12	tetrachlorobenzoquinone	0.398	0.383	2101.0±2	3.4	3.3	0.3	1.7	2109.7
13	tetrachlorohydroquinone	0.393	0.382	2199.3±2	3.4	2.9	0.5	1.7	2207.8

SUMMARY

Experimental methods have been developed for correcting heats of combustion of chlorinated compounds, determined with the "quartzwool" method. Such corrections were applied to values published from the Institute for Organic Chemistry of the University of Lund by E. Eftring, K. J. Karlsson, E. Schjånberg and G. Sjöström. Totally 128 heat values were corrected. In order to check the reliability of the corrections applied, six of the chloro compounds were also burned in a moving bomb.

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Paper Chromatographic Separation of Volatile Fatty Acids

A Study of a Number of Factors Involved

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Several authors [1–4] have described methods for the paper chromatographic separation of volatile fatty acids, without prior formation of derivatives. These methods appear to have been developed relatively independently, and they thus differ in certain details, although the principle used for the detection of the acid spots is common to all.

In the course of other investigations, we desired to identify, and if possible determine quantitatively [5], small amounts of volatile acids, and it thus became necessary to study the techniques for their chromatographic separation more closely, in order to find the most sensitive and reproducible method. The method evolved has subsequently been used successfully for the determination of volatile acids in cheese [7].

In principle, the analysis is carried out as follows: The acid mixture in the form of a salt solution is placed on the paper and development is carried out with, for example, butanol/water.

As the R_F values of the alkali salts are very low, ammonium or ethylamine salts must be used.

Because of the volatility of the acids and their tendency to undergo esterification, an excess of base must be present throughout the entire process. When the chromatogram is ready it is dried and the excess of base evaporates. By spraying with a suitable indicator, it is possible to detect the position of the acids as coloured spots on a background of another colour. Whether the acids spots appear in the acidic or basic colour of the indicator depends on the concentration of the alkali and the pK value of the indicator.

Brown and Hall [1] and Brown [2] start with the sodium salts which are subsequently converted to the ammonium salts in the high concentration of ammonia. Butanol or ethanol/butanol saturated with 1.5 N or 3 N ammonia is used as the solvent, and drying is carried out for one hour at room temperature, or for five minutes at 100°. The paper is

sprayed with a 0.04 % aqueous solution of bromothymol blue adjusted to pH 7.5 with NaOH, and the acid spots appear yellow on a blue background. 5 μg of acid can be detected.

Hiscox and Berredge [3] use a solution of the acids in butanol neutralised to pH 6—7 with ethylamine. Water-saturated *n*-butanol is used as solvent, and the bottom phase consists of a butanol-saturated ethylamine solution. The concentration of the ethylamine is said to be "not critical". The paper is dried in front of a fan and sprayed with bromocresol green, the acids then appearing as blue spots on a yellow-green background. 4 μl of 0.001 *N* acid can be detected, corresponding in the case of acetic acid to 0.24 μg.

Kennedy and Barker [4] use the ammonium salts of the acids. In common with the previous authors they use Whatman No. 1 paper, but wash it with oxalic acid and a large volume of water. This gives a purer background and more distinct spots.

Ethanol/ammonia, water/acetone/ammonia or water/butanol/ammonia are used as solvents, the papers are dried for 5 minutes at 100°, and sprayed with an aqueous solution of bromophenol blue containing citric acid.

These authors do not give the lower limit of sensitivity, but state that they worked with 1—2 μmolar solutions, *i.e.* with very much larger amounts than the other workers.

Experience gained in a number of preliminary experiments taken in conjunction with the fact that the Hiscox-Berredge procedure permits the use of lower concentrations of acids than the other methods, convinced us that this method would be the most satisfactory in respect of both sensitivity and reproducibility. We therefore concentrated on developing this particular method for reasons which will become more apparent from the following discussion.

EXPERIMENTAL

An initial series of failures showed clearly that the success of the analytical procedure was critically dependent on obtaining the right balance between a number of factors such as the type of paper, the ethylamine concentration, the method of drying the papers, the indicator concentration, the solvent for the indicator, the method of spraying etc. Each of the individual factors was found to exert a very great influence on the final result. Consequently it was necessary to investigate the influence of each variable separately, and repeated experiments were required, before it became possible to decide on the most satisfactory procedure.

Paper Quality. The following types of paper were tested:

Munktell OB (unwashed, washed with butanol, and washed with oxalic acid) [4]
Munktell 20
» 20: 150 g
Whatman No. 1
» » 4
» » 54
Schleicher and Schüll 1101 L
» » » 2043 a
» » » 2043 b
» » » 602 H:P
» » » 1507

It became apparent that the above papers behaved in very different ways. At a given ethylamine concentration, some papers such as S.S. 602 H:P and Munktell OB washed with oxalic acid gave a dark blue background on which no acid spots were discernible, while other papers such as Whatman 54, S.S. 2043b and Munktell 20:150 g gave very light backgrounds, but at the same time only very faint acid spots appeared.

Munktell 20, S.S. 1101 L, S.S. 2043a and S.S. 1057 could not be used because of their very bad capillarity.

In certain cases impurities from the paper collected in the neighbourhood of the advancing solvent front, and these partly or completely masked caprylic acid. This applied especially in the case of the fastest running paper, Munktell OB and although this difficulty could be overcome by washing the paper with butanol before use this treatment is time consuming and troublesome.

Whatman No. 1 paper gave a clean solvent front, a pure background and good contrast, and the chromatograms ran at a suitable speed, so that in the main this paper was employed. The solvent front advanced the full length of a 57 cm strip in 24 hours, but the chromatograms could be taken off after 16 hours, and thus it was convenient to let them run overnight.

For the separation of formic, acetic and lactic acids, which will be described later, the faster running paper Munktell OB could be used. By using a special technique, the impurities at the solvent front could be eliminated, and the background remained pure although somewhat darker in colour than in the case of Whatman No. 1 paper.

The Concentrations of Ethylamine and the Indicator. The purity of the background depends on both the kind of paper and on the concentration of ethylamine present. Even with Whatman No. 1 paper the background is quite blue if 0.1 *N* ethylamine is used and the subsequent spraying is carried out with too weak an indicator. The indicator which is an acid naturally neutralises the excess of ethylamine if the concentration of the indicator is sufficiently high, but at the same time one runs the risk that the salts produced will buffer the acid spots to such an extent that they will not be visible.

Thus it is not desirable to have more ethylamine than is necessary to prevent evaporation or esterification of the acids, and a suitable indicator concentration must be chosen. With an ethylamine concentration in the bottom phase of 0.5 %, an indicator concentration of approximately 2 % is appropriate if ethanol is the solvent. With 0.1 % ethylamine, the indicator concentration should be about 0.5—0.7 %. With Whatman No. 1 paper the best combination is 0.010—0.025 *N* ethylamine in the bottom phase and 0.25 % indicator. For Munktell OB paper the indicator concentration should be *ca.* 0.4 %.

If the concentration of ethylamine is below 0.005 *N* a number of curious effects such as yellowish streaks behind the spots, a diminished spot size etc. become noticeable. In this connection it is of interest to decide whether ethylamine should be present solely in the bottom phase or in the solvent as well.

Experiments showed that considerably better results were obtained if ethylamine was not present in the solvent but only in the bottom phase. When ethylamine was present in the solvent the R_F values were lower, the spots were deformed and the background was considerably darker. In order to get the normal shade of colour in the background the ethylamine concentration must be reduced considerably. For this reason it is more difficult to make use of the ascending technique which is normally more simple. The descending technique proved to be more satisfactory.

Sprayer and Solvent for the Indicator. When the indicator solution is transferred to the paper by means of a compressed air sprayer, its concentration is increased by an amount

which depends on the solvent, the size of the drops, the ratio of air to liquid, the distance from the sprayer to the paper etc. Thus the indicator concentration given is only applicable with the sprayer and solvent which was used.

In order to avoid evaporation as much as possible a high boiling solvent should be used. Butanol has not a sufficiently great solvent power to be used alone, but if the indicator is first dissolved in ethanol and the solution obtained is diluted with an equal volume of butanol good results are obtained.

The requirements of a suitable sprayer are very great. It should deliver a relatively rapid stream of small uniform droplets distributed evenly over a relatively large surface. If the droplets are large, or if there is a mixture of large and small droplets, "moth-ea ten" spots appear, *i.e.* the amount of indicator on the paper is so high in certain places that the blue colour of the acid spots is masked. If the spraying area of the spray is small it is difficult to spray large areas uniformly, and uniform spraying is essential if certain amounts of acids are to give spots of a definite size. Local overdosing or underdosing decreases or increases the area of the spots since the concentration of the acid in the spot is minimal at the perifery which is therefore especially easily masked. If the sprayer delivers a very slow stream of indicator it takes too long a time to cover a large area.

In all, seven different sprayers were tested. All were of the usual fixed jet type, but they differed in detail. The sprayer which proved to be the best was one of a type made at the Department of Biochemistry at Uppsala, and shown diagramatically in Fig. 1. The most suitable sprayer must be selected by testing a batch.

Using the technique described here, bromocresol green, bromocresol purple and bromophenol blue were the only indicators which could be used. This is in agreement with the findings of the authors referred to above.

Drying the Paper. When the chromatogram is dried after development the excess ethylamine is evaporated with the water/butanol mixture. After the drying has proceeded to a certain stage the acids also can theoretically be evaporated. It is therefore important that the drying should proceed until the excess of ethylamine has been removed, but no further. It is not easy to decide exactly when this stage is reached.

In actual practice the paper is dried for the length of time which gives rise to the greatest contrast between the acid spots and the background on subsequent spraying. If the chromatogram is dried in the air at room temperature about one hour is required: if the time is prolonged to two hours the spots become somewhat smaller and fainter. Nevertheless the time of drying is less critical than the other factors. In our experiments it proved more satisfactory to dry the chromatograms in air at room temperature than at 100° or in circulating air at 37°.

The Concentration of the Acid and the Volume of the Sample. The Degree of Separation. In our experiments it has been possible to detect 0.005 μ eq. of acid. The volume of the drops has been varied between 1 and 8 μl without any disadvantages becoming apparent. Thus the amount of acid which is most readily used is 0.10—0.30 μeq. *i. e.* 5—20 μg in the case of acetic acid. Larger amounts of acid can of course be analysed provided that the distance between the spots is sufficiently large. The separation is however not as good as the length of the paper becomes inadequate.

Lactic, formic and acetic acids move most slowly and form a single spot near the starting line. Caprylic, capric and lauric acids also form a single spot but the remaining acids move at intermediate rates and are well separated. Capric and lauric acids are normally separated during the preparation of the sample as they are insoluble in water so that caprylic acid can be distinguished even in their presence.

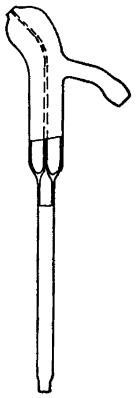

Fig. 1. Sprayer developed in the Department of Biochemistry, Upsala University.

In theory at least it should be possible to separate lactic, formic and acetic acids by virtue of the differences in R_F values. (See Table 1). Thus if the solvent front is allowed to run off the end of the paper the acids can move greater distances and differences in R_F values are more effectively utilised.

Our experiments on this line met with only partial success. It was sometimes possible to distinguish lactic acid and formic acid from one another if the single acids were used and were run side by side. Less frequently it was possible to separate formic acid and acetic acid.

As it seemed that unintential variations in the concentration of the ethylamine and the amount of acid might effect the results, these factors were subjected to a closer scrutiny. The effect of the water content of the butanol was also investigated. It transpired from these experiments that the separation is improved by increasing the concentration of the ethylamine, although it is still not entirely satisfactory. (See Fig. 2).

Even if the individual acids showed greater differences in R_{ac}* values, this was not so when they were mixed.

Clearly the acids are mutually soluble to some extent and this means that the R_{ac} values are not constant.

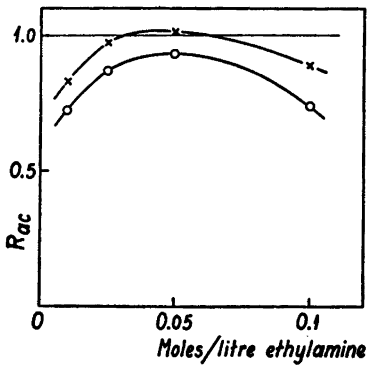

Fig. 2. The rates of travel of lactic and formic acids relative to acetic acid, as a function of the concentration of ethylamine.

——————— *Acetic acid*
—×——×— *Formic acid*
—O——O— *Lactic acid*

R_F value referred to acetic acid instead of the solvent front.

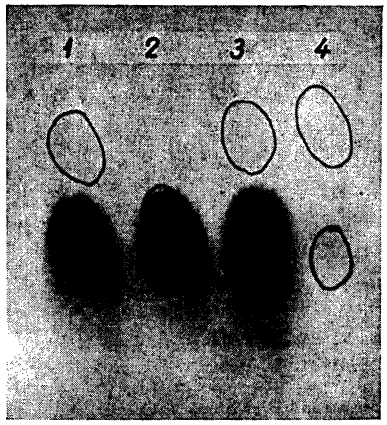

Fig. 3. Separation of lactic, formic and acetic acids using 0.1 N ethylamine in the bottom phase.

1) a mixture of all three acids.
2) » » » formic acid and acetic acid.
3) » » » » » » lactic acid.
4) » » » acetic » » » »

In the presence of large amounts of acids the R_F value of acetic acid decreases, as is apparent from the fact that the acetic acid spots in 1—3 are above those in 4.

However by making use of the technique below it is possible to detect the presence of lactic, formic and acetic acids in a mixture, even although the acids do not separate completely.

The acids are placed in the form of spots containing 0.5—1.5 μ eq. (total) on the quick running paper Munktell OB. A pad of Munktell paper or some other porous paper is attached to the lower end of the chromatogram as described by Miettinen and Virtanen [6] and the paper is run in parallel with an ordinary chromatogram. By virtue of the greater speed of running of the former, the separation of the acetic acid group is improved.

The paper is sprayed in the normal manner with indicator and the positions of the spots due to the lactic acid, the formic acid, and the acetic acid are marked round the outer edges. The paper is then sprayed on both sides with a silver nitrate reagent (equal parts of 0.1 N $AgNO_3$ and 5 N NH_3) and heated at 105° for 1—2 hours. After this treatment the location of the formic acid is indicated by a brown spot. By considering the position of this spot in relation to the outline of the spot due to the acid mixture as revealed when the indicator is washed away it is possible to decide if both acetic and lactic acids are present. Figs. 3 and 4 indicate the application of this technique.

It follows from Fig. 2 that it is advantageous to use 0.1 N or more concentrated ethylamine in order to obtain the best separation, but often the normal 0.025 N ethylamine is satisfactory with this technique.

When the concentration of the formic acid is high, the spot due to this material diffuses over the acetic acid spot and it may become difficult or impossible to decide whether both formic and acetic acids are present or not. (Fig. 3). This diffusion is

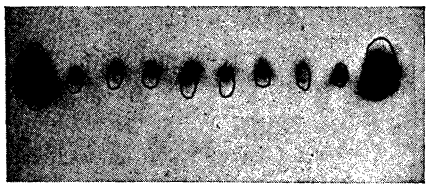

Fig. 4. The separation of formic and acetic acids in the distillate from various types of cheese. (Lactic acid is present only in very small amounts.) Ethylamine concentration in bottom phase, 0.025 N. Because of the low concentration of lactic acid, the acetic acid travels further than the formic acid, as is shown by the "empty space" under the formic acid spots. The controls, a mixture of all three acids seen at the edges, show an "empty space" above the formic acid spots due, in this case, to the lactic acid.

probably only apparent, being caused by the fact that the silver reaction is more sensitive than the indicator at the lowest acid concentrations.

The R_F values for the various acids should depend on several factors. It has already been mentioned that the concentration of the ethylamine and the amount of acid present effect the R_F values of formic and acetic acids, and this probably also applies in the case of the other acids. Table 1 lists the R_F values and R_{ac} values normally obtained.

Table 1. R_F and R_{ac} values of the acids

Acid	R_F, Whatman N:o 1	R_{ac}, Munktell OB
Lactic		0.75—0.94
Formic		0.82—1.00
Acetic	0.17	1.00
Propionic	0.25	
n-Butyric	0.37	
n-Valeric	0.49	
n-Caproic	0.63	
n-Caprylic	0.74	
n-Capric	0.75	
n-Lauric	0.73	

DISCUSSION

The method of detecting the fatty acids on the developed chromatograms is based on the fact that the acids are present in the form of their ethylamine salts which have a higher pH value, or at least a higher buffer capacity than their surroundings. When the indicator is sprayed on the paper this buffering capacity is utilised and the spots retain their pH while the surroundings assume a pH value closer to that of the acidic indicator. Thus the background appears in the acidic colour of the indicator and the spots in the basic tint.

The smaller the amount of indicator used the more clearly the spots stand out. If a large amount of indicator is used in order to keep the pH of the background low, the intensity of the colour in the spots decreases simultaneously. For this reason it is desirable to keep the acidic character of the background or its salt content low. All papers are more or less acidic and some contain salts as impurities. These papers retain ethylamine and thus acquire a high buffer capacity or already have such a high buffer capacity by virtue of their salt content that the background becomes blue. To eliminate this by adding acid to the indicator is wrong in principle, as by so doing one diminishes the chance of detecting small concentrations of acid. It would be better though still not satisfactory to increase the concentration of the indicator, as then one would at least increase the contrast to some extent.

The best way is to use clean paper and an ethylamine concentration which is no higher than absolutely necessary to avoid irreversible adsorption and to adjust the concentration of the indicator accordingly. Brown and Hall use such a large excess of alkali that the background acquires a higher pH than the spots. This is not very desirable as it implies that the relationship between the buffer capacities of the spots and the background is not as favourable as possible, and the sensitivity must be decreased.

From the foregoing remarks one is led to the conclusion that, *a priori*, the method developed by Hiscox and Berredge offers the greatest possibility for the detection of minimal amounts of acids.

If one wishes to work with higher concentrations, or wishes to suppress the appearance of traces of acids, there is no reason to suppose that the methods developed by Brown and Hall or by Kennedy and Barker will not give satisfactory results.

RECOMMENDED TECHNIQUE

In order to attempt quantitative experiments, it is essential that a standardised technique be employed. The following detailed procedure has been developed partly with this end in view, and partly in order to eliminate accidental irregularities which occasionally arise.

Preparation of the sample. The acids in the sample should be isolated by distillation neutralisation, evaporation, acidification with $NaHSO_4$ solution and extraction with butanol. The butanol solution is finally neutralised to bromophenol blue (pH 7) with 33 % ethylamine.

The concentration of the individual acids should be 0.025—0.050 *N*, and the amount of butanol used for the extraction is decided upon with the help of titration data and a knowledge of the origin of the material (the number of acids present must be known or estimated).

Solutions etc. 1) *n*-Butanol. Commercial 98—100 % primary *n*-butanol treated with *ca.* 10 g of KOH per litre and fractionated through an efficient column.

The fraction b.p. $\leqq$ 116° contains water and can be reworked. The fraction b.p. 116—118° is used for the chromatography. The fraction b.p. 118—119° can be used for preparing the sample.

2) Water-saturated butanol. The fraction of b.p. 116—118° is shaken vigorously with about 25 % of distilled water. The butanol is kept over water and can be used as soon as it is completely clear.

3) Butanol-saturated ethylamine. 33 % ethylamine is diluted to 0.025 *N* with butanol-saturated water, and the strength is checked by titration with 0.1 *N* HCl using methyl red as indicator.

4) Indicator solution. 2.5 g of bromocresol green is dissolved in 500 ml of ethanol and 500 ml of butanol (b.p. > 118° is suitable) is added.

5) Standard solutions of the acids. Approximately 0.1 eq. of acid is weighed into a 50 ml measuring flask, which is then filled to the mark with analytically pure methanol. The solution so obtained is titrated with 0.1 *N* alkali using phenolphthalein as indicator, and an appropriate amount to give a solution which will be eventually exactly *N* is transferred to a 50 ml flask. One drop of 1 % bromothymol blue is added followed by 33 % ethylamine until the colour turns blue, and the solution is diluted to the mark with butanol. (Lactic acid gradually decomposes and solutions of it change in concentration over a few weeks.) 0.1 *N*, 0.050 *N* and 0.025 *N* solutions are prepared from the *N* solutions by mixing the various acids and diluting with ethylamine-neutralised butanol. These figures refer to the individual acids, not to the total concentration. It is convenient to make two mixtures, one containing lactic, formic and acetic acids, and the other acetic, propionic, butyric, valeric, capric and caprylic acids.

6) Silver reagent. 50 ml 0.1 *N* $AgNO_3$ + 50 ml 5 *N* NH_4OH. The solution should be freshly prepared each day.

Method. 1) Ethylamine solution is placed in the bottom of the tank to a depth of about two cm and the lid is replaced.

2) The papers are cut and lined. A sheet of Whatman N:o 1 is used for the separation of the higher acids and a sheet of Munktell OB for the separation of formic, acetic and lactic acids. A pad of filter paper is attached to the lower edge of the latter sheet. This pad should weigh about five times as much as the chromatogram itself, and is folded together tightly and attached by means of a number of staples.

3) About 3 ul of the sample is spotted on to the paper, its position being marked with a small cross. The two outer positions on the paper are reserved for standard solutions, usually 0.050 *N* and 0.025 *N*.

4) The paper is hung in the through and kept in place with a glass rod, and the water-saturated butanol is run in. After 16—32 hours the papers are removed and allowed to dry in the air for one hour. (The pad should be removed immediately after the paper is removed from the tank.)

5) The papers are sprayed evenly with the indicator solution.

6) The acid spots apparent on the Munktell paper are marked around the edges, and the paper is sprayed with the silver reagent, and heated for 1—2 hours at 100—125°, during which time it should be allowed to hang freely. The indicator and the excess of silver reagent is removed by repeated washing with distilled water and the paper is dried in the usual way.

Preservation of the papers. The chromatograms obtained as above can only be kept for a few hours. In order to preserve them for longer periods they should be sprayed with a 5 % solution of paraffin in pure benzene. When the benzene has evaporated the paper is warmed until the paraffin melts and spreads evenly over the surface. The contrast is diminished by this procedure and unfortunately the weakest spots may disappear entirely.

If the treated chromatograms are kept between plain paper they may be preserved for up to several months but this method is really only satisfactory when large amounts of acids have been used. If a record is to be kept it is much better to photograph the chromatograms using panchromatic film or plates and a dense red-violet or orange filter.

SUMMARY

The methods available for the paper chromatographic analysis of volatile fatty acids have been investigated with a view to ascertaining which factors affect the sensitivity, the contrast between the spots and the background etc.

It has been shown that several factors must be carefully balanced one against the other if the best results are to be obtained. The variables concerned are the following: the quality of the paper, the concentration of volatile alkali in the atmosphere, the method of drying and the time of drying the paper, the concentration of the indicator and the solvent used, the construction of the sprayer, and finally the manner in which the spraying is carried out.

Detailed directions have been given of the most satisfactory procedure.

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Addition Compounds of Olefins with Metal Salts

I. The Reaction of Ethylene with Mercuric Ions in Aqueous Solution

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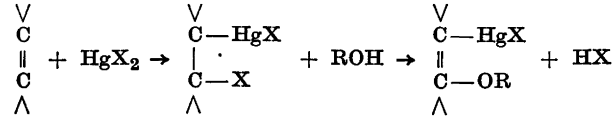
The first attempt to measure the equilibrium and rate constants for the reaction between a mercuric salt (mercuric chloride) and ethylene in aqueous solution was carried out by Sand and Breest [1] in 1907.

A very interesting result was obtained by the present authors when trying to repeat Sand and Breest's measurements: it was found impossible to detect any reaction at all between mercuric chloride and ethylene. Our experiments seem to show that no reaction takes place if the ethylene used is especially pure. Sand and Breest prepared their ethylene from ethanol and sulphuric acid, while ours was prepared from freshly distilled ethylene bromide and zinc at 40° C, whereby very pure ethylene was produced. When ethylene was prepared in a similar way to that of Sand and Breest, a reaction took place, but it was impossible to reproduce any of their results: every time a fresh quantity of ethylene was prepared, a new "equilibrium constant" which differed from the preceding one by a factor varying from 0.1 to 10 was obtained. Consequently, it seems that the results obtained by Sand and Breest are without value because of impurities in the ethylene used. With ethylene prepared in our way reproducible results using dissociated mercuric salts have been obtained.

Until 1935 most workers assumed that the reaction takes place as follows:



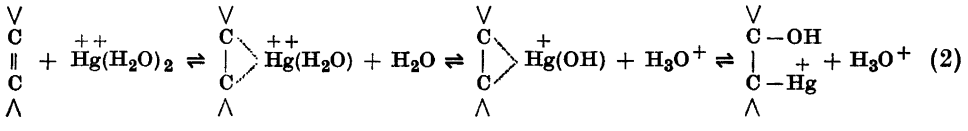
(1)

but more detailed views [2] on this point have also been advanced.

Wright *et al.*[3-4] have studied the kinetics of the reaction between mercuric acetate and various olefins in methanol and have put forward various possibilities for the mechanism.

The only work which elucidates the mechanism of the reaction in aqueous solution was carried out by Lucas, Hepner and Winstein [5], who investigated the reaction between cyclohexene and mercuric nitrate.

Like Lucas, Hepner and Winstein, we may assume that the formation of coordination compounds are produced by simple addition. As the aqueous mercuric ion is assumed by J. Bjerrum [6] to be mainly a diaquo ion and as ethylene only occupies one place in the coordination sphere we have assumed the existence of the following equilibria:



I II III

In the present work we have been able manometrically to show that the reaction is bimolecular with regard to the diaquo mercuric ion and ethylene, but kinetically we have not been able to prove the existence of the ions I and II, which are present only in very small concentrations. For the rate constant in 1 *M* perchlorate and 1 *M* nitrate medium, respectively, the following values were found:

$$\log_{10} k = 5.15 - \frac{1280}{T} \tag{3}$$

$$\log_{10} k = 4.94 - \frac{1260}{T} \tag{4}$$

where k is the absolute rate constant in mole per liter per second and T the absolute temperature.

The reaction is shown to be reversible and runs almost to an end. The uptake of ethylene is immediately followed by dissociation of a hydrogen ion. For the equilibrium constant:

$$K = \frac{([\mathrm{II}] + [\mathrm{III}]) \cdot [\mathrm{H}^+]}{[\mathrm{Hg}^{++}] \cdot [\mathrm{C_2H_4}]} \tag{5}$$

we have obtained the value $3.5 \cdot 10^6$ in 1 *M* perchlorate solution.

By measuring the partition coefficient of cyclohexene between mercuric nitrate solution and carbon tetrachloride Lucas, Hepner and Winstein [5] have determined both of the equilibrium constants*:

$$\frac{\left[{}^{++}_{Hg}{}^{C_6H_{10}}_{H_2O}\right]}{[Hg^{++}]\cdot[C_6H_{10}]} = 2.2\cdot 10^4 \text{ and } \frac{\left[{}^{+}_{Hg}{}^{C_6H_{10}}_{OH}\right]\cdot[H^+]}{[Hg^{++}]\cdot[C_6H_{10}]} = 5.0\cdot 10^4$$

By dividing the second equilibrium constant by the first one we get for the apparent acidic dissociation exponent of the aquo cyclohexene mercuric ion: $pK = -0.36$. Professor J. Bjerrum has suggested to the present writers that the reason why the aquo ion I behaves as a strong acid must be that the assumed equilibrium between the two isomeric ions II and III is displaced strongly in favour of the converted hydroxo ion III.

Hietanen and Sillén [7] have recently found the following values for the acidic dissociation exponents of the diaquo mercuric ion: $pK_1 = 3.70$ and $pK_2 = 2.60$. If we can disregard the electronegative influence of the olefin substituent the isomerization constant of the hydroxo mercuric ion:

$$K_i = \frac{[III]}{[II]} \tag{6}$$

appears to be of the order of magniture $\sim 10^4$.

Winstein and Lucas [8], Taufen, Murray and Cleveland [9], and Trueblood and Lucas [10] have investigated silver coordination complexes with olefins, and demonstrated that one silver ion combines with one molecule of olefin in analogy to the mercuric compounds, but in contrast hereto no measurable quantity of hydrogen ions are liberated. As, however, the aquo ethylene silver ion does not behave like an acid, it must be kept in mind that the diaquo silver ion is $\sim 10^8$ times weaker as an acid than is the corresponding aquo mercuric ion.

Winstein and Lucas [8] have determined the equilibrium constant:

$$\frac{\left[{}^{+}_{Ag}{}^{C_6H_{10}}_{H_2O}\right]}{[Ag^+]\cdot[C_6H_{10}]} = 81.2$$

and Trueblood and Lucas [10] have determined the equilibrium constant:

$$\frac{\left[{}^{+}_{Ag}{}^{C_2H_4}_{H_2O}\right]}{[Ag^+]\cdot[C_2H_4]} = 85.3$$

* Lucas *et al.* do not distinguish between the ions II and III.

In a single experiment the present writers have also determined the latter constant and, in agreement with Trueblood and Lucas, have obtained the value: 87.2.

The present experiments have also shown that if one of the water molecules in the ions: $Hg(H_2O)_2^{++}$ and $Ag(H_2O)_2^{+}$ is replaced by another ligand, *e.g.* chlorine ion, bromine ion or ammonia (in the case of silver only by ammonia) a reaction occurs with ethylene, whereas no reaction whatsoever takes place with mercury when both of the water molecules are replaced by halide ions or ammonia.

EXPERIMENTAL

Apparatus. All the experiments were carried out in a Brønsted shaking apparatus similar to that described by Hofman-Bang [11] with the following alterations: The cap with the little glass container was omitted, and through the bottom of the reaction flask a platinum wire was melted in for the sake of measuring the mercury-mercuric potential in the solution. As a rule, glass stoppers were used, but in the experiments where the mercury-mercuric potential had to be determined, a special glass stopper (a tube which nearly touched the bottom of the flask, ending in a glass filter plate and with a glass stopcock outside the flask) was placed in the reaction flask. The tube was filled with 1 *M* ammonium nitrate acting as bridge to the reference electrode, and sufficient mercury was placed in the reaction flask to cover the platinum wire completely.

The entire shaking apparatus, including the manometer which was filled with mercury, was lowered into a water thermostat, which usually was kept at 25.00° C ± 0.02° C.

The volume capacity of the apparatus was determined by measuring the pressure before and after addition, through a special funnel with a glass stopcock, of a known amount of mercury, and for the flask used in all the experiments for determination of the rate constant the average value: 74.51 ± 0.20 ml was found and for the special flask, only used for the determination of the equilibrium constant: 56.91 ± 0.20 ml was found.

Materials and solutions. The inorganic chemicals employed were all of analytical grade (Merck). The mercuric solutions were prepared by weighing mercuric oxide and dissolving it in 4 *M* perchloric or nitric acid in a volumetric flask and diluting with water and sufficient sodium perchlorate (or sodium nitrate) solution to obtain a total anion concentration of 1.000 mole per liter. The constants given here are therefore not the 'activity constants' but 'concentration constants' valid only under our specific conditions.

In this paper square brackets will be used for true concentrations and *C* for total concentrations, and all concentrations are given in mole per liter and all pressures in mm Hg.

Ethylene bromide and ethylene were prepared as described by Trueblood and Lucas [10] with only a few unimportant alterations. The ethylene was kept in a water gasometer and purified just before it was transferred to the apparatus by passing through two Friedrichs washing bottles containing concentrated sulphuric acid and two bottles with basic pyrogallol solution.

PROCEDURE AND CALCULATIONS

A definite amount of mercuric salt solution is transferred with a Krogh syringe pipette into the reaction flask, as well as into the compensation flask. The glass stoppers are greased and as soon as the flasks are closed both chambers are evacuated by means of an oil pump until the solutions bubble briskly. The stopcock between the reaction chamber and the compensation chamber is now closed and the stopcock connected with the gasometer containing ethylene is opened until the desired pressure is attained. The experiment is started by switching on the motor which works the shaking mechanism, and the ethylene pressure is then read at definite intervals.

The concentration of the ethylene consumed is calculated by means of the equation:

$$C_x = \beta \cdot (P_0 - P_\infty) - \alpha \cdot P_\infty \tag{7}$$

where P_0 is the initial pressure, P_∞ the pressure of ethylene when the equilibrium is established (both expressed in mm Hg), α the molar solubility of ethylene in water at 1 mm Hg pressure and β an apparatus constant.

The values of α are those determined by Grollman [12], who found: at 20° C, $\alpha = 7.37 \cdot 10^{-6}$; at 30° C, $\alpha = 5.55 \cdot 10^{-6}$ and at 40° C, $\alpha = 4.52 \cdot 10^{-6}$. The values for temperatures within these intervals are determined by graphical interpolation, the value at 25° C is found in this way to be: $\alpha = 6.34 \cdot 10^{-6}$. These figures are only valid in pure water solutions, but they are used in this work as we have no knowledge of the exact value in 1 M salt solution. The true value can only be a few per cent less than the one employed, and it will be very easy to recalculate the results when an exact value is determined, as α is a factor in all the equations used in the present work.

The apparatus constant β depends on the temperature and the volume of the gas in the reaction chamber, and is determined by the equation:

$$\beta = \frac{(V - v) \cdot 10^3}{v \cdot RT} \tag{8}$$

where V is the volume of the reaction chamber, v the volume of the solution, T the absolute temperature and R the gas constant which has the value: 62 359 when V and v are expressed in ml and pressure in mm Hg. The values of β in all the experiments for determination of rate constants are: at 20° C, $1.496 \cdot 10^{-4}$; at 25° C, $1.471 \cdot 10^{-4}$; at 30° C, $1.446 \cdot 10^{-4}$ and at 35° C, $1.423 \cdot 10^{-4}$ ($V = 74.51$ ml and $v = 20$ ml). The value of β at 25° C in the experiments for determination of the equilibrium constant is: $0.992 \cdot 10^{-4}$ ($V = 56.9$ ml and $v = 20$ ml).

The concentration of the hydrogen ion was measured with an ordinary glass electrode connected with a "Radiometer" valve potentiometer, model PHM 3i, and the true hydrogen ion concentration $[H^+]$ was determined in every case by standardizing against a solution containing the same concentration of mercuric nitrate and sodium nitrate and almost the same hydrogen ion concentration as the solution to be measured. When measuring the hydrogen ion concentration in a solution of the composition: $C_{Hg(NO_3)_2} = 0.0250$; $C_{HNO_3} = 0.0500$ and $C_{NaNO_3} = 0.9000$ the following average value of ten measurements was obtained; 0.0503 ± 0.0005. As a rule, with one measurement alone there is an uncertainty of about 3 %.

The concentration of the mercuric ion in the solutions was determined by measuring the mercury-mercuric potential with the same valve potentiometer as mentioned above. The folloving cell was used:

$$\mathrm{Hg} \;\Big|\; \begin{matrix} Hg^{++} \\ Hg_2^{++} \\ Hg_g^{+} \end{matrix} \;\Big|\; \begin{matrix} C_2H_4 \\ OH \end{matrix} \;\Big|\; \begin{matrix} 1\ M \\ NH_4NO_3 \end{matrix} \;\Big|\; \begin{matrix} Hg_2Cl_2 \\ 1\ M\ \ KCl \end{matrix} \;\Big|\; \mathrm{Hg}$$

As the diffusion potential may be considered constant for this cell, the mercuric ion concentration, when E° is known, may be calculated from the equation:

$$E = E^\circ + \frac{RT}{2F \cdot \log e} \cdot \log\,[Hg^{++}] \qquad (9)$$

As the calomel electrode was only a simple working electrode, it was necessary to standardize it against a solution with a known mercuric ion concentration for determination of E°. The standard solution was made from a solution with the following concentrations: $C_{Hg(ClO_4)_2} = 0.0250$, $C_{HClO_4} = 0.0500$ and $C_{NaClO_4} = 0.9000$ mole per liter, by shaking with mercury for nine hours to establish the equilibrium between mercurous and mercuric ions. The mercuric ion concentration is calculated from the equation:

$$[Hg^{++}] = \frac{C_{Hg}}{1 + K_{Hg}} \qquad (10)$$

where C_{Hg} stands for the total concentration of mercurous and mercuric ions, which in this case is the same as the initial concentration, and K_{Hg} is the equilibrium constant for the mercurous and mercuric ions. The value of K_{Hg} determined by Sillén [13] is: 129.2, and the concentration of the mercuric ion in the standard solution, in equilibrium with the mercurous ions, is calculated from equation (10): $1.920 \cdot 10^{-4}$ and together with the measured value of E gives us the value of E°.

PRELIMINARY EXPERIMENTS

Some experiments were carried out in order to determine the number of molecules of ethylene that combine with one mercuric ion. The total concentration of the solution used was: $C_{Hg(NO_3)_2} = 0.0250$; $C_{HNO_3} = 0.0500$ and $C_{NaNO_3} = 0.9000$ mole per liter. The average number $\bar{n}$ of ethylene molecules bound to a mercuric ion is indicated by:

$$\bar{n} = \frac{C_x}{C_{Hg^{++}}} \tag{11}$$

where C_x stands for the consumed ethylene according to (7) and $C_{Hg^{++}}$ is the initial concentration of mercuric ions. The results are given in Table 1 from which it is seen that one molecule of ethylene combines with one mercuric ion.

Table 1. Results of experiments showing that nearly one molecule of ethylene combines with one mercuric ion.

The stochiometric concentrations of the solution used were: $C_{Hg(NO_3)_2} = 0.0250$; $C_{HNO_3} = 0.0500$ and $C_{NaNO_3} = 0.9000$ mole per liter. P_0 is the initial pressure of ethylene and P_∞ the pressure when equilibrium is established at 25° C.

Expt. No.	P_0	P_∞	$P_0 - P_\infty$	C_x	$\bar{n}$
1	233.5	61.4	172.1	0.02493	0.995
2	221.1	49.1	172.0	0.02500	1.000
3	537.2	352.1	184.9	0.02497	0.998

Other experiments were carried out in order to determine the number of hydrogen ions that are liberated for every molecule of ethylene consumed. The solution used was the same as described above. The average number $\bar{\nu}$' of hydrogen ions liberated for every molecule of ethylene consumed is designated by:

$$\bar{\nu} = \frac{[H^+]_\infty - C_{HNO_3}}{C_x} \tag{12}$$

where $[H^+]_\infty$ is the measured hydrogen ion concentration when the equilibrium is established, and the difference, $[H^+]_\infty - C_{HNO_3}$, is an expression for the increase of hydrogen ion concentration.

From Table 2 it will be seen that nearly one hydrogen ion is liberated for every molecule of ethylene consumed, in accordance with (2). In Expts. Nos. 2 and 3 there was an excess of ethylene, and Expt. 2 was interrupted before the equilibrium was established, thus causing the uncertainty in this experiment to be greater than that of the other.

DETERMINATION OF THE EQUILIBRIUM CONSTANT K

The expression for the equilibrium constant K is already given (5), and the experimental results are shown in Table 3. The average value is found to be: $3.5 \cdot 10^6 \pm 0.2 \cdot 10^6$.

Table 2. Results of experiments showing that nearly one hydrogen ion is liberated for every molecule of ethylene consumed.

The solution and temperature are the same as in Table 1.

Expt. No.	P_0	P_∞	C_x	$[H^+]_\infty$	ν
1	75.4	0.0	0.01109	0.06141	1.028
2	241.0	103.5	0.02231	0.07421	1.075
3	205.9	36.1	0.02498	0.07488	0.998

The concentration of the mercuric ion is calculated from equation (9).

As the equilibrium is strongly shifted in favour of the complex ion, the initial concentration of the mercuric ion has been used as the best approximation for the concentration of the complex ion instead of the calculated value of C_x from equation (7).

The concentration of the hydrogen ion is calculated as the sum of the initial concentration and the concentration of the complex ion.

As the solubility of ethylene is slight we may assume that Henry's law is undoubtedly fulfilled and the concentration of free ethylene may therefore be calculated from the equation:

$$[C_2H_4] = a \cdot P$$

where the value of a is $6.34 \cdot 10^{-6}$ at 25° C, when P is expressed in mm Hg.

Expts. Nos. 1, 2, 3a and 4a were carried out as described above by shaking a solution of mercuric perchlorate with ethylene (the reaction proceeded from left to right according to equation (2)).

It was shown in two different ways that the reaction is reversible.

Table 3. Survey of experiments for the determination of the equilibrium constant K at 25° C.

The stochiometric concentrations of the solution used were: $C_{Hg(ClO_4)_2} = 0.0250$; $C_{HClO_4} = 0.0500$ and $C_{NaClO_4} = 0.9000$ mole per liter.

Expt. No.	P_∞	$E-E^\circ$	C_x	$[H^+]_\infty$	$[Hg^{++}]_\infty$	$[C_2H_4]_\infty \cdot 10^4$	$K \cdot 10^{-6}$
1	39.8	− 0.1676	0.0250	0.0750	$2.12 \cdot 10^{-6}$	2.52	3.5
2	191.4	− 0.1873	0.0250	0.0750	$4.68 \cdot 10^{-7}$	12.1	3.3
3a	230.7	− 0.1919	0.0250	0.0750	$4.02 \cdot 10^{-7}$	14.6	3.2
3b	233.7	− 0.1548	0.0167	1.800	$5.78 \cdot 10^{-6}$	14.8	3.5
4a	297.7	− 0.2224	0.0250	0.0750	$2.95 \cdot 10^{-8}$	18.9	3.4
4b	40.2	− 0.1684	0.0250	0.0750	$1.99 \cdot 10^{-6}$	2.55	3.7
5	278.8	− 0.1923	0.0250	0.0750	$3.09 \cdot 10^{-7}$	17.7	3.4
6	113.9	− 0.1821	0.0250	0.0750	$6.85 \cdot 10^{-7}$	7.23	3.8

After equilibrium was established in Expt. No. 3a (which takes about nine hours) a definite amount of 4 *M* perchloric acid was added to the reaction mixture (this amount was calculated so that the salt concentration was still 1 *M*). The entire apparatus was now evacuated, then ethylene admitted to the reaction vessel until the pressure was as near as possible to the equilibrium pressure which had been established before the addition of acid. It was seen that the reaction now proceeded in the reverse direction, as the pressure of ethylene slowly increased (after nine hours the total rise was 1.7 mm Hg) and the concentration of the mercuric ion also increased (at zero it was $0.31 \cdot 10^{-6}$ after one hour it was $1.43 \cdot 10^{-6}$, after four hours $2.82 \cdot 10^{-6}$ and after nine hours it reached the highest value when the equilibrium was established again, *viz.* $5.78 \cdot 10^{-6}$) and the value of the equilibrium constant was satisfactory.

In Expt. No. 4b (which was started similarly to Expt. No. 3b) the pressure of ethylene had been decreased from 279.7 to 25.6 mm Hg after equilibrium was established. When equilibrium had been established again, the ethylene pressure had increased from 25.6 to 40.2 mm Hg and the mercuric ion concentration had also increased from $0.03 \cdot 10^{-6}$ to $1.99 \cdot 10^{-6}$. The value of the equilibrium constant was again satisfactory.

MEASUREMENT OF THE RATE OF REACTION

In Table 4 an example is given of an ordinary experiment, from which it will be seen that it takes about three minutes before an equilibrium is established between the solubility rate of ethylene and the velocity of the reaction with mercuric ions. In these first three minutes there is not enough ethylene in the solution (even though the solubility rate is very high) as the rate of reaction is extremely high as long as the mercuric ion concentration is of the same order as the initial concentration. As will be seen, nearly 50 % of the total amount of mercuric ions had already reacted with ethylene in these first three minutes.

Then, for about half an hour, the reaction takes place at such a rate that it is possible to measure the pressure exactly at the desired time, and the rate constant in this interval is almost a true constant. From this juncture until the equilibrium is established the reaction takes place very slowly.

The calculation of the rate constant, on the basis of the assumption that the reaction is bimolecular, is made by means of the equation:

$$-\frac{\mathrm{d}\,[C_2H_4]}{\mathrm{d}t} = k \cdot [Hg^{++}] \cdot [C_2H_4]$$

Table 4. Measurement and calculation of an ordinary experiment.

This experiment is the same as No. 1 in Table 7.

Time in minutes	P	$P_0 - P$	$\log \frac{P \cdot C_{Hg}^{++}}{P_0(C_{Hg}^{++} - \beta(P_0 - P))}$	$k = \frac{\beta \cdot \log e}{\alpha(P_0\beta - C_{Hg}^{++})} \cdot \log \frac{P \cdot C_{Hg}^{++}}{P_0(C_{Hg}^{++} - \beta(P_0 - P))}$
0	91.9	0		
2.5	52.1	39.8	0.128	276
3	46.9	45.0	0.156	270
3.5	42.3	49.6	0.184	264
4	37.9	54.0	0.215	263
4.5	34.2	57.7	0.246	263
5	30.9	61.0	0.274	260
5.5	27.9	64.0	0.308	262
6	25.1	66.8	0.342	263
6.5	22.9	69.0	0.372	263
7	20.9	71.0	0.403	262
7.5	18.9	73.0	0.437	263
8	17.0	74.9	0.475	266
8.5	15.7	76.2	0.503	265
9	14.1	77.8	0.542	268
9.5	12.9	79.0	0.574	268
10	11.9	80.0	0.604	267
10.5	10.9	81.0	0.637	267
11	10.0	81.9	0.671	268
12	8.5	83.4	0.733	267
13	7.1	84.8	0.807	270
14	6.2	85.7	0.859	266
16	4.9	87.0	0.954	258
18	3.9	88.0	1.047	250
20	3.3	88.6	1.117	250
40	0.9			
60	0.5			
540	0.0			

The integrated form of this equation is:

$$\log \frac{\dfrac{(\beta(P_0 - P_\infty) - \alpha P_\infty) \cdot \log e}{\alpha P_0(\beta(P_0 - P_\infty) - \alpha P_\infty) - \alpha C_{Hg}^{++}(P_0 - P_\infty)} \cdot \dfrac{(P_0 - P_\infty) \cdot P \cdot C_{Hg}^{++}}{P_0 \left(C_{Hg}^{++}(P_0 - P_\infty) - (P_0 - P)(\beta(P_0 - P_\infty) - \alpha P_\infty)\right)}}{} = k \cdot t \qquad (13)$$

For pressures less than about 175 mm Hg there is a deficit of ethylene, so that pressure P_∞ when equilibrium is established is so small (less than 0.1 mm Hg) that it can be neglected and the integrated form can be simplified to:

$$\frac{\beta \cdot \log e}{\alpha(\beta \cdot P_0 - C^{++}_{Hg})} \cdot \log \frac{P \cdot C^{++}_{Hg}}{P_0 (C^{++}_{Hg} - (P_0 - P))} = k \cdot t \qquad (14)$$

As a rule the rate constant was determined from the slope of the straight line plot of the logarithmic expression against t. In all the tables in this paper the rate constants are given in mole per liter per minute.

In most of the experiments a deficit of ethylene was used because it was easier to calculate the rate constant from the more simple expression (14) and it also provided a check of the purity of the ethylene used since the pressure P_∞ must be very near zero. Where this was not the case, the value of the pressure when equilibrium had been established was used to calculate how much atmospheric nitrogen was mixed with the ethylene, and then this value was used for correction of the pressure. Small amounts of atmospheric air are always present in the ethylene when it is kept in a gasometer, as the water that enters when the gas is let out is saturated with air.

In all the experiments, except one, the composition of the solution when working in nitrate medium was the same as described under preliminary experiments, and in perchlorate medium it was: $C_{Hg(ClO_4)_2} = 0.0250$; $C_{HClO_4} = 0.0500$ and $C_{NaClO_4} = 0.9000$ mole per liter. As the hydrogen ion concentration in all the experiments in this paper is greater than 0.0250, the hydrolysis of the aquo mercuric ion is less than 1 $^0/_{00}$ in all the solutions [7].

In Expt. No. 12 (Table 7) another initial concentration was employed because mercurous nitrate was used instead of mercuric nitrate. Almost the same rate constant was obtained in this case as in all the other experiments in nitrate solution. It is only possible to make small alterations in the mercuric ion concentration in pure mercuric solutions, as the capacity of the apparatus is limited, and a certain fall in pressure of ethylene is necessary in order to obtain the least possible deviation.

The initial pressure of ethylene was varied from 66 to 712 mm Hg without any influence on the rate constant being observed. The results of these tests are shown in Table 5.

Table 5. Variation of ethylene pressure at 25° C.

The solution used was the same as in Table 1.

Expt. No.	P_0	k
1	66	317
2	90	307
3	167	282
4	213	287
5	283	308
6	537	287
7	712	282

Table 6. Variation of initial hydrogen ion concentration at 25° C.

The initial concentrations were: $C_{Hg(NO_3)_2} = 0.0250$ and $C_{NaNO_3} = 0.9000$ mole per liter.

Expt. No.	C_{HNO_3}	k
1	0.0250	326
2	0.0500	332
3	0.200	332

Table 6 gives the results of a few experiments made with different initial concentrations of the hydrogen ion. These concentrations lie between 0.0250 to 0.200 mole per liter. As there is nothing to indicate any influence on the rate constant, this shows that the reaction takes place between an ethylene molecule and the diaquo mercuric ion and not with the hydroxo mercuric ion as suggested by some of the earlier authors.

In the experiments with 1 M nitrate medium, several difficulties were encountered. The rate constant showed some inexplicable dependence on the purity and age of the ethylene. Thus, freshly prepared ethylene gives about 5—10 % higher values than older ethylene, and a minimum value is obtained after about four days and remains constant. We may assume that the dependence on the purity of ethylene in this case is of the same type as already mentioned with the experiments analogous to those of Sand and Breest, employing mercuric chloride. In 1 *M* perchlorate solutions there was no significant dependence on the purity and age of ethylene.

A summary of experiments made at different temperatures is given in Tables 7 and 8. Employing Arrhenius' equation in 1 *M* perchlorate and 1 *M* nitrate medium respectively, all the results may be expressed as follows:

$$\log_{10} k = \log_{10} A - \frac{E_A}{RT \cdot \log e} = 5.15 - \frac{5.810}{4571 \cdot T} \qquad (15)$$

$$\log_{10} k = 4.94 - \frac{5.855}{4571 \cdot T} \qquad (16)$$

From the above it will be seen that the value of energy of activation (expressed in kcals.) and the value of the frequency exponent are exceedingly small, and less than usually found in rapid complex reactions [14]. The value of the rate constant given in the last column is calculated from these equations, and there is a very good agreement with the experimental average value.

The difference between the rate constants with nitrate or perchlorate as medium may be due to the tendency of mercuric ion to associate with the nitrate ion. According to Sillén [13], it is about 50 % associated in 1 *M* nitrate

Table 7. Results of experiments at various temperatures for determination of the rate constant in 1 M sodium nitrate solution.

The stochiometric concentrations of the solution used in all the experiments except Nos. 12 and 13 were: $C_{Hg(NO_3)_2} = 0.0250$; $C_{HNO_3} = 0.0500$ and $C_{NaNO_3} = 0.9000$ mole per liter. In Expt. No. 12 mercurous nitrate was used and the initial concentration of the mercuric ion was only: 0.0001935. In Expt. No. 13 three per cent of the nitrate ions were exchanged with perchlorate ions. In Expts. Nos. 22, 23 and 24 freshly prepared ethylene was used. The value of k in the last column is calculated from equation (4).

Expt. No.	Temperature in °C	k	k average	k calculated
1	20°	265	266	266
2	20°	267		
3	25°	317		
4	25°	318		
5	25°	315		
6	25°	315		
7	25°	315		
8	25°	317	316	314
9	25°	313		
10	25°	320		
11	25°	319		
12	25°	306		
13	25°	313		
14	27°	335	335	335
15	30°	369		
16	30°	367		
17	30°	365	366	366
18	30°	364		
19	30°	364		
20	35°	419	429	427
21	35°	440		
22	25°	338		
23	27°	361		
24	35°	461		

solution, whereas the perchlorate ion is supposed to have a very little tendency to associate with mercuric ion. If an ordinary experiment is calculated on the basis of an initial concentration of only 60—65 % of the total, a rate constant is obtained which agrees with the corresponding value in perchlorate medium. Thus it seems that ion association explains most of the effect of the medium [15]. One experiment (No. 13, Table 7) in which 3 % of the nitrate ions were exchanged with perchlorate ions was carried out, in order to ascertain whether the perchlorate ion might have a catalytic effect, but the rate constant was exactly the same as in pure nitrate solutions.

Table 8. Results of experiments at various temperatures for determination of the rate constant in 1 M sodium perchlorate solution.

The stochiometric concentrations of the solution used in all the experiments were: $C_{Hg(ClO_4)_2} = 0.0250$; $C_{HClO_4} = 0.0500$ and $C_{NaClO_4} = 0.9000$ mole per liter. The value of k in the last column is calculated from equation (3).

Expt. No.	Temperature in °C	k	k average	k calculated
1	20°	371		
2	20°	361		
3	20°	372	366	369
4	20°	364		
5	20°	363		
6	25°	430		
7	25°	437		
8	25°	441	436	434
9	25°	430		
10	25°	436		
11	25°	454		
12	30°	510		
13	30°	523	510	510
14	30°	508		
15	30°	499		

The value of the rate constant in dilute aqueous solution must be of the same order of magnitude as the one found in 1 M salt solution, because the reaction occurs between an ion and a neutral molecule [16].

MEASUREMENT OF THE RATE CONSTANT WITH OTHER IONS

In the literature, only one example has been mentioned in which a reaction takes place between mercurous ion and an unsaturated compound, *viz.* by Biilmann [17] who investigated the reaction between mercurous nitrate and allyl alcohol.

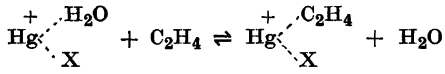
One experiment was made using mercurous nitrate and ethylene (Expt. No. 12, Table 7). As there was an uptake of ethylene and the velocity appeared to be about 10—20 times less than in the experiments with mercuric nitrate (after 3 minutes only about 10 % had reacted, after 30 minutes only 30 % and after two hours 60 %), it was assumed at first that the mercurous ion had reacted with ethylene, but when calculating the rate constant from equation (13) it was obvious that this could not be the case, as no straight line was formed when plotting the lograrithmic expression against t. When we calculate the rate constant asuming that only the mercuric ion in the mercurous nitrate

solution reacts with ethylene, the value obtained is nearly the same as found in the experiments with pure mercuric solutions. In making the calculation we assumed that the mercuric ion is formed instantaneously from mercurous ions.

As previously demonstrated by Biilmann, the reaction occurs with precipitation of mercury which is readily seen, since the solution becomes grey and muddy after about half an hour. The initial concentration of mercurous ion is: 0.0250 and the initial concentration of mercuric ion calculated according to equation (10) is: 0.0001935.

It has also been shown that no reaction takes place when two or more ligands are bound to the mercuric ion, *e.g.*, $HgCl_2$; $HgCl_3^-$ $HgCl_4^{--}$; $HgBr_2$; $Hg(NH_3)_2^{++}$; $Hg(NH_3)_3^{++}$.

In solutions with maximum concentration of monocomplex, *i.e.*, about 30 % of the ion: $HgCl^+$; $HgBr^+$ or $Hg(NH_3)^{++}$, a reaction takes place with almost the same velocity as for the mercuric ion. For this reason the reaction with these ions is assumed to occur mainly according to:



(17)

IV

Since some mercuric ions are present in the solution, a reaction according to (2) runs parallel to (17) and therefore an equilibrium between the ions II and IV may be assumed. After a few hours a white precipitate is formed, thus making it difficult to carry out further investigations with these ions. Only in a few cases was it possible to calculate the rate constant for these complex

Table 9. Results of experiments for determination of the rate constant at 25° C with different complex ions and salts.

Expt. No.	Complex ion	k
1	Hg_2^{++}	0
2	$HgCl^+$	263
3	$HgBr^+$	258
4	$Hg(NH_3)^{++}$	(300)
5	$HgCl_2$	0
6	$HgBr_2$	0
7	$Hg(NH_3)_2^{++}$	0
8	$HgCl_3^-$	0
9	$HgCl_4^{--}$	0
10	$Hg(NH_3)_3^{++}$	0
11	$Hg(NH_3)_4^{++}$	0

ions. In the calculation, correction is to be made for the reaction with the mercuric ions as well as for the displacement of the equilibrium:

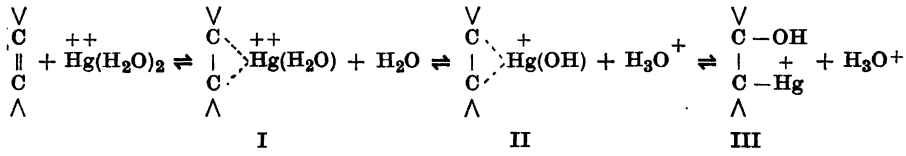
$$Hg^{++} + HgCl_2 \rightleftharpoons 2\ HgCl^+ \qquad (18)$$

which has been thoroughly investigated by Sillén [13].

A list of these experiments is given in Table 9. The values for the monohalide ions were only determined from one experiment and the value may therefore only be considered as preliminary. This is particularly the case in the experiment with the monoammine mercuric ion, where a precipitate was formed after a few minutes.

SUMMARY

Kinetic investigations have been carried out on the reaction between mercuric salts and ethylene. It has been shown that the reaction is bimolecular with regard to the aquo mercuric ion and ethylene and that the uptake of ethylene is instantaneously followed by dissociation of a hydrogen ion. The following equilibria are suggested:



The equilibrium between the ions II and III must be well to the side of the organic form. This explains why the coordination compound I behaves as a strong acid.

It has been shown that the reaction is reversible and the value of the equilibrium constant is found to be:

$$K = \frac{([II] + [III]) \cdot [H^+]}{[Hg^{++}] \cdot [C_2H_4]} = 3.5 \cdot 10^6$$

The relation between the rate constant (time in seconds) and the absolute temperature in 1 M perchlorate medium has been found to be:

$$\log_{10} k = 5.15 - \frac{1280}{T}$$

The rate constant at 25° C is found to be: 7.23 mole per liter per second.

Some experiments have been made to investigate the reaction with other mercuric salts and with mercurous nitrate.

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The Degradation of Pyrimidines for Tracer Work

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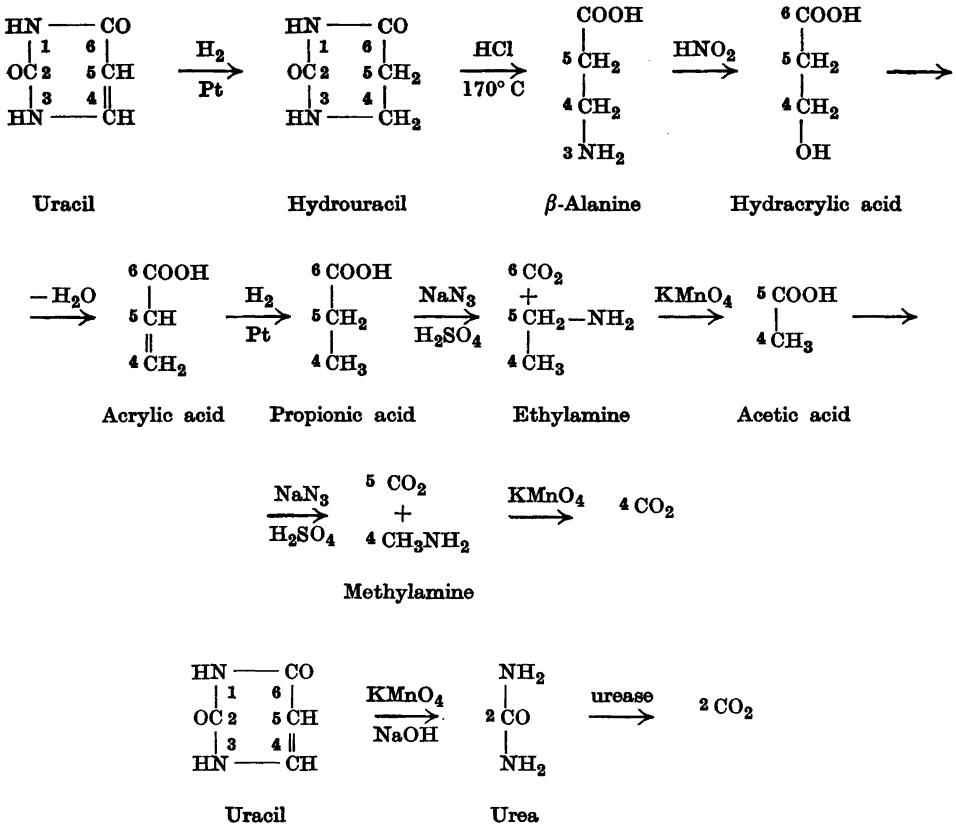
During the past few years much work has been done on the metabolism of the pyrimidines. In order to secure full understanding of these problems it is necessary to be able to determine the distribution of isotope within the pyrimidine ring. In a preliminary report [1] a degradation of uracil was outlined in which oxalic acid and urea were obtained after oxidation with potassium permanganate. Following the work of Offe [2] oxalic acid was thought to be representative of carbon atoms 5 and 6. A similar degradation was published at the same time by Heinrich *et al.*[3] However, by degrading uracil labeled in position 5 with C^{13} and in position 6 with C^{14} it could be shown that oxalic acid was also formed to a certain extent from carbon atoms 4 and 5. A new degradation method for uracil has therefore been worked out.

The main steps in this degradation are as follows: After hydrogenation of uracil to hydrouracil according to Brown *et al.*[4] and subsequent hydrolysis with hydrochloric acid, nitrogen atom 3 and carbon atoms 4, 5 and 6 where obtained as β-alanine [5]. Deamination of this compound led to hydracrylic acid and dehydration to acrylic acid [6], which upon subsequent hydrogenation yielded propionic acid. This substance was degraded with two Schmidt decarboxylations according to Phares [7].

Carbon atom 2 was obtained in essentially the same way as described before [1]. After oxidation with potassium permanganate and hydrolysis with alkali carbon dioxide was liberated from the resulting urea with urease.

This degradation procedure makes it possible to determine the isotope concentration in every carbon atom directly. It has been tested using labeled uracil and was found to give results in good agreement with the theoretical values.



EXPERIMENTAL

Degradation procedure. Uracil (0.8—1 millimole) was dissolved with warming in 10 ml of a freshly prepared 0.5 % solution of gum arabic. To this was added 0.2 ml of a 10 % solution of chloroplatinic acid, and the reaction vessel was freed of air and filled with hydrogen to a pressure of 2.5 atmospheres. The sample was hydrogenated with shaking at 75° C for 24 hours. By that time the light absorbtion had completely disappeared, indicating that the hydrogenation was complete.

Hydrolysis of hydrouracil. After the hydrogenation the solution was evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml concentrated hydrochloric acid and transferred to a bombtube. The tube was sealed and heated at 170° C for 24 hours. The tube was opened and the contents were centrifuged and the precipitate washed twice with a few ml of water. The supernatant plus washings was evaporated to dryness *in vacuo*.

Chromatography of the hydrolysate. The residue from the evaporation was taken up in 3 ml + 1 ml + 1 ml of 2 *N* HCl and applied to the top of a Dowex 50 column (200 mm × 10 mm). The column was eluted with 2 *N* HCl and the position of the peak determined with ninhydrin according to Moore and Stein [8]. Under these conditions β-alanine and

ammonia were obtained together in a single peak that appeared after an elution volume of about 20 ml. An identical chromatogram was obtained with a sample of authentical β-alanine. The β-alanine from the hydrolysate was further identified by its chromatographic behaviour on starch using 2 : 1 propanol — 0.5 *N* HCl as eluant. Finally, a sample of the β-alanine from the hydrolysate was mixed with known β-alanine and subjected to chromatography on starch in the same way. A single apparently homogenous peak was obtained. The yield of β-alanine after chromatography on both Dowex 50 and starch was about 60 % calculated from the nitrogen value after Kjeldahl combustion. The yield after chromatography on Dowex 50 only, calculated in the same way after removal of the ammonia by steam distillation, was about 80 % indicating that the β-alanine at this stage of purification was contaminated with a nitrogen-containing substance other than ammonia. This contamination, however, did not affect the accuracy of the degradation, as can be seen in the experiment with labeled uracil.

The transformation of β-alanine to acrylic acid. The chromatographic fractions containing β-alanine were pooled and repeatedly evaporated to dryness *in vacuo* to remove HCl as completely as possible. The residue was dissolved in 5 ml of water, neutralized and 2 g of sodium nitrite were added. The flask was equipped with a dropping funnel and an outlet tube connected with a mercury valve. The air was replaced with carbon dioxide and 25 ml of 2 *N* H_2SO_4 introduced through the funnel. After 3 hours 800 mg urea were added to destroy the remaining sodium nitrite. The test for nitrite with potassium iodide-starch was negative after about one hour. The solution was transferred to a 500 ml double necked, round-bottom flask and diluted to 75 ml. An equal volume of concentrated H_2SO_4 was added with cooling in ice water. The flask was connected to a condenser and equipped with a dropping funnel. Distillation over a free flame was maintained until 500 ml of distillate had been collected. During the distillation water was dropped into the solution to keep the volume constant. The operation took at least two hours.

Hydrogenation of acrylic acid to propionic acid. Two and one-half grams of gum arabic were dissolved in the distillate and 1 ml of a 10 % solution of chloroplatinic acid added. A slow stream of hydrogen was passed through the solution for 3 hours during which time it was refluxed on a boiling water bath. After neutralization the volume was reduced to about 50 ml *in vacuo*. After acidification with an excess of 5 *N* H_2SO_4, a little silver sulfate was added and the propionic acid distilled over with steam. The distillate was neutralized and evaporated to dryness. It was acidified with a slight excess of 10 *M* H_2SO_4 and extracted with 5 ml + 2 ml + 2 ml chloroform. The extract was applied to the top of a Celite column (200 mm × 20 mm), and the column was eluted first with 100 ml of chloroform saturated with 0.5 *N* H_2SO_4 and then with chloroform — 1 % butanol saturated with 0.5 *N* H_2SO_4 as described by Mosbach *et al.*[9] The yield of propionic acid was 30 % calculated on the basis of uracil.

The degradation of propionic acid. Phares' method[7] with two Schmidt decarboxylations was used. Starting with 0.9 millimole uracil at least 0.1 millimole $BaCO_3$ for isotope analysis was obtained in the final step.

The degradation of uracil to urea. Uracil (0.1—0.15 millimole) was dissolved in 3 ml water with warming. The solution was cooled to roomtemperature, and a 5 % solution of $KMnO_4$ was added dropwise until the permanganate colour persisted slightly. A drop of 0.1 *N* H_2SO_4 was added now and then to facilitate the precipitation of MnO_2. The excess $KMnO_4$ was destroyed with hydrogen peroxide and the resulting precipitate centrifuged down and washed twice with a few ml of water. One ml of 5 *N* NaOH was added to the combined supernatant and washings, and the sample was hydrolyzed for 15 minutes on

the boiling water bath. The solution was neutralized with 5 *N* H_2SO_4 with phenol red as indicator and 0.5 ml H_2SO_4 added in excess. After a rapid aeration for 5 minutes the solution was neutralized with carbonate-free 5 *N* NaOH until it became faintly red. A small amount of urease, in solution, was added, and the mixture allowed to stand for 30 minutes at 42° C. It was then acidified with 5 *N* H_2SO_4 and aerated for half an hour with warming to 100° C. The CO_2 was precipitated as $BaCO_3$. Yield 75 % calculated on the basis of uracil.

The degradation of labeled uracil. Uracil labeled in position 5 with C^{13} and in position 6 with C^{14} was synthesized from aspartic acid labeled in the β-carbon atom with C^{13} and in the β-carboxyl group with C^{14} *. The aspartic acid was deaminated to malic acid with an excess of potassium nitrite in glacial acetic acid. The resulting solution was acidified with concentrated H_2SO_4, the precipitate of potassium sulfate filtered off and the filtrate extracted with ether. The acetic acid was removed by distillation *in vacuo* and the remaining syrup used directly for the synthesis of uracil according to Davidson *et al.*[10]. Yield 25 %, calculated on the basis of aspartic acid.

Labeled uracil (0.9 millimole) was degraded as described above. The result is given in Table 1.

Table 1. Comparison of the theoretical values for C^{13} and C^{14} in labeled uracil with actual values found by degradation. The theoretical values were calculated from the C^{13}- and C^{14}-values of the total uracil molecule. They agreed quite satisfactorily with the values for the aspartic acid used in the synthesis of the uracil. The C^{14}-values are reported as counts/min. at infinite thickness. All samples were counted as $BaCO_3$.

Carbon atoms	Theoretical values		Values obtained from degradation	
	C^{14} counts/min.	C^{13} % excess	C^{14} counts/min.	C^{13} % excess
C_4	—	—	0	0.00
C_5	—	0.17	0	0.16
C_6	600	—	604	0.00

The degradation outlined in a preliminary report [1], in which carbon atoms 5 and 6 were supposed to be obtained as oxalic acid, was also tested by degrading uracil labeled as described above. The $BaCO_3$ obtained from oxalic acid was analysed for C^{13} and C^{14}. The value for C^{13} was 0.083 % excess, compared to the theoretical value 0.085 %. The value for C^{14}, however, was 210 counts/min. compared to the theoretical value 300 counts/min. Since the uracil was labeled in position 5 with C^{13} and in position 6 with C^{14}, it appears that oxalic acid was formed not only from carbon atoms 5 and 6 but also to an appreciable extent (30 %) from carbon atoms 4 and 5. This degradation is, therefore, quite unsatisfactory for use in tracer studies.

* I am greatly indebted to Dr. Gösta Ehrensvärd for his generous gift of the labeled aspartic acid.

SUMMARY

A degradation of uracil for tracer work has been described. Carbon atoms 4, 5 and 6 were split off as β-alanine, which after conversion to propionic acid, was degraded by two Schmidt decarboxylations according to Phares. Carbon atom 2 was obtained as urea.

Using this method every carbon atom in the ring can be analyzed directly for its isotope content. The degradation has been tested by degrading labeled uracil; the values obtained were found to agree quite satisfactorily with the theoretical ones.

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Pyrazole Studies

V. The Oxidation by Air of Some 3,4-Dialkylsubstituted 1-Phenylpyrazolones-5

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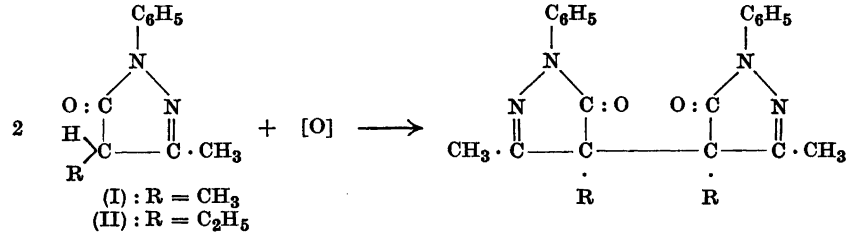
In a previous paper [1] the potentiometrical titration of 5-pyrazolones with bases was described and it was mentioned that even when monosubstituted in position 4 the pyrazolones are sufficiently acid to give off a proton during the addition of sodium or barium hydroxide to their ethanolic solution.

It was found, however, that if the ethanolic solution of a 4-monosubstituted pyrazolone had been kept for some days before titrating it, it would not consume the calculated amount of base, and if the solution was not too diluted it would, in the course of 8—14 days, deposit crystals of a new substance, less soluble in ethanol that the pyrazolone and absolutely neutral to sodium hydroxide. We have now examined more closely the behaviour of 1-phenyl-3-methyl-4-methyl- (or ethyl-)pyrazolone-5 by isolating and analysing the new substances.

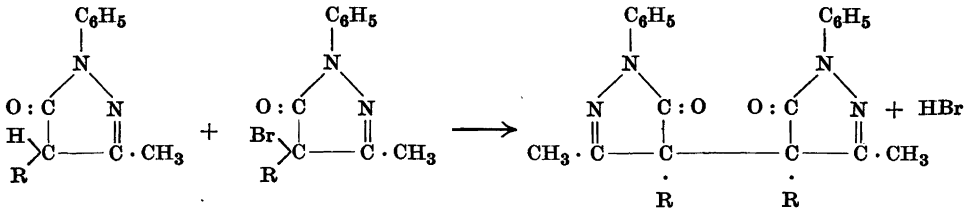
First we thought them to be isomers of the ordinary pyrazolones as the nitrogen content of the new substances are, within the limit of the experiment, identical with the nitrogen content of the pyrazolones. A closer examination proved, however, that the neutral substances are oxidation-products of the pyrazolones, 4,4′-bis-pyrazolones. The oxidation is caused by the atmospheric oxygen and the reaction is strongly catalysed by cupric ions (*cf.* Smith [2]). If the ethanolic solutions are kept in completely filled flasks no oxidation takes place, not even if a trace of cupric sulfate is added to the solution.

The overall-reaction seems to be:



(I) : R = CH_3
(II) : R = C_2H_5

The structure of the neutral substances are proved by their preparation from 4-alkylsubstituted pyrazolone and 4-alkyl-4-bromopyrazolone (Westöö [3])



and by the fact that the ultraviolet absorption spectra of the substances prepared in this way are identical with the spectra of the oxidation products isolated.

An oxidation takes place not only in solution but in the solid state too. Preparations of 1-phenyl-3,4-dimethyl-pyrazolone-5 or 1-phenyl-3-methyl-4-ethyl-pyrazolone-5, kept in cork-stoppered flasks for some months were found to have been partially oxidised, and when heated for 3—4 days to 100° oxidation also takes place. Treated in the last mentioned way two different oxidation products of the pyrazolones may be isolated. By recrystallisation of the heated pyrazolone from ethanol the bis-pyrazolone may easily be isolated as a slightly soluble substance. M.p. for the two pyrazolones mentioned 165° and 161—162° respectively. When the mother liquors from the bis-pyrazolones are slowly concentrated at room temperature two other substances with m.p. 113° and 117° respectively may be isolated. These substances, too, are neutral to sodium hydroxide. By elementary analysis we found that they contain one atom of oxygen more than the pyrazolones, and it may be shown that this oxygen is contained in a hydroxyl group, as the substances with *m*-nitrobenzazide form *m*-nitrophenylcarbamic esters (*cf.* Veibel and Lillelund [4]). At room temperature only the lower melting oxidation products seem to be formed.

As for the position of the hydroxyl group the only possibility seems to be at C_4. The low-melting neutral substances are therefore considered to be 1-phenyl-3-methyl-4-methyl-(or ethyl-)4-hydroxy-pyrazolone-5. In agreement with this assumption the substance formed by oxidation of (I) is transformed into 1-phenyl-3,4-dimethyl-4-chloro-pyrazolone-5 by treatment with phosphorous trichloride.

A comparison of the ultraviolet absorption spectra of the hydroxy-compounds and of the corresponding 4-chloro- and 4-bromo-substituted pyrazolones (these spectra have been examined by Westöö [5]) corroborates the assumption of the hydroxy compounds being 4-hydroxypyrazolones.

EXPERIMENTAL PART

(I) and (II) were prepared as indicated by Knorr and Blank [6] and showed the physical properties indicated in the literature.

1. Oxidation by air of the pyrazolones in ethanolic solution. A solution of (II) in ethanol was placed in a thermostat at 30°. The flask was stoppered with a cork-stopper. At intervals samples to 50 ml were titrated with barium hydroxide.

In a parallel experiment smaller flasks with ground glass-stoppers were completely filled with the solution, placed in the thermostat and a new flask opened for each sample. In this series, the air had no access to the solution (Table 1).

Table 1.

Sample taken after hours	a) Access of air ml $Ba(OH)_2$	b) No access of air ml $Ba(OH)_2$
0	7.60	—
168	6.50	6.90
336	5.60	6.75
480	4.80	6.75
672	4.00	6.75

The experiment proves that the oxidation is caused by the air, not by *e. g.* acetaldehyde or other impurities in the ethanol.

2. Catalytic action of cupric ions on the oxidation of the pyrazolones. The following solutions were prepared. At intervals samples to 50 ml were titrated with barium hydroxide (Table 2).

c) 20 ml water, filled up to 250 ml with a 0.033 *M* ethanolic solution of (II).
d) 20 ml 1.176 *N* hydrochloric acid, filled up to 250 ml with the same solution as in c.
e) 20 ml 0.01 % $CuSO_4$, $5H_2O$ in water, filled up to 250 ml with the same solution as in c.
f) 20 ml 0.1 % $CuSO_4$, $5H_2O$ in water, pyrazolone as in c—e.

These solutions are seen to be about 0.03 *M* with regard to pyrazolone.

Table 2.

Sample taken after hours	50 ml solution consume ml $Ba(OH)_2$, 0.2144 *N*			
	c	d	e	f
0	7.17	7.17	7.17	7.17
120	6.65	6.50	4.2	1.8
192	—	—	1.5	—
336	5.10	5.35	—	—

The amount of barium hydroxide used by hydrochloric acid subtracted.

The catalysis caused by cupric ions is seen to be strong. It increases with the concentration of cupric ions. Hydrogen ions up to a concentration about 0.1 *N* are seen to have only a slight decreasing effect upon the oxidation of the pyrazolone, but if the concentration of hydrogen ions is increased to 1.6 *N* the oxidation is completely suppressed, both in the absence and in the presence of cupric ions (Table 3). The amount of barium hydroxide used by the sulfuric acid subtracted.

Table 3. g) 100 ml 4 N sulfuric acid, 5 ml 0.1 % $CuSO_4$, $5H_2O$ in water, 0.06 M ethanolic solution of (II) to 250 ml.

Sample taken after hours	ml $Ba(OH)_2$
0	8.55
72	8.35
144	8.33
216	8.63

When the figure for the sample after 216 hours is greater than for the previous samples it is due to the fact that when the sulfuric acid has been neutralised, the oxidation is strongly catalysed by the cupric ions. During the titration air is introduced into the solution by mechanical stirring, and the oxidation is therefore much faster than when air is only accessible by diffusion. The titration of the 3 first samples was carried out slowly, but for the last one, where the consumption of barium hydroxide was known, it was possible to reduce the time used for the titration to a minimum.

That the copper-catalysed oxidation is caused by air is shown in the experiment recorded in Table 4.

Table 4. h) 15 ml 0.1 % $CuSO_4$, $5H_2O$ in water; Ethanolic solution of (II) to 250 ml; 4 Flasks with ground glass-stoppers were filled completely with this solution and placed in the thermostat at 30°. One flask was opened for each titration.

Sample taken after hours	ml $Ba(OH)_2$
0	11.5
48	11.8
96	12.0
144	12.1

The increase in the figures means, as above, that the titration is carried through in a shorter time when the total amount of barium hydroxide necessary has been determined by previous titrations.

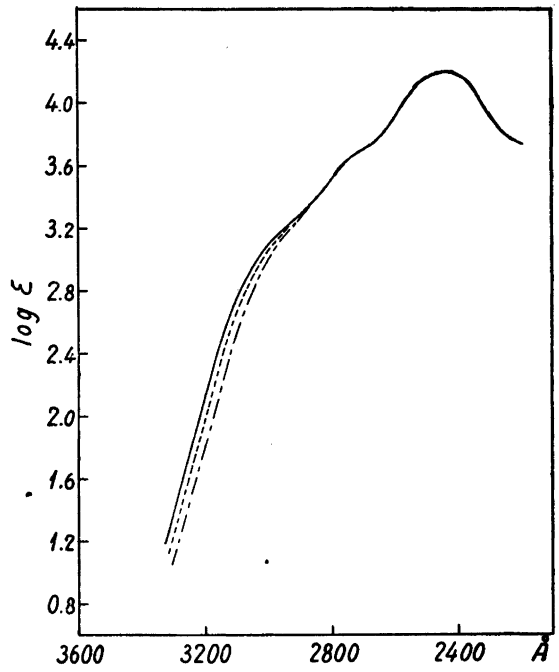


Fig. 1. Ultraviolet absorption spectra of bis-(I) (— - — - — - —), bis-(II) (————), and bis-1-phenyl-3,4-dimethylpyrazolone-5 (- - - - - - - - -).

3. Identification of the substances obtained by the oxidation in ethanolic solution. Not too diluted ethanolic solutions of (I) and (II), kept at room temperature for some time will deposit crystals, from (I) with m.p. 165°, from (II) crystals sintering at 152° and melting with slight decomposition at 161—162°. A substance with m.p. identical with that of the substance deposited from (I) has been prepared by reaction between (I) and brominated (I), bromine entering in position 4 (Westöö [3]), and mixed melting point of the isolated and the synthesised substance does not show depression. From this it may be concluded that the substances deposited from the ethanolic solutions are the bis-pyrazolones. Knorr [7] indicates for the bis-pyrazolones from (I) and (II) m.p. 164° and 160°. The bis-pyrazolone of (II) may possibly exist in dimorphous forms, a phenomenon not unknown in the pyrazolone-group. Elementary analyses agree with the assumption of bis-pyrazolones.

bis-I. $C_{22}H_{22}N_4O_2$ (374.4)	Calc.	C = 70.6 %,	H = 5.9 %		
	Found	C = 70.5 »,	H = 5.9 »,		
bis-II. $C_{24}H_{26}N_4O_2$ (402.5)	Calc.	C = 71.6 »,	H = 6.51 »,	N = 13.92 %	
	Found	C = 71.7 »,	H = 6.45 »,	N = 13.85 »	

The identity of the two substances isolated in the way described with the bis-pyrazolones was further proved by comparison of their ultraviolet absorption spectra, meas-

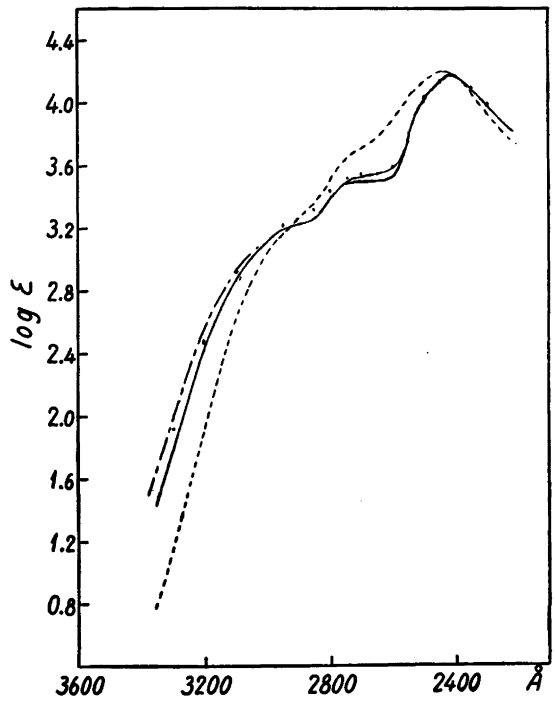


Fig. 2. Ultraviolet absorption spectra of (III), (————), (IV) (— - — - — - —), bis-1-phenyl-3,4-dimethylpyrazolone-5 (- - - - - - - - -), and some determinations of the absorption of (III) in acid ethanolic solution (· · · ·)

ured with a Beckman-U.V.-spectrograph, with the absorption spectrum of a sample of bis-(I), prepared and measured by Westöö [3]. The absorption curves are given in Fig. 1.

No doubt the oxidation in ethanolic solution leads to the formation of the bis-pyrazolone. As mentioned above the reaction is catalysed by cupric ions. Further investigation of the cupric-ion catalysed reaction has shown that also the substances (III) and (IV) mentioned below may be formed.

4. Identification of the substances obtained by keeping the pyrazolones at 100° for some days. (I) and (II) are both practically insoluble in ether, but when they have been kept for some months at room temperature or for a couple of days at 100° they have been partially transformed into ether-soluble substances. The hydrobromide of (I), on the other hand, may be kept at least for a year at room temperature without transformation. Parallel to the transformation the amount of sodium or barium hydroxide used to neutralise the pyrazolone is reduced. The transformation products are evidently neutral substances in distinction to the pyrazolones. By evaporation the ethereal solution leaves crystals which by fractionated crystallisation from ethanol may be separated into two fractions, one, sparingly soluble in cold ethanol, being the above mentioned bis-pyrazolone, the other, easily soluble in both ethanol and ether, being neutral as the bis-pyrazolone but containing per pyrazolone-nucleus one atom of oxygen more than the bis-

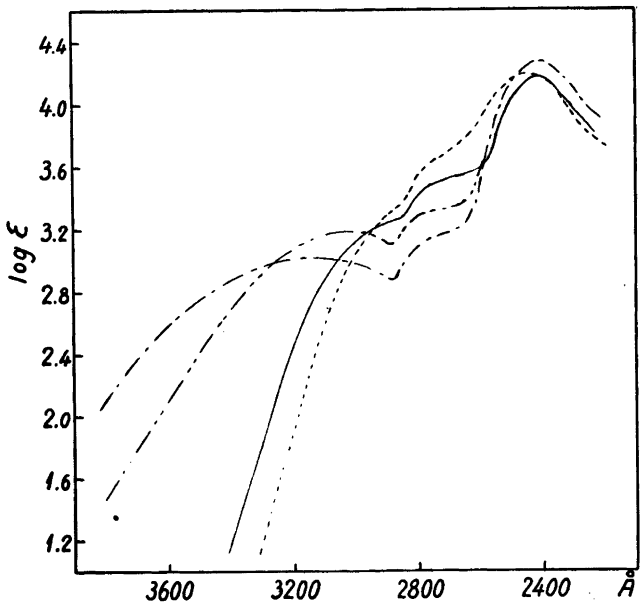


Fig. 3. Ultraviolet absorption spectra of 1-phenyl-3,4-dimethyl-4-bromo-pyrazolone-5 (— · — · — · —), 1-phenyl-3,4-dimethyl-4-chloro-pyrazolone-5 (— — — — —), 1-phenyl-3,4-dimethyl-4-hydroxy-pyrazolone-5 (————), and bis-1-phenyl-3,4-dimethyl-pyrazolone-5 (· · · · · · · · ·).

pyrazolone. M. p. of the substance from (I) 113°, from (II) 117°. Elementary analyses give the formulas $C_{11}H_{12}O_2N_2$ (III) and $C_{12}H_{14}O_2N_2$ (IV).

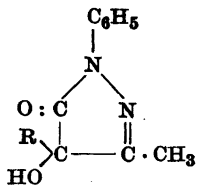
III.	$C_{11}H_{12}O_2N_2$ (204.2)	Calc.	C = 64.68 %,	H = 5.92 %,	N = 13.72 %
		Found	C = 64.9 »	H = 5.95 »	N = 13.88 »
IV.	$C_{12}H_{14}O_2N_2$ (218.2)	Calc.	C = 66.04 »	H = 6.47 »	N = 12.84 »
		Found	C = 65.8 »	H = 6.38 »	N = 12.91 »

The extra oxygen atom is contained in a hydroxyl group. By treatment with PCl_3 (III) was transformed into 1-phenyl-3,4-dimethyl-4-chloro-pyrazolone-5 (V), identified through analysis, m.p. (68°) and mixed m.p. with an authentical sample of V which showed no depression.

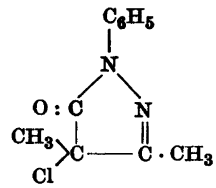
V.	$C_{11}H_{11}O\,N_2Cl$ (222.7)	Calc.	C = 59.3 %,	H = 5.0 %
		Found	C = 59.5 »	H = 5.2 »

A further proof of the presence of a hydroxyl group was obtained by reaction of (IV) with *m*-nitrobenzazide. Hereby a substance (VI) was isolated, the elementary analysis of which corresponds to a *m*-nitrophenylcarbamic ester of (IV):

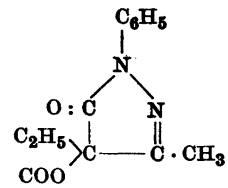
VI.	$C_{19}H_{18}O_5N_4$ (382.4), m.p. 139°	Calc.	C = 59.6 %,	H = 4.7 %,	N = 14.7 %
		Found	C = 59.7 »	H = 4.5 »	N = 14.9 »



III : R = CH_3
IV : R = C_2H_5



V



VI

The only other possible position of an oxygen atom seems to be at *N* in position 2, transforming (I) and (II) into amine oxides or into N-hydroxy compounds. Such compounds will hardly be of neutral character, and the absorption curves, shown in Fig. 2 and Fig. 3, corroborate the formulas (III) and (IV). In Fig. 2 is shown that the absorption of (III) is the same in acid as in neutral solution. This behaviour is shown by pyrazolones with two substituents at C_4, *e. g.* 4-alkyl-4-halogeno-5-pyrazolone (Westöö [5]), whereas antipyrine, with a substituent at N_2, shows another absorption spectrum in acid than in neutral solution (Valyashko and Bliznyukov [8]).

In Fig. 3 the absorption spectra of the hydroxy-compounds are compared with the absorption spectra of 4-alkyl-4-halogeno-5-pyrazolones. For all compounds an absorption band with maximum $\sim$ 2 400 Å is found. For the halogeno-compounds the absorption in the region 2 500 Å $-$2 900 Å is diminished, most pronounced for the bromo-compound, and a broad secondary maximum about 2 900 Å$-$3 300 Å may be seen. Our substances (III) and (IV) now fit well into this series, in accordance with the slightly electronegative character of OH as compared with Cl and Br. From the broad absorption band about 2 900 Å$-$3 300 Å only a shoulder at 2 900 Å remains, which in the bis-pyrazolone is further reduced to an inflexion point. The decrease in absorption in the region 2 500 Å$-$2 900 Å is only 1/3 of that of the bromo-compound, 1/2 of that of the chloro-compound. For further discussion of the spectra see Westöö [5].

Knowing that substances of the general formula (V) with I or Br instead of Cl are able to couple with (I) and (II) with formation of the bis-pyrazolone (*cf.* Smith [2]) it would be natural to postulate that the formation of the hydroxy-compounds (III) and (IV) are intermediate steps in the formation of the bis-pyrazolones. This assumption has, however, to be abandoned, as solutions of (I) and (III) or (II) and (IV) in ethanol may be kept for a long time without formation of bis-pyrazolones if the access of air is prohibited, whereas the formation of bis-pyrazolones starts as soon as air is admitted. This, too, fits well into the series of the halogeno-compounds as the velocity of coupling diminishes in the order I > Br > Cl, the difference in velocity between the bromo- and the chloro-compound being considerable. We, therefore, have to admit two reactions, proceeding simultaneously, one leading to the formation of bis-pyrazolones, the other to the formation of the hydroxy-compound. Both reactions take place in the solid phase as well as in solution, and at all events in solution both reactions are catalysed by cupric ions, the first mentioned very strongly. The oxydation is inhibited by hydrogen ions.

SUMMARY

Solutions of 3,4-dialkylsubstituted 5-pyrazolones in ethanol were found to be oxidised by air with formation of two different oxidation products, one being the corresponding 3,4-dialkylsubstituted bis-pyrazolones, the other

being the 3,4-dialkyl-4-hydroxypyrazolones. The formation of the bis-pyrazolones is strongly catalysed by cupric ions, the catalysis being inhibited by hydrogen ions. Also the formation of the hydroxy-compounds is catalysed by cupric ions.

In the solid phase the oxidation proceeds more slowly than in solution. The influence of cupric ions upon the velocity of oxidation in the solid state has not been investigated.

The ultraviolet absorption spectra of the bis-pyrazolones and the hydroxy-pyrazolones have been determined and compared with the absorption spectra of an authentical sample of the bis-pyrazolone and of 4-bromo- and 4-chloro-substituted 3,4-dialkylpyrazolones.

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Bifunctional Amines and Ammonium Compounds
I. Preparation of β-Tertiary-Aminoesters and Ammonium Halides of Dibasic Acids

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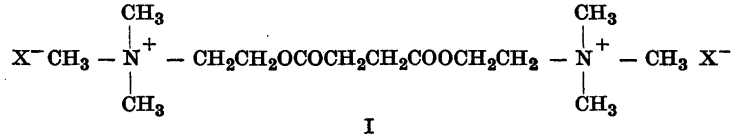
The discovery of synthetic curarizing substances with a potency comparable to or higher than that of *d*-tubocurarine [1–3] has created a great deal of interest in bifunctional 'onium' compounds [4–6]. One of the practical results of this interest has been the introduction into therapy of the bis-methohalides of bis-(β-dimethylamino)ethyl succinate (I) ('succinylcholine' salts) as short acting muscular relaxants for use as adjuvants in anaesthesia [7].

This paper describes a novel method for the preparation of succinylcholine and related substances together with their precursors, the bis-tertiary-amino-esters. The method has proved generally applicable to acids capable of forming cyclic anhydrides, and further makes possible the preparation of 'hybrid' esters, *i.e.* esters containing different groups in the two ends of the molecule.



I

A study of the biological properties of such 'hybrid' compounds should add to our understanding of the pharmacodynamics of agents of this general type.

Another feature of the method is that it enables us the preparation of half-aminoesters and their corresponding ammonium compounds, reported metabolic breakdown products of the bis-quaternary esters [8].

After this work was already completed Rice and coworkers [9] have reported that certain half-aminoesters of dibasic acids have hypotensive properties,

and that one bis-tertiary ester, bis-(β-diethylamino)ethyl cis-Δ^4-tetrahydrophthalate, also was capable of lowering the blood pressure of normotensive dogs.

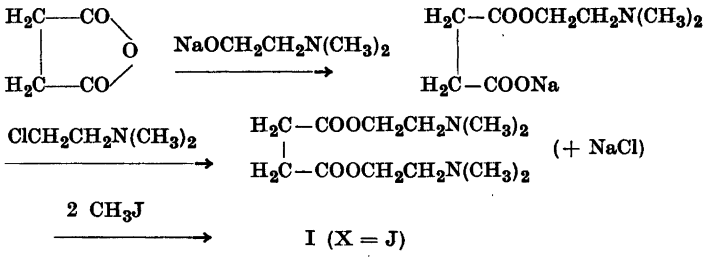
Succinylcholine, which was first reported by Hunt and Taveau [10] in 1911 (no experimental details), and its homologues, has hitherto been prepared by four different routes.

Le Heux [11] prepared it by reacting the chloride of choline with succinoyl chloride. Fusco and coworkers [4] esterified *tert.*-aminoalcohols with succinoyl chloride, isolated the hydrochloride of the bis-ester and quaternized the free ester by heating it in sealed tubes with the proper alkyl halide, while Phillips [12] reported poor to moderate yields of the bis-tertiary aminoester by an ester interchange method. Finally Walker [6] esterified succinic acid with ethylene bromohydrin and subsequently reacted the bis-β-bromoester with trimethylamine.

Phthalylcholine has been reported by Hunt and Taveau [10] while Pyman [13] prepared the corresponding bis-tertiary ester by reaction of phthaloyl chloride with β-diethylaminoethanol.

The half-esters reported by Rice and coworkers [9] were prepared by reacting one mole of β-diethylaminoethanol with one mole of a cyclic anhydride. One of these half-esters was in turn transformed to an acid chloride with oxalyl chloride and then allowed to react with β-diethylaminoethanol to give the desired bis-ester of tetrahydrophthalic acid.

The principle of the method used in the present work will appear from the following diagram (using the preparation of succinylcholine iodide as an example).



The reaction is carried out in an inert solvent such as toluene. The second stage proceeds without isolation of the sodium salt except when a half-ester is desired. In this case the second stage is not carried out. The bis-ester is recovered by distillation and subsequently transformed into the salt of a quaternary ammonium hydroxide by treating it with a reactive alkyl halide.

It has also been found that in production the isolation of the bis-ester is often unnecessary, for instance the bis-methoiodide prepared from the crude

Table 1.

$(R_1)_2NCH_2CH_2OCO(A)COOCH_2CH_2N(R_2)_2$

(A)	R_1	R_2	B.p. °C	Press. mm	Yield %	Empirical formula	Analyses % N calc.	found
succinic	CH_3	CH_3	135—8[a]	1.0—2.0	48	$C_{12}H_{24}O_4N_2$	10.68	10.61
succinic	C_2H_5	C_2H_5	160—5[b]	2.0	62	$C_{16}H_{32}O_4N_2$	8.94	8.96
succinic	CH_3	C_2H_5	147—151[c]	2.0	70	$C_{14}H_{28}O_4N_2$	9.73	9.78
maleïc	CH_3	CH_3	145[d]	1.5	21[e]	$C_{12}H_{22}O_4N_2$	10.85	9.80
phthalic	CH_3	CH_3	178[f]	1.5—2.0	42	$C_{16}H_{24}O_4N_2$	9.19	9.04
phthalic	C_2H_5	C_2H_5	195—200[g]	1.8—2.2	58	$C_{20}H_{32}O_4N_2$	7.70	7.65
d-camphoric	CH_3	CH_3	176—180[h]	2.0—2.2	53	$C_{18}H_{34}O_4N_2$	8.19	7.97
d-camphoric	C_2H_5	C_2H_5	192—8[i]	1.5—2.2	58	$C_{22}H_{42}O_4N_2$	7.04	6.87

[a] Literature[4] 131° C/1—2 mm Hg. [b] Literature[4] 152° C/1—2 mm Hg; picrate, m.p. 128° C (from abs. ethanol). [c] Picrate, m.p. 107° (recryst. lowers m.p.). [d] Picrate, m.p. 164—8° (from abs. ethanol). [e] A considerable amount of insoluble polymer is formed in this condensation. After the distillation a colorless precipitate separates from the oil. This precipitate melts at 95—8° and contains only traces of nitrogen. [f] Picrate, m.p. 158—61° (recryst. lowers m.p.). [g] Picrate, m.p. 145—8° (recryst. lowers m.p.); literature[12] 146—7°. [h] Picrate, m.p. 227—9° (from abs. ethanol). [i] Picrate, m.p. 163—5° (from abs. ethanol).

Table 2.

$X^-R_3(R_1)_2\overset{+}{N}CH_2CH_2OCO(A)COOCH_2CH_2\overset{+}{N}(R_2)_2R_3X^-$

(A)	R_1	R_3	R_2	X	M.p. °C	Recryst. from[a]	Yield %[b]	Empirical formula	Analyses % X calc.	found.
succinic	CH_3	CH_3	CH_3	J	255—6[c]	A—W(20:1)	76	$C_{14}H_{30}O_4N_2J_2$	46.60	46.61
—	CH_3	CH_3	C_2H_5	Br	148	A—E(2:5)	90	$C_{16}H_{34}O_4N_2Br_2$	33.44	32.65
—	C_2H_5	C_2H_5	CH_3	J	142—4[d]	A	73	$C_{18}H_{38}O_4N_2J_2$	42.31	42.22
—	C_2H_5	C_2H_5	C_2H_5	Br	185—7	A[e]	64	$C_{20}H_{42}O_4N_2Br_2$	29.90	29.60
—	CH_3	C_2H_5	CH_3	J	180—3	M—Ac(3:2)	83	$C_{16}H_{24}O_4N_2J_2$	44.38	44.36
—	CH_3	C_2H_5	C_2H_5	Br	148	M[e]	79	$C_{18}H_{38}O_4N_2Br_2$	31.57	31.14
maleïc	CH_3	CH_3	CH_3	J	264	M—W(5:1)	57	$C_{14}H_{28}O_4N_2J_2$	46.75	46.25
—	CH_3	CH_3	C_2H_5	Br	206	A	36	$C_{16}H_{32}O_4N_2Br_2$	33.57	33.15
phthalic	CH_3	CH_3	CH_3	J	128	A—M(1:1)	71	$C_{18}H_{30}O_4N_2J_2$	42.85	41.72
—	CH_3	CH_3	C_2H_5	Br	144—7	A	86	$C_{20}H_{34}O_4N_2Br_2$	30.35	29.21
—	C_2H_5	C_2H_5	CH_3	J	177—9	A	72	$C_{22}H_{38}O_4N_2J_2$	39.15	38.85
—	C_2H_5	C_2H_5	C_2H_5	Br	189		60	$C_{24}H_{42}O_4N_2Br_2$	27.44	27.38
d-camphoric	CH_3	CH_3	CH_3	J	229	A—M(2:1)	76	$C_{20}H_{40}O_4N_2J_2$	40.55	40.76
—	CH_3	CH_3	C_2H_5	Br	220	Ac[f]	89	$C_{22}H_{44}O_4N_2Br_2$	28.54	28.36
—	C_2H_5	C_2H_5	CH_3	J	205	A	84	$C_{24}H_{46}O_4N_2J_2$	37.19	36.81
—	C_2H_5	C_2H_5	C_2H_5	Br	205	A[e]	65	$C_{26}H_{52}O_4N_2Br_2$	25.92	25.87

[a] A : ethanol; E : ether; Ac : acetone; M : methanol; W : water [b] after recrystallization [c] with decomposition. Literature[4] 237° C. [d] Literature[4] 142° C. [e] precipitated by addition of ether. [f] Purified by suspension in several portions of boiling acetone.

solution of bis-(β-dimethylamino)-ethyl succinate being generally of 95 per cent purity or better.

In this study the following anhydrides have been subjected to bis-esterification: Succinic, maleïc, phthalic and *d*-camphoric. The alkoxides were prepared from β-dimethylaminoethanol and β-diethylaminoethanol. The halides were β-dimethylaminoethyl chloride and β-diethylaminoethyl chloride. The bis-esters were transformed into the ethobromides and the methoiodides. The esters are summarized in Table 1 and the corresponding quaternary compounds in Table 2. In addition a half-ester of phthalic acid was prepared.

The pharmacological properties of the compounds described in this paper are being studied by Dr. J. G. A. Pedersen of this laboratory. Dr. Pedersen will report his results elsewhere.

EXPERIMENTAL *

Bis-(β-dialkylamino)ethyl esters. The compounds listed in Table 1 were prepared by essentially the procedure described for *bis-(β-dimethylamino)ethyl succinate.* To a solution of 217 g of freshly distilled β-dimethylaminoethanol (2.44 mole) in 1 830 ml of dry toluene in a 10 l three necked flask equipped with stirrer, reflux condenser, drying tube, and dropping funnel is added 56.1 g of sodium (2.44 atom) at once. After the spontaneous reaction has subsided the mixture is refluxed for 12 hours and cooled to room temperature. At this point 244 g of succinanhydride (2.44 mole) is added in small portions while stirring. The reaction is exothermic. When addition is complete the mixture is left standing overnight and later refluxed for 2 hours. In the meantime 422 g of β-dimethylaminoethylchloride hydrochloride (2.44 mole plus an excess of 20 %) moistened with 300 ml of toluene is treated with a solution of 146 g of sodium hydroxide dissolved in 1 250 ml of water while kept under the water tap. The two layers are separated (if there is emulsion formation addition of potassium carbonate will hasten separation into two layers) the aqueous layer is extracted with two extra portions of 300 ml each of toluene. The toluene extracts of the free amine are united and dried over potassium carbonate (100 g). The dry extract is added to the supension of the half-ester sodium salt in toluene from the dropping funnel while the mixture is stirred and kept gently refluxing. The precipitate soon changes appearance (sodium chloride). When addition is finished the reaction mixture is refluxed for further 4 hours.

Sodium chloride is removed by filtration, toluene by evaporation through a 60 cm Widmer column, and the residue distilled in vacuum. After a small amount of lower-boiling material the main fraction, consisting of bis-(β-dimethylamino)ethyl succinate distils at 135—142° C at 1 to 2 mm Hg. Redistillation gives 325 g (48 % yield) of a light yellow oil which turns dark upon standing, b. p. 135—139° C at 1 mm Hg.

The bis-methoiodides of the bis-(β-dialkylamino)ethyl esters listed in Table 2 were prepared essentially according to the method used for the preparation of *bis-methoiodide*

* All boiling points and melting points are uncorrected. Analyses are macro determinations by Mrs. E. Ifversen and Mrs. G. Speggers of this laboratory.

of bis-(-dimethylamino)ethyl succinate (I, X = J) (succinylcholine iodide). In a three-necked flask equipped with stirrer, reflux condenser, and dropping funnel 200 g of bis-(β-dimethylamino)ethyl succinate (0.77 mole) is dissolved in 1 500 ml of dry acetone. The flask is placed in a crushed ice bath and during stirring 144 ml (2.31 mole) of methyl iodide (freshly distilled) is added from the dropping funnel during one hour. The mixture is then allowed to stand at room temperature overnight. The precipitate is filtered and dried. Recrystallized from 2 000 ml of ethanol and 100 ml of water it melts at 256°. Yield 318 g (76 %).

The bis-ethobromides of bis-(β-dialkylamino)ethyl esters listed in Table 2 were prepared essentially as described for the preparation of *bis-ethobromide of bis-(β-dimethylamino) ethyl succinate.* Fifty-two grams of bis-(β-dimethylamino)ethyl succinate (0.2 mole) is dissolved in 500 ml of dry acetone, 55 g of ethyl bromide added, the mixture placed in a steel pressure-tank of 750 ml volume and heated at the steam bath for 36 hours. The tank is cooled and the contents removed (sometimes it is necessary to dissolve the crystalline cake out with dilute ethanol and later recover the products from this solution). Recrystallized from ethanol-ether (2 : 5) there is obtained 86.0 g (90 %) of white hygroscopic plates, m. p. 148°.

For the preparation of smaller portions it is more convenient to use sealed glass tubes for the preparation of ethobromides.

Diethylaminoethyl phthalate. This compound was prepared from 23.4 g of diethylaminoethanol (0.2 mole), 4.6 g (0.2 atom) of sodium, and 29.6 g of phthalic anhydride (0.2 mole) in 200 ml of toluene essentially as described earlier with the exception that the mixture is only refluxed for 6 hours after addition of anhydride. The precipitate is then removed by filtration and dried in vacuo. This sodium salt is hygroscopic and has no definite melting point. It was dissolved in ethanol and dry hydrogen chloride was added until 0.2 mole had been absorbed. The precipitated salt was removed by filtration and the solution evaporated to one third of its original volume. Addition of dry ether precipitates diethylaminoethyl phthalate, but only after several days. The precipitate was removed, recrystallized from acetone. Yield 12 g (22.8 %). M. p. 114° C (Rice *et al.*[9] reports a m. p. of 119—20° C).

SUMMARY

A new synthesis of bis-(β-dialkylamino)alkyl esters of dibasic acids is described.

Using this method eight esters have been prepared and transformed into sixteen corresponding methoiodides and ethobromides.

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Bifunctional Amines and Ammonium Compounds
II. Bis-β-dialkylaminoethyl Ethers and their Quaternary Ammonium Halides

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The present paper describes the preparation of bis-β-trialkylammonium-ethyl ether halides (I) by various routes. These compounds were evaluated with respect to their action on the transmission in autonomic ganglia.

$$X^-R_1R_2R_3\overset{+}{N}CH_2CH_2OCH_2CH_2\overset{+}{N}R_1'R_2'R_3'X^-$$

I

The finding that succinylcholine (II), which can be considered as a 'twin'-molecule of acetylcholine linked through the acetyl groups had curarizing properties comparable to these of decamethonium compounds (III)[1,2] has provided a great stimulus to the search for new curarimimetic agents among the 'twin' or 'bolaform' molecules [3,4,5].

$$X^-(CH_3)_3\overset{+}{N}CH_2CH_2OCOCH_2CH_2COOCH_2CH_2\overset{+}{N}(CH_3)X^-$$

II

$$X^-(CH_3)_3\overset{+}{N}CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2\overset{+}{N}(CH_2)_3X^-$$

III

$$X^-(CH_3)_3\overset{+}{N}CH_2CH_2CH_2CH_2CH_2\overset{+}{N}(CH_3)_3X^-$$

IV

This work has shown that, in general, introduction of one or more, 'hetero' atoms in the alkylene chain between the two charged nitrogen atoms does not greatly influence the biological activity of the molecule.

Although the similarity between the action of succinylcholine and decamethonium is doubtless very superficial the same line of reasoning would lead one to believe that, since bis-trimethylammoniumpentane halides (IV) and certain bis-trialkylammoniumhexane halides (hexamethonium halides) are ganglion blocking agents, the bis-trialkylammonium-oxa-pentane halides (I), which can be considered as 'twin' molecules of choline and its homologues should also possess ganglionic blocking activity. Although this view is not easy to reconcile with the observation by Dale [6] that bis-trimethylammoniumethyl ether had weak muscarinic properties (of the same order of magnitude as the activity of choline), it was substantiated through the present work, a prerequisite for appearance of ganglion blocking activity being that one of the methyl substituents on each nitrogen be exchanged for a higher alkyl group *e.g.* ethyl.

This is in line with newer results from this laboratory [7] showing bis-trialkylammonium-thia-pentane halides with at least one ethyl group on each nitrogen to be more potent ganglionic blocking agents than bis-trimethylammonium-thia-pentane halides, which were recently reported by Bergel [8] to possess such activity. A similar relation exists for the bis-trialkylammoniumhexane halides themselves [9] and for the bis-trialkylammonium-aza-pentane halides [10].

The preparation of bis-ammoniumethyl ethers was attempted first by the classical route [11] from bis-β-haloethyl ether and tertiary amine. This method leaves little choice in the combination of N-alkyl groups and it soon became apparent that the method also had other shortcomings. The most serious of these was the necessity of using bis-iodoether if yields of a higher order were required. The use of the more readily accessible β-chloroether, due to the more strenous reaction conditions, consistently resulted in low yield of the desired quaternary compound and a high proportion of by-products, presumably due to ring closure. An exception to this was the preparation of bis-pyridiniumethyl ether chloride, where no alkyl split with subsequent ring closure can take place.

It was later found that bis-ammonium ethers could be prepared conveniently by alkylation of the bis-tertiary amines (V) obtained by a Williamson condensation of a β-dialkylaminoethyl chloride with the sodium alkoxide of a β-dialkylaminoethanol in an inert medium. This method makes possible a much greater variety in the combination of N-alkyls, including 'hybrid' ethers, *i.e.* ethers containing different groupings in the two ends of the molecule.

$$(R_1)_2NCH_2CH_2OCH_2CH_2N(R_2)_2$$

V

Table 1.

$$X^-R_3(R_1)_2\overset{+}{N}CH_2CH_2OCH_2CH_2\overset{+}{N}(R_2)_2R_3X^-$$

Code no.	R_1	R_2	R_3	X	M.p. °C	Recryst. from [a]	Yield, % [b]	Emp. formula	Anal. calc.	%X^- found
As-3553	CH_3	CH_3	CH_3	J	305	—	88	$C_{10}H_{26}ON_2J_2$	57.10	56.85
As-3558	CH_3	CH_3	CH_3	CH_3SO_4 [c, d]	199	A	46	$C_{12}H_{32}O_9N_2S_2$		
As-3554	CH_3	CH_3	C_2H_5	Br	277	—	69	$C_{12}H_{30}ON_2Br_2$	42.30	42.31
As-3501	CH_3	CH_3	n-C_3H_7	Br	189	A-Ac(1:5)	64	$C_{14}H_{34}ON_2Br_2$	39.35	39.12
As-3502	CH_3	CH_3	n-C_4H_9	Br	222	A-Ac(1:5)	79	$C_{16}H_{38}ON_2Br_2$	36.80	36.94
As-3504	CH_3	CH_3	C_3H_5	Br	179	A-Ac(2:1)	41	$C_{14}H_{30}ON_2Br_2$	39.75	39.55
As-3508	CH_3	CH_3	CH_2Ph	Cl [d]	194	A-Ac(1:3)	42	$C_{22}H_{34}ON_2Cl_2$	17.15	17.18
As-3509	CH_3	CH_3	CH_2COOEt	Cl [d]	169	A-Ac(1:5)	78	$C_{16}H_{34}O_3N_2Cl$	17.48	17.34
As-3659	C_2H_5	C_2H_5	CH_3	J	259	—	96	$C_{14}H_{34}ON_2J$	50.75	50.19
As-3689	C_2H_5	C_2H_5	C_2H_5	Br	235	A	41	$C_{16}H_{38}ON_2Br_2$	36.80	37.01
As-4179	C_2H_5	CH_3	CH_3	J	262	M-A(1:1)	58	$C_{12}H_{30}ON_2J_2$	53.75	53.83
As-4178	C_2H_5	CH_3	C_2H_5	Br	261	—	68	$C_{14}H_{34}ON_2Br_2$	39.25	39.18
As-3594	pyridinium			Cl	156	—	62	$C_{14}H_{18}ON_2Cl_2$	23.60	23.04

[a] A:ethanol; Ac:acetone; M:methanol.
[b] after recrystallization.
[c] % N calc. 6.79; % N found 6.84.
[d] hygroscopic.

Thus from the three possible ethers having two ethyl groups or two methyl groups on nitrogen *viz.* bis-β-dimethylaminoethyl ether (V) ($R_1 = R_2 = CH_3$), bis-β-diethylaminoethyl ether (V, $R_1 = R_2 = C_2H_5$), and bis-(β-dimethyl-β'-diethyl)aminoethyl ether (V, $R_1 = CH_3$; $R_2 = C_2H_5$) six different ammonium compounds having only ethyl and methyl substituents on nitrogen together with a number of compounds with other alkyl substituents on nitrogen have been prepared. These ammonium compounds are summarized in Table 1.

The extension of the method to the preparation of ethers in which the nitrogen is part of a heterocyclic ring system and to the preparation of ethers containing other alkylene groups than ethylene will be the subject of subsequent papers.

The quaternary bis-ammonium halides (I) were examined for ganglionic blocking activity on the cervical ganglion of the cat (nictitating membrane contraction).

Some preliminary data are shown in Table 2.

A more detailed report on the pharmacology of these compounds will appear elsewhere.

Table 2.

Compound	G.B.A. [a]	LD_{50} [c] mg/kg
Hexamethonium bromide [b]	100	38
As 3553	7	1450
As 3554	40	215
As 4178	140	46

a) Expressed in per cent of hexamethonium bromide activity.

b) In doses of 0.25 — 1.00 mg/kg i.v.

c) Mice, intraperitoneal route.

EXPERIMENTAL *

Bis-β-chloroethyl ether. The only quality of bis-(β-chlorethyl) ether available at the outset of this work contained rather large amounts of halogenated impurities, which could only be removed by laborious methods. Accordingly a laboratory method for preparation of the bis-chloroether was developed using bis-β-hydroxyethyl ether (diethylene glycol, Mo & Domsjö) as starting material. Diethylene glycol, 106 g (1 mole) was dissolved in 212 g of pyridine (2.66 mole). To this was added 238 g (2 moles) of thionyl chloride under stirring during two hours. After one night at room temperature, the mixture was refluxed for one hour, cooled, and poured into 2 l of a 5 % solution of sodium hydroxide in water. This mixture was extracted with ten portions of 300 ml ether, the ether extract washed with approximately 100 ml of 4 *N* sulfuric acid, then twice with 500 ml of water, and dried over calcium chloride. The ether was removed and the residue distilled at reduced pressure. Yield: 88 g (62 %). B.p. 65° C at 15—16 mm Hg. Redistillation at atmospheric pressure gives a b.p. 176—177° (Literature [12] 177—178°).

$C_4H_8Cl_2O$ (M : 143) Calc. Cl 49.6 Found Cl 50.06 (Stepanow)

Bis-β-iodoethyl ether. Prepared form 29.7 g (0.1 mole) of mercuric sulfate and an excess of ethylene by the method of Sand [13] as modified by Schoeller [14]. Yield 23 g (74 %) of crude product.

Condensations with bis- chloroethyl ether, bis-(β-pyridinumethyl) ether dichloride. (As-3594). Dry pyridine, 16 g (appr. 0.2 mole) was mixed with 14.2 g of β-dichloroethyl ether (0.1 mole) and heated under reflux for twenty minutes (*cf.*[15]). After cooling down to room temperature the mixture separates into two layers of which the lower crystallizes on standing. The reaction mixture is dissolved in absolute ethanol which, upon evaporation, leaves only crystals. These are recrystallized twice from ethanol-acetone (1 : 2). M.p. 155—7° C, Yield 18.5 g (62 %) (see Table 1). Similar attempts of preparing bistrialkylammoniumethyl ethers by heating equimolar amounts of the reagents in a solvent (*i. e.* ethanol or isopropanol) to 100° C in sealed tubes for periods of time varying from two to twenty four hours afforded in every case a very small amount of crystalline material. The main products were liquid and distillable, but no further attempt of elucidating their structure was made.

* All melting and boiling points are uncorrected. The nitrogen and halogen values are macro determinations by Mrs. E. Ifversen and Mrs. G. Speggers of this laboratory. Carbon and hydrogen values are micro determinations by Mr. A. Grossmann, University of Copenhagen.

Bis-(β-trimethylammonium)-ethyl ether diiodide (As-3553). Prepared according to Ewins [11] from 15.6 g (0.05 mole) of bis-β-iodoethyl ether and 100 ml of ethanol containing approximately 10 g of trimethylamine. Yield 6.5 g (32 %) of plates, m.p. 301° (from ethanol), (Lit. [11] 275°). No depression of m.p. when mixed with a specimen prepared by the other method.

Bis-β-dimethylaminoethyl ether ($V, R_1=R_2=CH_3$). An alkoxide is prepared from 89.1 g of β-dimethylaminoethanol (1 mole) dissolved in 300 ml of dry toluene by addition of 23 g of clean sodium metal. The alkoxide soon begins to separate. It is necessary to heat under reflux for approximately 8 hours to ensure complete reaction. Any unreacted sodium is removed before proceeding. In the meantime a solution of β-dimethylaminoethyl chloride in toluene is prepared by hydrolyzing 144.1 g of the hydrochloride of β-dimethylaminoethyl chloride (1 mole) with a solution of 60 g sodium hydroxide in 150 ml of water, extracting the free chloroamine three times with 300 ml of toluene, and drying the combined toluene extracts with 150 g of potassium carbonate. This solution is filtered and added to the alkoxide suspension. Heating is continued for another 8 hours, when the salt formed in the reaction is removed by filtration, air-dried, and weighed for control. The filtrate is distilled through a 60 cm Widmer column for removal of toluene. The residue is distilled *in vacuo* which gives 105 g (66 %) of the desired ether, b.p. 79—83° C at 12—16 mm Hg. Redistillation to prepare a sample for analysis affords 83 g (52 %) of a middle fraction, colorless oil which darkens on standing, b.p. 79—81° C at 15 mm Hg. n_D^{25} 1.4290.

$C_8H_{20}N_2O$ (160)	Calc.	C 60.00	H 12.55	N 17.48
	Found	» 59.62	» 12.45	» 16.89

The hydrochloride was prepared by passing a stream of dry hydrogen chloride through the reaction mixture in toluene. The brown mass formed was crystallized from benzene-ethanol (1 : 1), m.p. 163°.

$C_8H_{22}N_2OCl_2$ (233.2) Calc. Cl 30.4, found 30.4. Picrate, m.p. 140°

Bis-β-diethylaminoethyl ether (V, $R_1=R_2=C_2H_5$). Prepared exactly like the bis-dimethyl ether from 117.2 g of diethylaminoethanol (1 mole), 23 g of sodium, and 172.1 g of diethylaminoethyl chloride, HCl. Heating period 12 h. Yield 132 g (60 %) of colorless oil, b.p. 121—23° C at 15 mm Hg. n_D^{23} 1.4389.

$C_{12}H_{28}N_2O$ (216.4)	Calc.	C 66.60	H 13.06	N 12.95
	Found	» 66.55	» 12.91	» 12.77

Hydrochloride, m.p. 214°

$C_{12}H_{30}N_2OCl_2$ (289.3) Calc. Cl 24.5, found Cl 24.82. Picrate, m.p. 130° (recryst. lowers m. p.).

Ethyl-β-diethylaminoethyl ether. Condensation was also carried out in ethanol as a solvent. The major part of the reaction product in this case destilled at 55—60° C at 15 to 16 mm Hg, indicating that in alcohol sodium ethoxide reacts in preference to the amino-alkoxide.

Bis-(β-dimethyl-β'-diethyl)-aminoethyl ether (V,$R_1=CH_3$, $R_2=C_2H_5$). Prepared exactly as above from 117.2 g of diethylaminoethanol, 23 g of sodium, and 144.1 g of dimethylaminoethyl chloride, HCl. Heating period 18 h. Yield 83 g (44 %) of colorless oil, b.p. 103° C at 15 mm. n_D^{25} 1.4337.

$C_{10}H_{24}N_2O$ (188.3)	Calc.	C 63.8	H 12.8	N 14.90
	Found	» 64.06	» 12.69	» 14.85

Hydrochloride, m.p. 180°.

$C_{10}H_{26}N_2OCl_2$ (261.2) Calc. Cl 27.15, found Cl 27.44. Picrate, m.p. 106°.

Bis-(β-trialkylammoniumethyl ether halides. (Table 1.) The quaternary compounds were prepared by mixing the amine, the appropriate alkyl halide (or dimethyl sulfate) in a slight excess, and the tenfold volume of dry acetone. If no precipitate had formed after one night the mixture was heated for 6 h., if necessary in a sealed tube. The precipitate was then removed by filtration and recrystallized. The quaternary derivatives were formed very readily from the bis-dimethylaminoethyl ether, the methoiodide, methosulfate and allobromide in a violent reaction, while the mixed ether reacted less readily and the bis-diethylether showed little tendency to react. The physical constants of these derivatives are summarized in Table 1.

SUMMARY

A convenient synthesis (Williamson condensation) for the preparation of bis-β-dialkylaminoethyl ethers is described.

The preparation of thirteen bis-β-trialkylammoniumethyl ether salts is described.

These compunds have a paralyzing action on the transmission in autonomic ganglia. The 'asymmetric' members shows the higher order of activity.

The authors are indebted to Messrs. Mo och Domsjö A/B for a generous gift of diethyleneglycol.

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Studies on the Chemistry of Lichens

III *. Disaccharides from *Umbilicaria pustulata* (L.)Hoffm.

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In Part II of this series the isolation of an arabitol galactoside from the lichen *Umbilicaria pustulata* (L.) Hoffm. was reported. The name umbilicin was proposed for the substance, and as the result of periodate oxidation experiments it was concluded that it might be either 3-D-arabitol β-D-galactopyranoside or, less likely, a galactofuranoside. Further investigations have shown, however, that the first suggestion is incorrect, and there is evidence in favour of the furanoside structure. In order to continue these investigations we required larger amounts of umbilicin, and as the method for its isolation previously described is rather tedious an alternative was sought. Fractionation of the carbohydrate mixture from the acetone extract on a carbon column, as described by Whistler and Durso [1] proved to be very satisfactory and further amounts of umbilicin could be obtained by continuing the extraction with methanol and fractionating the carbohydrates on a carbon column, using the gradient elution method [2]. In this way arabitol, mannitol and umbilicin were isolated in a pure state, together with smaller amounts of α, α-trehalose and sucrose. The last two substances have not been isolated from lichens previously, but as sucrose occurs almost universally in the plant kingdom and α,α-trehalose is a common constituent of fungi, these results are not particularly surprising. From the amounts of carbohydrates isolated the following approximative percentages could be estimated:

Arabitol 0.6 %, mannitol 0.8 %, α,α-trehalose 0.2 %,
umbilicin 2.5 %, sucrose 0.2 %.

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** Svenska Sockerfabriks A.B. Researsch Fellow 1951—1952.

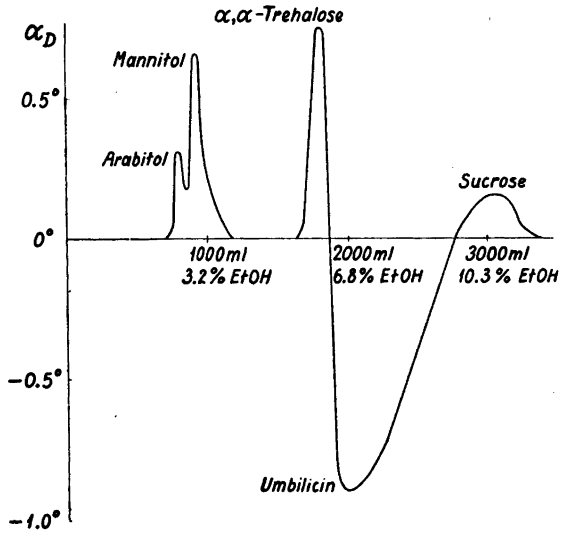

Fig. 1. Fractionation of the methanol extract on a carbon-Celite column.

EXPERIMENTAL

Air-dried, ground *Umbilicaria pustulata* (550 g) was extracted in a continuous extractor with ether for one week, then with acetone for one week and finally twice with methanol for the same time. The acetone and methanol extracts were worked up similarly. The solvent was removed under reduced pressure, the residue treated with water and undissolved material was removed by filtration. An excess of basic lead acetate was added to the aqueous solution, the precipitate formed was filtered off, and the excess of lead was precipitated with hydrogen sulfide. Coloured impurities were partly removed by passing the solution through a short column of aluminium oxide, and the eluate was evaporated to dryness. From the acetone extract and the two methanol extracts the residues 7 g, 21 g and 0.5 g respectively were obtained.

The carbohydrate fraction (7 g) from the acetone extraction was dissolved in 1 % ethanol (70 ml) and absorbed on to the top of a column (23 cm × 3.3 cm) of equal parts (by weight) of "carbo animalis pro analysi" (J. P. Riedel & E. de Haën) and Celite. The absorbent had been filled into the tube as a thick slurry in 1 % ethanol. The column was washed successively with 1 % ethanol (1 000 ml), 10 % ethanol (500 ml) and 50 % ethanol (1 000 ml). The 1 % ethanol eluted a crude mixture of arabitol and mannitol (2.5 g) and the 10 % alcohol crude umbilicin (2.8 g), which, however, did not crystallize. When this material was acetylated with acetic anhydride in pyridine, pure umbilicin octaacetate, corresponding to 1.8 g of umbilicin, was obtained.

The methanol extract was worked up in batches and in a typical run 6 g in 60 ml of 1 % ethanol was put on a carbon-Celite column (35 cm × 4.5 cm). The carbohydrates were eluted with aqueous ethanol (4 000 ml) the concentration of which was continuously increased from 1 % to 15 %. The elute was divided into fractions by means of an auto-

matic fraction collector, and the optical rotation of each fraction was determined (2 dm tube). The fractions with zero rotation were examined for arabitol and mannitol by investigating their rotation in acidified molybdate solution [3]. (5 ml solution, 5 ml 10 % aqueous ammonium molybdate, 2.5 ml *N* sulfuric acid, 2 dm tube). The results obtained are given in Fig. 1. The fractions were also investigated paper chromatographically and the intermediate fractions, containing two substances, were discarded. When the pure fractions were concentrated, five crystalline substances, corresponding to the peaks in Fig. 1, were obtained. These were further purified by recrystallization from ethanol or aqueous ethanol and identified as the following.

Arabitol: 0.2 g, m.p. 97—99°. (All melting points are uncorrected.)

Manitol: 0.8 g, m.p. 162—163°.

α,α-Trehalose: 0.3 g, m.p. 94—95°. Non-reducing, yielded only glucose on hydrolysis. Acetate, m.p. 75—78°.

Umbilicin: 3.0 g, m.p. 136—137°. $[\alpha]_D^{20}$ —80° (water, $C = 2$). Acetate, m.p. 84—85°. $[\alpha]_D^{20}$ —22° (chloroform, $C = 2$).

(As larger amounts of umbilicin and its acetate have been available, we consider these values more accurate than those previously recorded.)

Sucrose: 0.35 g, m.p. 178—180°. Non-reducing, yielded glucose and fructose on hydrolysis. Acetate, m.p. 87—88°.

All the substances were compared with authentic specimens and no melting point depressions were obtained.

The fractions prior to those containing arabitol, contained some unidentified material (0.4 g) which gave a strong colour reaction with ninhydrin, and when the column was washed with 50 % ethanol, a further small amount of material (0.75 g) was obtained.

SUMMARY

In addition to the previously known constituents arabitol, mannitol and umbilicin, α,α-trehalose and sucrose have been isolated from the lichen *Umbilicaria pustulata* (L.) Hoffm.

An improved method for the isolation of umbilicin from this lichen has been developed.

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Separation of Sulphate and Hydrogen Sulphate Ions from Interfering Substances by Adsorption on Aluminium Oxide, Prior to Sulphate Determination

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The difficulties in satisfactorily precipitating barium sulphate from solutions containing certain strongly adsorbable ions or, generally, from solutions containing large amounts of foreign salts or acids are well known. A method of previously isolating the sulphate as free as possible from these interfering substances seems to be highly desirable. Using the commercially obtainable ion exchange resins it is in most cases possible to separate sulphate from cations and uncharged substances, but not from other anions, in a way sufficiently simple for practical analysis. Such separations will, however, be laborious to perform in the presence of larger amounts of foreign substances; large ion exchanger volumes will be necessary involving large volumes of solutions, large consumption of reagents for regeneration, and appreciable loss of time. The necessity of using large ion exchanger volumes is of course a consequence of the unspecificity of sulphate adsorption on these resins because in quantitative adsorption sulphate ions are accompanied by most other anions. The authors found, however, that *aluminium oxide* as used for chromatographic purposes will, under certain conditions, meet the required specificity for sulphate ions — particularly hydrogen sulphate ions — to a high degree; moreover, elution of adsorbed sulphate and regeneration of the adsorbent are easily carried out.

Aluminium oxide has been used by Schwab & Dattler [1] for chromatographic separation of anions. As a result of a series of qualitative experiments, in each of which two kinds of anions were introduced into the adsorption column, these authors could arrange a sequence of adsorption for the different anions.

Later on a few additional results were obtained by Kubli [2,3], whose sequence is the following

$$OH^- - PO_4^{3-} - C_2O_4^{2-} - F^- - \left\{ \begin{array}{l} SO_3^{2-} \\ [Fe(CN)_6]^{4-} \\ CrO_4^{2-} \end{array} \right\} - S_2O_3^{2-} - SO_4^{2-} - \left\{ \begin{array}{l} [Fe(CN)_6]^{3-} \\ \\ Cr_2O_7^{2-} \end{array} \right\} -$$

$$\left\{ \begin{array}{l} NO_2^- \\ \\ CNS^- \end{array} \right\} - J^- - Br^- - Cl^- - NO_3^- - MnO_4^- - ClO_4^- - CH_3COO^- - S^{2-}$$

The anions are arranged in order of decreasing adsorption. These experiments were carried out with dilute solutions containing only the sodium or potassium salts of the different anions. Kubli also performed some quantitative separations: determination of K in K_2SO_4 and $K_2Cr_2O_7$ after adsorption of the anions, and separation of Fe and Mg from PO_4 and SO_4 followed by determination of the different constituents.

Dean [4,5] has used aluminium oxide for the separation of fluoride and sulphate from potassium in silicate analysis as well as for the isolation of the nitroso-R-salt complex of cobalt in the analysis of alloys.

The reaction mechanism at adsorption has been outlined as an anion exchange, *e.g.*,

$$\begin{array}{l} -Al-O \\ \qquad\quad Al^+X^- \\ -Al-O \end{array} + Y^- = \begin{array}{l} -Al-O \\ \qquad\quad Al^+Y^- \\ -Al-O \end{array} + X^-$$

The reaction generally proceeds towards the formation of the most difficultly soluble basic aluminium salt. Provided X^- is a hydroxyl ion and the not too weak acid H^+Y^- is added, the reaction will proceed to the right on account of formation of water. As all other anions are removed by an excess of hydroxyl ion (see adsorption sequence above) this gives an often applicable method of introducing different Y^- groups in the aluminium oxide.

EXPERIMENTAL

Preparations used.

All experiments cited below have been performed using Merck's "*Aluminiumoxyd nach Brockmann*". A few experiments not recorded here show that Riedel — de Haen's corre-

Table 1. Maximal capacity of the aluminium oxide for some different anions.

Anion	Anion added as	Capacity mmole anion/ml of column
ClO_4^-	$HClO_4$	0.17; 0.17
»	$NaClO_4$	0.17
Cl^-	HCl	0.21
»	NaCl	0.16
NO_3^-	HNO_3	0.20
»	$NaNO_3$	0.16
F^-	1 *M* NaF	0.10
»	0.1 *M* NaF	0.12
»	0.1 *M* NaHF	0.43
CH_3COO^-	CH_3COOH	0.22
»	0.1 *M* CH_3COONa	0.13
$H_2PO_4^-$	H_3PO_4	0.25
»	KH_2PO_4	0.21; 0.21
HSO_4^-	H_2SO_4	0.17
SO_4^{2-}	K_2SO_4	0.083

sponding preparation had essentially the same properties. For the column experiments the finer particles were removed by washing; the batch experiments were carried out with the original preparation.

All chemicals used were of reagent grade quality.

The maximal capacity of the aluminium oxide for some anions.

The maximal capacity of the aluminium oxide for some anions was determined. An adsorption tube was prepared from a glass tube, constricted at the lower end and plugged with Pyrex wool. It was filled by wet packing with a slurry of the adsorbent, forming an adsorption column of height 50 mm, volume about 2 ml. The adsorbent was converted into the desired anion form by washing with 10 ml of 0.1 *M* NaOH, 3 × 5 ml water, 5 ml of the 1 *M* acid in question, and finally with 3 × 5 ml of water. Gentle suction was applied.

In some cases the adsorbent was converted into the desired anion form by washing its perchlorate form with a neutral or slightly acidic solution of the anion.

The adsorbed anion was eluted by washing with 10 ml 0.1 *M* NaOH followed by 3 × 5 ml of water, and determined through titration of the eluate with 0.1 *M* HCl. Eluting a second time in the same way proved the elution to be quantitative.

The results (Table 1) show that the maximal capacity is variable. Thus it is generally higher when an acid rather than a neutral salt has been used for saturating the adsorbent, indicating a certain adsorption of acid molecules in addition to the ion exchange. While no such effect is observed for the noncomplex-forming perchloric acid, it is specially marked in the case of the strongly associative hydrofluoric acid.

Low values of the capacity have been obtained when treating the adsorbent with neutral salts of weak acids, *e.g.*, sodium fluoride or acetate. In these cases the hydroxyl ion concentration and the buffer capacity are sufficiently high for the conversion of an appreciable part of the adsorbent into its hydroxyl form. Qualitative experiments also show a considerable decrease in pH when neutral salt solutions are added to perchlorate-saturated aluminium oxide.

If we disregard these cases, the capacity is about 0.17 mmole/ml for univalent anions and half as large for bivalent (SO_4^{2-}). Consequently, the adsorbent is more advantageously used in acidic solution for sulphates.

Equilibrium experiments

The results published by Schwab and Dattler and Kubli elucidate only the order of adsorption of the different anions. Quantitative relations between the strength of adsorption of the anions may be obtained from equilibrium experiments. A small amount of the anion under investigation is allowed to compete with a fairly large, and therefore practically constant, concentration of a standard anion, and the adsorbed amount determined. Perchlorate ion was chosen as a proper standard, since it is one of the most weakly adsorbed.

10.0 g of the aluminium oxide was introduced into a 100 ml centrifuge tube and converted to its perchlorate form by washing with 0.5 *M* Na_2CO_3, water, 1 *M* $HClO_4$, and water. The water remaining after centrifuging was determined by weighing. A solution containing the competing anion and sodium perchlorate or perchloric acid was added and made up to a final volume of 90 ml. The concentration of the two latter constituents were kept at 1 *M*. The mixture was stirred for 10 minutes with a motor driven stirrer, centrifuged, and a convenient amount of the clear solution pipetted off for analysis. Chloride was determined argentometrically according to Mohr, nitrate as ammonia after reduction by Devarda's alloy, phosphate colorimetrically according to Scheel [6], chromate colorimetrically, and sulphate gravimetrically as barium sulphate.

Table 2. Partition coefficients, K, of some anions by competition with 1 M sodium perchlorate or perchloric acid. K = (millimoles of anion in 10 g of aluminium oxide) / (millimoles of anion in 90 ml of solution). Percent adsorbed = 100 K / (1 + K). Total capacity of 10 g aluminium oxide about 2.3 milliequiv.

Millimoles of anion added in 90 ml of solution	Partition coefficient, *K*, in 1 *M* $HClO_4$					1 *M* $NaClO_4$ (pH 4)				
	NO_3	Cl	$\frac{1}{2}\ Cr_2O_7$	PO_4	SO_4	NO_3	Cl	$\frac{1}{2}\ Cr_2O_7$	PO_4	SO_4
2	0.35	0.47	—	2.3	5.2	0.09	0.12	—	—	—
1	0.48	0.71	3.6	13	76	0.10	0.13	3.1	240	4.8
0.5	0.61	0.95	7.7	58	180	0.12	0.15	9.7	510	29
0.25	0.71	1.4	17	110	>250	—	0.17	22	2500	99
0.125	—	—	34	—	>250	—	—	41	—	>250

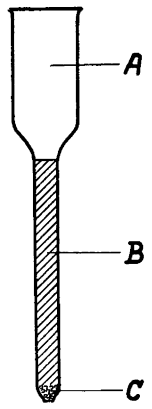

Fig. 1. Adsorption tube. A: receptacle for sample solution, volume 50 ml. B: adsorption column, height 120 mm, diameter 11 mm. C: plug of Pyrex wool.

The different anions had been added as the corresponding acids to the perchloric acid solution, and as the sodium or potassium salts ($NaNO_3$, NaCl, K_2CrO_4, KH_2PO_4, K_2SO_4) to the sodium perchlorate solution. In the latter case, the pH of the mixture was brought to about 4 by addition of perchloric acid in order to avoid the otherwise ensuing differences in hydroxyl ion adsorption.

Phosphate and sulphate hold an exceptional position among the anions investigated on account of their strong adsorption (Table 2). At pH 4 phosphate is much more strongly adsorbed than sulphate, in strongly acid solution the order is reversed — possibly due to the increasing ratio between the concentrations of HSO_4^- and $H_2PO_4^-$. Hydrogen sulphate ion is more strongly adsorbed than sulphate ion. As will be shown below, hydrogen sulphate ions may be quantitatively adsorbed and separated from large amounts of chloride and, at least, small amounts of dichromate; consequently it should be possible to separate hydrogen sulphate ions from the anions succeeding dichromate in the order of adsorption. Of course the break-through capacity for sulphate is increased when washing with solutions of anions situated in the lower end of the adsorption sequence. When separating sulphate from large amounts of metals, these should preferably be present as perchlorates.

The partition coefficient, K, increased with decreasing concentration of the adsorbed anion, although in a considerably higher degree for those which are most strongly adsorbed. This makes the adsorbent specially adapted for the isolation of traces; in any case only half the total capacity of the adsorbent should be used.

APPLICATIONS

The adsorption tubes (Fig. 1) used for all the following experiments were filled to a volume of 10—12 ml with washed aluminium oxide having a total capacity of about 2 milliequiv. The tube was placed in the neck of a filtering flask with the aid of a rubber gasket and suction applied to obtain a rate of flow of 10—15 ml/min. During this treatment gas bubbles often formed in the column, but they were disregarded.

Preliminary experiments proved that the elution could be performed with sodium carbonate as well as with the hydroxide. The carbonate has a smaller dissolving action on the adsorbent and was accordingly preferred. It was later found that ammonia would also be equally good which may be convenient if the eluate has to be further concentrated by evaporation.

The adsorption part of the experiments was performed in the following way unless otherwise indicated. An adsorption column which had not been previously used was washed with 50 ml 0.5 *M* sodium carbonate followed by 2 × 10 ml of water; washing until negative alkaline reaction is unnecessary. The adsorbent was now converted to its perchlorate form by sucking through 10 ml 1 *M* perchloric acid and washed with 2 × 10 ml of water; washing until negative acidic reaction is superfluous and may be disregarded in most cases.

The sample solution, generally having a volume of 100—200 ml, was now sucked through and the column washed with 2 × 10 ml 1 *M* perchloric acid and 3 × 5 ml of water. The adsorption tube was fitted into a clean filtering flask and the adsorbed anions eluted by washing with 5 ml 0.5 *M* sodium carbonate followed by 6 × 5 ml 0.05 *M* sodium carbonate and 2 × 10 ml of water. The eluate was transferred to a 250 ml beaker for analysis. After acidifying with perchloric acid, the column is ready for a new sample. Our adsorption columns did not show any changes in performance after 30—40 analyses each.

The eluate was properly acidified and the sulphate determined gravimetrically as barium sulphate, taking the usual precautions when precipitating in the presence of alkali salts.

Isolation of sulphate from pure solutions of acids and salts

Sulphate was isolated from the solutions recorded in Table 3 and determined as described above. Blanks were run and applied as corrections; no blank exceeded 0.5 mg $BaSO_4$.

In addition to sulphate the column will, after the adsorption process, contain small amounts of other anions which, such as for instance nitrate or chromate, may coprecipitate with barium sulphate. Those anions may generally be removed by washing, preferably with solutions of more strongly adsorbable anions. Thus nitrate is easily removed by washing with 10 ml of 1 *M* hydrochloric acid. Chromate is difficult to remove in this way on account of its strong adsorbability. It may, however, be transformed to cations by reduction to the tervalent state and then easily washed out. Reduction was carried out by washing the column with 20 ml 1 *M* perchloric acid containing about 3

Table 3. Determination of sulphate after isolation from pure acid or salt solutions. 0.499 milliequiv. H_2SO_4, corresponding to 58.2 mg $BaSO_4$, adsorbed from 100 ml of solution. Adsorption column 10—12 ml.

Solution	$BaSO_4$ found mg	Diff., mg
0.1 *M* $HClO_4$	59.0	+ 0.8
1 *M* »	58.2	± 0.0
2 *M* »	58.2	± 0.0
4 *M* »	58.2	± 0.0
0.1 *M* HCl	58.3	+ 0.1
1 *M* »	58.0	− 0.2
2 *M* »	58.1	− 0.1
4 *M* »	38.0	− 20.2
0.1 *M* HNO_3*	59.0	+ 0.8
1 *M* »	60.0	+ 1.8
2 *M* »	59.4	+ 1.2
4 *M* »	55.7	− 2.5
0.1 *M* NaCl, 0.1 *M* HCl	58.3	+ 0.1
0.9 *M* » 0.1 *M* »	58.6	+ 0.4
1.9 *M* » 0.1 *M* »	58.5	+ 0.3
0.1 *M* $NaNO_3$, 0.1 *M* HNO_3	58.1	− 0.1
0.9 *M* » 0.1 *M* »	58.1	− 0.1
1.9 *M* » 0.1 *M* »	58.2	± 0.0
0.01 *M* K_2CrO_4 1 *M* $HClO_4$	58.2	± 0.0
0.02 *M* » 1 *M* »	58.0	− 0.2
0.05 *M* CrO_3, 1 *M* $HClO_4$	58.2	± 0.0
0.1 *M* » 1 *M* »	58.2	± 0.0
0.2 *M* » 1 *M* »	58.2	± 0.0

* Adsorbed nitrates not removed by washing with hydrochloric acid; coprecipitation of nitrate!

millimoles of hydroxylamine hydrochloride, and the chromic salts washed away with 10 ml 1 *M* perchloric acid followed by water. The last traces of chromate are difficult to remove even with this procedure and it requires prolonged standing with an excess of the reducing solution in the column. These traces do not, however, interfere with the sulphate determination if they are reduced by adding a drop of 1 *M* hydroxylamine hydrochloride solution before the sulphate precipitation. The recorded analyses were carried out in that way.

As expected, the break-through capacity for sulphate increases in the order $HCl—HNO_3—HClO_4$ of accompanying anions. The break-through capacity in

acid chromate solutions is large enough to permit the use of the adsorption method for isolating sulphates from solutions of chromium steels.

Determination of sulphate in water

Water samples are usually concentrated by evaporation for the more accurate determination of their sulphate contents. This is more conveniently accomplished by adsorption on aluminium oxide. In this special case ion exchange resins can be used, but aluminium oxide has the advantage of more rapid elution.

A sample of the laboratory tap water was analyzed for sulphate. Amounts of 1 000 ml were made 0.1 *M* in perchloric acid, run through the adsorption column, and the adsorbed sulphate determined. For comparison, 1 000 ml of the water was evaporated in a platinum dish and sulphate determined according to a standard method. The results, expressed in mg SO_4/l, were: Adsorption method, 36.2, 36.2; Standard method, 36.9.

Determination of sulphur in biomaterials

Sulphur in ash containing biomaterials is generally determined as barium sulphate after ashing a mixture of the sample and some alkaline oxidizing agent. Thus the 6. Ed. of "Methods of Analysis" of the A.O.A.C. includes two official methods for the determination of sulphur in plant materials, *viz.*, after ashing with sodium peroxide or with magnesium nitrate. In both methods the resulting solution will be rather rich in salts; isolation of sulphate before its precipitation is therefore indicated.

Both methods were run on a sample of air dry hay with and without preliminary isolation of sulphate. The sample was ground to pass a 2 mm sieve. 1 g was weighed off for the magnesium nitrate method, 2 g for the sodium peroxide method. The solutions designed for adsorption of sulphate were prepared with perchloric instead of hydrochloric acid but otherwise after the manner of the standard method. The magnesium nitrate ashing gave 0.194 and 0.206 % S according to the official method and 0.196 and 0.192 % S according to the adsorption method. The corresponding results obtained after sodium peroxide ashing were 0.202 % S and 0.188 % S. The discrepancy between the latter two results is probably due to difficulties in controlling the ashing.

It has been suggested that sulphur could be determined in organic substances after wet ashing by nitric and perchloric acids, *e.g.*, in feed and biomaterials (Toepfer and Boutwell [7]) and rubber (Kahane [8]). Probably such a method would also be applicable for determination of total sulphur in soils. Provided the

oxidation of the sulphur compounds to sulphate proceeds quantitatively at these digestions, the subsequent analysis should be improved in speed and accuracy by applying the adsorption method as an intermediate step. A large excess of perchloric acid does not interfere.

Determination of sulphur in dolomite and glass

The U.S. Bureau of Standards' samples of dolomite and soda-lime glass were analyzed for sulphur using the adsorption method. The sample solution of the soda-lime glass was prepared exactly according to the method published in the certificate of analysis. The sample of dolomite (5 g) was dissolved in conc. nitric acid and bromine as in the certificate, but it was then evaporated with 20 ml of perchloric acid (sp.gr. 1.67) until the appearance of white fumes and diluted with water to about 100 ml. The following results were obtained: *Dolomite, No. 88.* Certificate values on total S: 0.033 % and 0.027 % (0.035 % SO_3 + 0.013 % S), found by adsorption method: 0.031 %; *Soda-lime glass, No. 80.* Recommended value on SO_3: 0.41 % (highest result: 0.49 %, lowest result 0.36 %). Found by adsorption method: 0.38 % and 0.38 %.

Determination of sulphur in iron and steel

The "umpire" method for the determination of sulphur in iron and steel involves dissolving the sample, usually 4.57 g, in nitric acid, conversion of the nitrates to chlorides by evaporation with hydrochloric acid, reduction of the iron to the bivalent state, and determination of the sulphate as barium sulphate. The procedure is quite time-consuming and requires very skillful handling for accurate results.

Application of the adsorption method should simplify the procedure considerably. No reduction of the iron should be necessary. The nitric acid solution could possibly be run directly through the adsorption column but should preferably be evaporated with an excess of perchloric acid to oxidize carbon and chromic salts. The latter will, at least in larger amounts, interfere with the sulphate adsorption by formation of complexes. Evaporation with perchloric acid until the appearance of white fumes may be performed on a hot plate, thus avoiding the time-consuming steam bath. The final determination in the presence of only small amounts of sodium chloride should be more accurate.

On account of these considerations some preliminary experiments were performed. A sample of 4.57 g of electrolytic iron was dissolved in dilute perchloric acid, oxidized with nitric acid, and evaporated to white fumes with a total of 50 ml perchloric acid (sp.gr. 1.67). The residue was dissolved in

Table 4. Determination of sulphate in synthetic iron solutions.

Red. of chromate	S added, mg $BaSO_4$	S found, mg $BaSO_4$	Diff., mg
Before adsorption	58.2	58.0	− 0.2
» »	58.2	57.7	− 0.5
» »	11.64	11.57	− 0.07
» »	11.64	11.41	− 0.23
After »	58.2	58.3	+ 0.1
» »	58.2	58.0	− 0.2
» »	11.64	11.57	− 0.07
» »	11.64	11.59	− 0.05

water to a volume of about 125 ml. This solution was purified from traces of sulphate by passing through an adsorption column. After washing the column with 1 *M* perchloric acid, the solution amounted to a volume of 150 ml. To this sulphate-free solution was now added 1.003 millimoles of sulphuric acid corresponding to 23.41 mg $BaSO_4$ and the sulphate was determined by the adsorption method. Four determinations gave 23.45, 23.19, 23.23, 23.47, average 23.35 mg $BaSO_4$ (blanks: 0.38 and 0.36 mg $BaSO_4$).

In further experiments, sulphate-free pure iron solutions were prepared in exactly the same way as above, and solutions of the common constituents of iron and steel added. The salts used were K_2CrO_4, $MnCl_2$, KH_2PO_4, $NiCl_2$, Na_2MoO_4, $Co(NO_3)_2$, NH_4VO_3, $CuCl_2$ and Na_2HAsO_4. The samples corresponded to a steel with 0.1 % Cr, 0.5 % Mn, 0.03 % P, 0.1 % Ni, 0.01 % Mo, 0.01 % Co, 0.001 % V, 0.2 % Cu, and 0.05 % As. After addition of measured amounts of sulphuric acid, the solution was again evaporated to white fumes, diluted with water to 150 ml and the sulphate determined by the adsorption method. In some experiments the chromate was reduced before adsorption by addition of ferrous chloride; in the other experiments chromate was reduced in the column by the hydroxylamine procedure previously described.

The results (Table 4) show good agreement between added and found amounts of sulphur. At this low percentage of chromium there is no significant difference between results obtained when chromate is reduced before or after adsorption. A further study on sulphur determinations in iron and steel will be published in another paper.

SUMMARY

The adsorption properties of aluminium oxide have been investigated with special reference to its use for isolating small amounts of certain anions from fairly concentrated salt or acid solutions. Experiments show that sulphate, hydrogen sulphate and dihydrogen phosphate ions (and, it is concluded from other papers, also fluoride ions) are exceptionally strongly adsorbed and may easily be isolated from most of the other common anions. This paper deals with the isolation of sulphates only.

Adsorbed sulphate is easily eluted with small amounts of dilute sodium hydroxide, sodium carbonate, or ammonia. This fact, together with the specificity of adsorption, makes aluminium oxide preferable to ion exchange resins for the isolation of small amounts of sulphate.

Some practical applications have been demonstrated; others have been indicated. Thus the adsorption method has been applied to the determination of sulphur or sulphates in pure salt or acid solutions, in water, in biomaterials, in dolomite, in glass, and in iron and steel. The results are generally good and show the practical applicability of the method.

The authors wish to thank Miss Ulla Friberg for valuable help with part of the experimental work.

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On the Crystal Structure of Tungsten Trioxide

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An investigation of the structure of tungsten trioxide was carried out in 1931 by Braekken [1], who described the structure as being triclinic (pseudo-monoclinic) having two angles very nearly = 90° and the third showing a slight deviation from this value. He determined the positions of the four tungsten atoms in the unit cell with trial and error methods, and also indicated the probable positions of the twelve oxygen atoms. In fact, this structure corresponds to a monoclinic or orthorhombic symmetry as was also pointed out in the Strukturbericht [2]. However, later reports on this structure vary. When Magnéli [3] interpreted the powder photographs of tungsten trioxide, he did not find any deviation from a monoclinic quadratic form, whereas Wyart and Foëx [4] by measuring single crystals optically came to the conclusion that the symmetry was triclinic. The cell dimensions given by the above mentioned authors are shown in Table 2.

These inconsistencies have made a renewed study of the symmetry of tungsten trioxide desirable. Because work on other tungsten oxides as well as on polytungstates was being carried out in this institute, it also seemed valuable to determine the tungsten positions with Fourier methods.

EXPERIMENTAL

Tungsten trioxide was investigated both with the powder and with the single crystal method. Powder photographs were taken with a Guinier focusing camera using monochromatic Cu-$K\alpha$-radiation. Several preparations of various origins, which had been recrystallized at about 850° C (Merck puriss., Baker analysed, and British Drug Houses) were investigated. They all gave identical powder patterns.

For the single crystal method, minute crystals were obtained from trioxide melts. Rotation and Weissenberg photographs were taken with Cu-K-radiation and with the axes [100] (layer lines 0–4) and [001] (layer lines 0–2) as rotation axes. Multiple film technique was used and the intensities were estimated visually against a scale consisting

of reflections of known relative intensities. The values of the Θ factors were calculated with Lu's curves [5] and the Fourier calculations were facilitated with Hägg-Laurent's calculating machine [6].

SYMMETRY AND UNIT CELL DIMENSIONS

In Weissenberg photographs no deviation from orthorhombic symmetry could be observed. In order to determine the symmetry, the powder photographs were analyzed in detail. The values of the unit cell dimensions given by Magnéli corresponding to monoclinic symmetry, and those given by Wyart and Foëx corresponding to triclinic symmetry, were tested. It was found that all lines in the powder photographs could be indexed by means of a monoclinic quadratic form (*Cf.* Table 1).

In Table 2 the values of the unit cell dimensions obtained in the present investigation are compared with those given by earlier investigators.

The Laue symmetry of WO_3 must clearly be $2/m$. Reflections $(h0l)$ were all absent when $h = 2n + 1$, and reflections $(0k0)$ were only observed when $k = 2n$; these absences are characteristic of the space-group $C_{2h}^5 - P2_1/a$. In fact, Braekken's co-ordinates correspond to this symmetry.

ATOMIC POSITIONS

A calculation of the structure factors, based on Braekken's co-ordinates for the tungsten positions, showed good agreement with the observed data. The signs of the F values obtained in this connection were used together with the intensities now obtained from the Weissenberg photographs for a calculation of the electron density function. The x and y parameters were determined from the projection of the electron density along the c-axis, and the z parameter and a control of the y parameter were obtained from the section $\varrho\ (\frac{1}{4}, y, z)$. The data thus obtained completely confirm Braekken's values, as can be seen from Table 3.

The tungsten atoms are thus in principle arranged as in the ReO_3 structure, but their distances show certain variations, *viz.*

3.705 Å approximately in the direction of the a-axis
3.79 Å » » » » » » b- »
3.835 Å in the direction of the c-axis

The positions of the oxygen atoms could not be determined from X-ray data. If the symmetry of the arrangement of the oxygen atoms is the same as that deduced for the tungsten atoms, 4 oxygen atoms must occupy the parameter-free positions 2 (a) and 2 (c). Concerning the other oxygen atoms, no

Table 1. Powder photograph data for tungsten trioxide Cu-Ka-radiation.

h k l	I_{rel}	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc	h k l	I_{rel}	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc
001	100	.0405	.0404	421	35	.2644	.2645
020	95	.0420	.0421	$13\bar{2}$	25	.2663	.2663
200	100	.0448	.0448	132			.2690
011	5	.0511	.0510	$31\bar{2}$	60	.2691	.2691
120	50	.0532	.0533	340			.2692
$11\bar{1}$	50	.0615	.0615	312	35	.2771	.2771
111	50	.0628	.0628	$23\bar{2}$	10	.2986	.2986
021	75	.0825	.0825	$32\bar{2}$	5	.3008	.3006
$20\bar{1}$	60	.0839	.0839	051	20	.3037	.3035
201	90	.0868	.0866	232			.3039
220			.0869	$34\bar{1}$	25	.3077	.3076
$12\bar{1}$	35	.0930	.0930	341	25	.3114	.3116
121	40	.0944	.0944	520	10	.3222	.3222
$22\bar{1}$	50	.1260	.1260	$51\bar{1}$	5	.3277	.3278
221	60	.1286	.1287	042	25	.3299	.3300
031	10	.1351	.1351	511	25	.3356	.3345
320	40	.1430	.1429	$40\bar{2}$			.3356
$13\bar{1}$	30	.1456	.1457	$14\bar{2}$	10	.3399	.3399
131	25	.1471	.1470	142	10	.3427	.3426
$31\bar{1}$	35	.1499	.1498	402	45	.3475	.3464
311	30	.1538	.1538	440			.3476
002	50	.1617	.1617	$33\bar{2}$	20	.3534	.3532
040	50	.1683	.1683	332	15	.3609	.3613
400	75	.1794	.1793	$24\bar{2}$	15	.3720	.3722
140			.1796	242	30	.3777	.3776
$11\bar{2}$	40	.1822	.1821	$42\bar{2}$			.3777
112	50	.1848	.1848	$11\bar{3}$	20	.3833	.3836
$20\bar{2}$	60	.2038	.2038	$44\bar{1}$	20	.3851	.3854
022			.2038	113	20	.3875	.3876
041	55	.2088	.2088	422			.3884
202			.2092	160	40	.3898	.3900
240	50	.2132	.2132	441			.3907
$40\bar{1}$	40	.2172	.2171	600	20	.4033	.4034
$14\bar{1}$	25	.2193	.2193	$53\bar{1}$	5	.4119	.4119
141	65	.2214	.2207	531	5	.4187	.4186
420			.2214	260	10	.4242	.4236
401			.2224	052			.4247
$33\bar{1}$	10	.2341	.2340	$34\bar{2}$	5	.4266	.4269
331	10	.2380	.2380	$16\bar{1}$	20	.4293	.4297
$22\bar{2}$	30	.2460	.2459	$43\bar{2}$	25	.4306	.4304
222	40	.2513	.2513	161			.4311
$24\bar{1}$		.2522	.2523	$15\bar{2}$	10	.4346	.4346
241	25	.2548	.2549	342			.4349
$42\bar{1}$	45	.2592	.2591	152	5	.4369	.4373

Table 1. continued.

$h\ k\ l$	I_{rel}	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc	$h\ k\ l$	I_{rel}	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc
$60\bar{1}$	15	.4397	.4398	360	20	.4790	.4796
432	5	.4412	.4411	$45\bar{1}$			.4801
620	30	.4453	.4455	313	30	.4809	.4812
601			.4479	$62\bar{1}$			.4819
540	25	.4483	.4485	451			.4854
033	20	.4587	.4585	$54\bar{1}$	5	.4858	.4856
$25\bar{2}$			.4669	621	10	.4895	.4900
$13\bar{3}$	10	.4671	.4677	541	15	.4918	.4923
$31\bar{3}$	20	.4689	.4692	630			.4981
133			.4718	$23\bar{3}$	10	.4983	.4993
252	25	.4714	.4722				

definite conclusions can be drawn. They may be situated half way between neighbouring tungsten atoms in accordance with Braekken's assumption. In this connection it may be mentioned that, in molybdenum oxides where MoO_6 octahedra exist which do not share edges with adjacent octahedra, the former are rather distorted. This has been demonstrated in the oxides Mo_8O_{23} and Mo_9O_{26} [8].

Table 2. Unit cell dimensions.

Authors	a Å	b Å	c Å	α	β	γ
Braekken [1]	7.28	7.48	3.82	90°	~90°	90°
Magnéli [3]	7.29	7.54	3.85	90°	90.9°	90°
Ueda and Ichinokawa [7]	7.278	7.460	3.838	90°	~90°	90°
Wyart and Foëx [4]	7.21	7.44	3.80	90.25°	90.02°	90.50°
The present investigation	7.285	7.517	3.835	90°	90.90°	90°

Table 3. Atomic parameters for tungsten.

Authors	4W in 4 (*e*) x	y	z
Braekken [1]	0.250	0.031	0.063
The present investigation	0.250	0.032	0.062

SUMMARY

The crystal structure of WO_3 was investigated. The symmetry has been found to be monoclinic (pseudoorthorhombic) and belonging to the space-

group $C_{2h}^5-P2_1/a$. The positions of the tungsten atoms given by Braekken have been verified with Fourier methods.

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An Interferometric Method for Recording the Refractive Index Derivative in Concentration Gradients

III. The Construction of the Optical Differentiators and an Experimental Test of the Method

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In the previous article on this subject [1], one of the authors described an arrangement for optical differentiation by Rayleigh interferometry of the refractive index function in a cell with stratified solutions. The theoretical foundation of the method was also given. At that time, only a makeshift for adjusting and keeping the inclined glass plates in position was available. These plates have now been built into two rigid mechanical devices called optical differentiators, which can be easily adjusted and are convenient in use. These differentiators will now be described, and an experimental test of the new method will be given.

DESCRIPTION OF THE OPTICAL DIFFERENTIATORS (L.-A.L.)

A photograph of one of the differentiators is shown in Fig. 1, one side wall being removed for better viewing. The metal frame consists of the bottom plate *a* with three adjusting screws, two side walls *b*, and a roof *c*. Two axes *d* go from one side wall to the other and are the centres of rotation of the movable glass plates. They are held in place by two 1 mm thick metal plates, *e* and *f*. The glass plates are inserted between two ebonite stoppers *g* fixed to the metal plates by screws. At one end of the plates, the holes for the screws are a little oblong, which makes it possible for the ebonite stoppers to be fixed in such a position that a slight pressure is exerted on the glass plates in their

lengthwise direction. Glass and metal plates are in contact with each other. All glass plates are 20 mm thick in the direction of the optic axis and are plano-parallel and homogeneous to within a small fraction of a wave-length. The outer plate, *h*, is 10 mm thick laterally, the inner one (*i* in Fig. 2, not visible in Fig. 1) is only 1 mm. The metal plates are at one end fixed to each of the two axes *d*. At the other end there is an elongation finishing with a holder for an adjusting screw *j* with a locking nut and a tip. On the bottom and roof plates, there are two metal blocks *k* acting as stoppers for the movable arms when the glass plates are in their vertical positions. In order to give the two plates exactly the same inclination in opposite directions, separate exchangeable gauge blocks *l* are inserted between the fixed stoppers *k* and the tips of the adjusting screws *j*. A set of 20 such gauge blocks of 5 different thicknesses have been made. The individual blocks in each set of four pieces have been worked to the same thickness with a high degree of accuracy, but the absolute thickness is not important since the blocks can easily be calibrated in terms of their vertical displacement of light. The tips of the arms are kept in contact with the gauge blocks, one due to the gravitational force, the other due to the action of the spring *m*. Close to the remote side wall, there is a third glass plate *n*, (in one of the differentiators; in the other it is a double prism with a very small refracting angle) 25 mm in breadth and fixed in a vertical position by two ebonite stoppers attached to the side wall. The glass plate or prism is kept immovable by two screws *o* acting on a thin piece of ebonite on top of the glass. The function of these two pieces of glass will be explained later in the text.

THE OPTICAL SYSTEM

In its main features, the optical system was described already in the previous article. However, that description referred to the method of making interferometric derivative records only, whereas the construction of the optical differentiators just described allows simultaneous photography of integral fringes, derivative fringes, and reference fringes. A more detailed description of the optical system will therefore be given here, with reference to Fig. 2. The symbols already defined have the same significance in Figs. 1 and 2. In the latter figure, we have in addition the diffusion cell in its proper position in relation to the differentiators. The diffusion chamber is denoted by *p*, and *q* is a reference chamber with a constant refractive index close to that in the cell. The cell has two outer and one partition wall, *r*, and beside the cell, there is a double-slit diaphragm *s*.

The light pencils β_1 and β_2 pass through the inclined plates *h* and *i* on either side of the metal plates *e*. Since they both pass through the cell at slightly

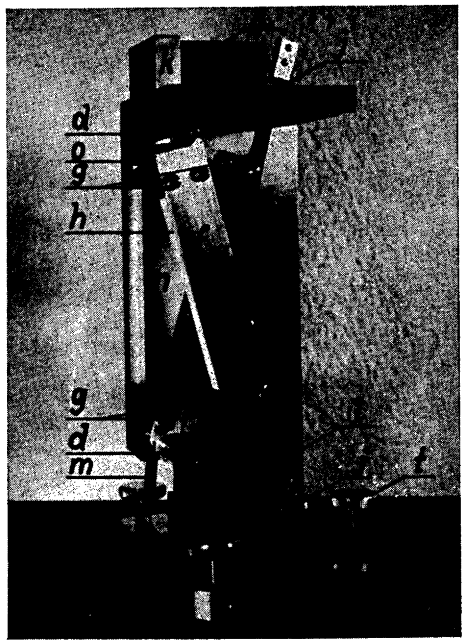

Fig. 1. An optical differentiator with one side wall removed.

different levels, they will form derivative fringes on the plate F. These pencils suffer no lateral deflection, so the derivative fringes will fall around the optic axis on the plate. The light pencils α_1 and α_2 pass through the vertical plate n_2, then α_1 passes through the cell and α_2 through the reference cell, and finally both pencils traverse one half of the double prism n_1. These pencils will form integral Rayleigh fringes on the plate, and due to the action of the prism these fringes will be laterally displaced and will not superimpose on the derivative fringes. The pencils γ, finally, pass entirely outside the cell through the same medium and will consequently form a system of rectilinear fringes which are useful as reference lines for the accurate measurement of the other fringes. These pencils pass through the other half of the double prism n_1 and are consequently deflected laterally towards the other end of the plate. The separation between the two coherent pencils in every couple of interfering rays is in all cases 2 mm, consequently the spacings between the interference fringes is the same in all three systems of fringes. Thanks to this circumstance, the raster method of producing multi-fringe interference patterns can be used (Svensson [2]). The possible number of lines in the raster and in the resulting interferograms depends on the refracting angle of the double prism. In the present arrangement, which was designed already before the raster method was

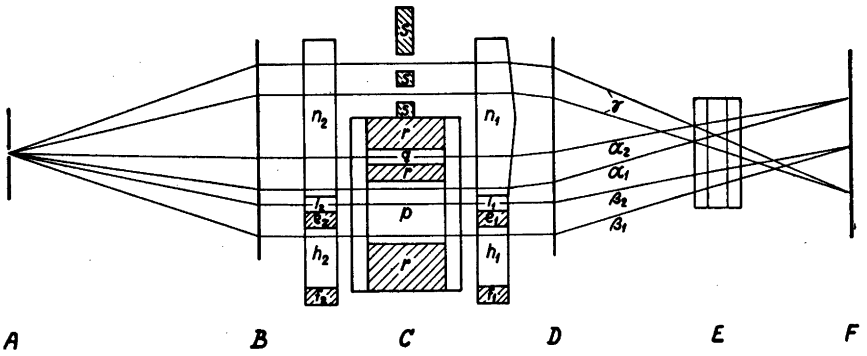

Fig. 2. Optical system for simultaneous interferometric recording of the refractive index and its derivative.

invented, this angle is rather small, and the number of lines has to be limited to about 10 if overlapping between the three interferograms is to be avoided.

The arrangement depicted in Fig. 2 requires an internal lateral dimension of the cell of at least 5 mm.

ADJUSTMENT AND CALIBRATION

The light source slit or raster A is first adjusted to the focal plane of the lens B (Fig. 2). Then it is replaced by a leadline hanging as nearly as possible in the same plane. An optical image of the string is formed on the plate F in the presence of the cylindrical lens E. To obtain this image, a longitudinal adjustment of the plate and an angular adjustment (turning round the optic axis) of the cylindrical lens are necessary. These adjustments are made alternately until the image of the string is perfectly sharp. One is thus sure that the axis of the cylindrical lens is strictly horizontal. The string is then removed, and the slit or raster is again put into place. If the string were hanging in another plane, the plate is readjusted longitudinally until a sharp image is restored. The slit or raster is turned round the optic axis until its image gets its maximum of sharpness; it is then known to be strictly vertical.

The next step in the adjustment is the sharp-focusing of the middle of the cell. A very narrow horizontal slit is placed in the cell stand in the middle of the water-bath, which has previously been filled with water and heated to the standard temperature to be used later in the measurements. The differentiator with the plane glass n_2 is placed between the water-bath and the lens D, Fig. 2, and the light from the slit in the water-bath is allowed to pass through this glass. The cylindrical lens is now shifted longitudinally until a sharp image of the slit in the bath is seen on the plate. If the slit is not strictly horizontal, no sharp image can be found, but this obstacle is removed either by reducing the lateral extension of the slit or by making it horizontal by turning the cell stand round the optic axis. After the sharp image has been found, the slit is opened to at least 5 mm, and a photograph of it is taken. Before the slit is taken out of the bath, its

longitudinal position is measured accurately, and after that its width is measured to within a few microns. The corresponding measurement on the photograph just mentioned then gives the optical cell magnification, which is an important apparatus constant. The optical image of the light source is now inspected once more. If it has become blurred, it must depend on a small angular deterioration of the cylindrical lens brought about during its longitudinal adjustment. In such a case, the lens is turned round the optic axis until a perfect optical image of the light source is restored. The longitudinal position of the cell in the water-bath can now be calculated from the position the horizontal slit had. It is, however, necessary to take into account that the cell has a glass window between its centre and the lens system. The cell position has to be corrected for the difference in geometric-optic thickness of this window and of an equally thick layer of water. Alternatively, an equally thick glass plate can be placed in the water-bath in front of the slit during the sharp-focusing of the slit.

The most important adjustment of the differentiator is that which is to secure absolute parallelism between the movable glass plates when no gauge blocks are used. (It is, on other hand, not important that the plates are strictly vertical.) We have found that the most accurate way of doing this is to put the cell in position and to make a very sharp boundary in it. It was pointed out in the preceding paper of this series that only one differentiator is required to give derivative fringes if the vertical extension of the light source is cut down sufficiently. Fringes formed in this way will therefore show a small peak in one direction or the other at the site of the diffusion boundary, except when the two movable glass plates are exactly parallel with each other. The test is the more sensitive the greater the refractive index gradient. The procedure to be used is consequently to reduce the vertical extension of the light source, to produce a great refractive index gradient in the cell, to screen off the plate n_2 and 9 mm of plate h_2, and to adjust the cell stand laterally until the derivative fringes appear with a maximum of contrast. With no gauge blocks in the differentiator, one of the tip screws j is then adjusted until the small peak in the fringes disappears and the fringes proceed strictly rectilinearly through the diffusion boundary. Both tip screws are then locked, and the fringes are controlled once more. When this procedure has been finished for the differentiator with the plate n_2, it is replaced by the differentiator with the double prism n_1, which is treated in the same way. The cell is then removed from the water-bath.

Now some simpler adjustments remain to be done. The vertical extension of the light source is again increased, but its lateral extension is reduced to the order of 1 mm. The differentiator is turned round a vertical axis until the shadow of the metal plate e, projected on a sheet of white paper behind the differentiator, becomes as thin as possible. By the use of a lead-line hanging down from the roof, the differentiator is further adjusted vertically in the plane perpendicular to the optic axis. The adjustment in a vertical plane parallel with this axis is not very important; it is quite sufficient to adjust the levelling screw t as indicated by a small water-level placed on top of the differentiator.

The differentiator with the plate n_2 is then placed on the other side of the water-bath, and the same adjustments are carried out. In addition to this, a lateral adjustment is required to make the position of the second differentiator exactly corresponding to that of the first. This is carried out by screening off both the n and 9 mm of the h glass blocks. The lateral adjustment is then very simple, the proper position being indicated by a maximum of contrast and definition of the interference fringes on the plate.

The adjustment of the differentiators is then completed, and the only remaining adjustment is that of the diffusion cell in the lateral direction. It is carried out in the same

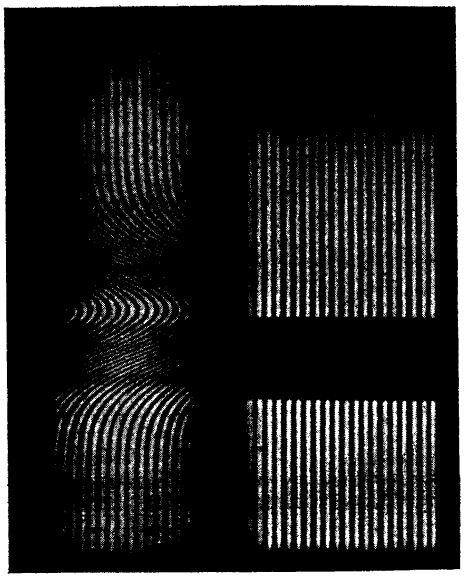

Fig. 3. A photograph of derivative interference fringes together with reference fringes.

way as described just above, if only derivative fringes are concerned. If one wants all three systems of fringes, the masks for the plates n have to be removed.

For calibration of the gauge blocks directly in terms of vertical shifts of light pencils, the cell is removed, and the narrow horizontal slit used before is again placed in the plane conjugated to the plate. This slit is photographed through the differentiator plates h and i, using the different gauge blocks, one set after the other. All exposures are made on the same plate. For each set of gauge blocks, two images of the slit are obtained. Division of the distance between them by the magnification factor gives the quantity Δx to be used in the calculation of the first approximation of the refractive index derivative, $\Delta n/\Delta x$.

A QUANTITATIVE EXPERIMENTAL TEST

The diffusion cell described in an earlier communication [3] was used. Since its internal lateral dimension is only 3 mm, it was impossible to get photographs of derivative and integral fringes in the same exposure. The two systems of fringes could however, be exposed on the same plate at a negligible time interval (about 15 seconds) by shifting the cell stand laterally a small distance after the derivative fringes had been exposed. The diffusion proceeded during 4.5 hours, and exposures were taken at intervals of 45 minutes. The gauge blocks were chosen so as to give about the same number of fringes across the boundary in the differential interferogram in all exposures. A suitable number of fringes from base to top is 20 per cent of the number of fringes in the integral interferogram.

The measurements were restricted to the maximum derivative. The plates were placed on the table of a comparator with a cross-motion device. They were turned on the table so as to make the reference fringes parallel with the cross motion. The table was then adjusted so that the hair-cross of the microscope coincided with one of the derivative fringes well outside the boundary. On moving the table cross-wise until the hair-cross

Table 1.

	Time sec.	$\left(\frac{\Delta x}{\Delta n}\right)^2 \cdot 10^6$	$[n'(x)]^{-2} \cdot 10^6$	$[n'(x)]^{-2} \cdot 10^6$ calculated	Discrepancy $d \cdot 10^6$
Integral fringes	1 800	88.85	86.87	87.26	−0.39
	3 600	184.58	180.47	180.60	−0.14
	6 300	324.00	316.78	320.63	−3.84
	8 100	425.56	416.08	413.97	+2.11
	10 800	574.61	561.81	553.99	+7.82
	13 500	704.53	688.84	694.02	−5.18
	16 200	852.64	833.65	834.04	−0.39
				$\sqrt{\Sigma d^2/7} = 3.9$	
Derivative fringes	1 800	94.03	90.40	88.88	+1.52
	3 600	182.09	178.44	182.07	−3.63
	6 300	338.11	323.44	321.85	+1.59
	8 100	432.98	418.27	415.04	+3.23
	10 800	566.65	551.91	554.82	−2.91
	13 500	708.49	693.73	694.60	−0.87
	16 200	850.23	835.45	834.39	+1.07
				$\sqrt{\Sigma d^2/7} = 2.3$	

came into the centre of the boundary, the number of fringes passing the cross was counted. The fractional part of the fringe number from base to top of the curve was then obtained by moving the table back to the last counted fringe by the micrometer screw and by dividing this distance by the distance between two neighbouring fringes in the same direction (this distance is everywhere the same). Due to a small curvature of the cell windows, the rectilinear parts of the derivative fringes were not absolutely parallel with the reference fringes. In order to apply corrections for this lens action of the cell, exposures were also taken from the cell with a homogeneous filling, for all sets of gauge blocks.

The height of the derivative curve can be obtained much more conveniently and with a negligible systematic error by aligning the plate in the direction of the slanting derivative fringes, *i. e.* by making the hair-cross coincide with one of the fringes on both sides of the boundary.

The maximum derivative was now computed from the integral fringes as described in an earlier report [4] from this laboratory, and directly from the height of the derivative fringes. The results are given in Table 1. The first column gives the time from the start of the diffusion. The second column gives the square of the reciprocals of the uncorrected derivatives, the third the same after the corrections have been applied. For each method of evaluation, the best-fitting straight line connecting the values in column 3 with those in column 1 was calculated by the method of least squares. The ordinates of these lines are found in column 4, and the discrepancies are given in the last column. The standard error of estimate is 0.47 per cent of the maximum ordinate for the integral fringe, and 0.28 per cent for the derivative fringe method. The slopes of the two lines coincided much better than what corresponds to these figures; they were $51.86 \cdot 10^{-9}$ and $51.77 \cdot 10^{-9}$, respectively.

DISCUSSION

We regard the above result of the very first quantitative experimental test of the new method as conclusive evidence that the theoretical background of the derivative fringe method is sound and that its accuracy is at least as good as that obtainable by numerical differentiation of the integral fringes. Moreover, the direct measurement of the height of the derivative curve is much more convenient and can be made in a small fraction of the time required for numerical differentiation. The same applies, of course, to all parts of the curve. The method should consequently be of value even for methods of evaluation which are dependent on the knowledge of the derivative in each point, *e. g.* the Boltzmann method.

As already pointed out[1] Vallet[5] has described another method of obtaining interferometric records of the refractive index derivative. In its use of polarized light this method is very ingeneous indeed, and possibly shows a way where one could find still better solutions to the problem of optical differtation. In its present form Vallet's arrangement has, however, too little flexibility; the vertical shift of one ray relative to the other is fixed by the optical components, and so the sensitivity cannot be changed.

SUMMARY

The construction of optical differentiators for use in an interferometric method of recording refractive index derivatives has been described. The construction is such that simultaneous exposures of derivative, integral, and reference fringes can be taken if a diffusion cell with at least 5 mm internal lateral dimension is available. The optical differentiation has been compared with numerical differentiation in a quantitative test experiment. It was found that both methods gave the same result within the experimental errors, and that these were smaller in the optical differentiation. This method is also much more convenient and rapid.

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

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Bacterial Growth Factors Related to Vitamin B_{12} and Folinic Acid in some Brown and Red Seaweeds

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The biological value of the seaweeds to some extent used as food and feed is scarcely explicable on the basis of analytical data concerning the normal major constituents. Recently the amino acids of some brown seaweeds have been investigated in this laboratory [1]. The present experiments with seaweed extracts using different Lactobacilli as test organisms for microbiological growth factors, have demonstrated the natural occurrence of several water-soluble substances related to the animal protein factors and hematopoietic factors identified as vitamin B_{12}, folinic acid (citrovorum factor, CF) and folic acid (FA).

The seaweeds studied in the present investigation belong to the following two classes of algae, *viz.*, the *Phaeophyceae* and the *Rhodophyceae*. Three species from each class have been investigated, — from the *Phaeophyceae: Sphacelaria arctica* (of the order *Sphacelariales*), *Laminaria saccharina (Laminariales)*, *Fucus vesiculosus (Fucales)*, from the *Rhodophyceae: Furcellaria fastigiata* (of the order *Gigartinales*), *Polysiphonia nigrescens* and *Rhodomela subfusca (Ceramiales)*. *Laminaria saccharina* was harvested on the west coast of Sweden near Fiskebäckskil (Skager Rack) during November. *Sphacelaria arctica*, *Fucus vesiculosus*, *Rhodomela subfusca* and *Furcellaria fastigiata* were collected at Simpnäs in the Åland Sea (the Baltic) in October, and *Polysiphonia nigrescens* was collected south of Bullerö in the Stockholm archipelago (the Baltic) in November 1951.

The algae were carefully cleaned and dried at room temperature as soon as possible after harvesting. 2–5 g of the dried samples were ground and extracted with 100 ml boiling water for thirty minutes to release the growth factors. The solutions were filtered and evaporated on a waterbath to 25 ml, filtered again, if necessary, and divided into two parts. One portion was autoclaved in 0.2 *N*. NaOH for 15 minutes at 120° C to destroy

any vitamin B_{12}, possibly present. Any residual activity on *Lb leichmannii* and *Lb lactis* Dorner must then be due to compounds other than vitamin B_{12}, such as desoxyribosides[2,3]. These two solutions from each algal sample were then used for the estimation of vitamin B_{12} and other growth factors.

Lactic acid bacteria utilised in the agar cup plate method have been used as test organisms in the following order: *Lactobacillus leichmannii* 313 (ATCC 7 830), *Lactobacillus lactis* Dorner 10 697, and *Lactobacillus lactis* Dorner 8 000. In this way a threefold assay of vitamin B_{12} type of activity was securd. *Leuconostoc citrovorum* 8 081 and *Streptococcus faecalis* 8 043 served for the folinic acid (CF) and folic acid (FA) test respectively.

The agar cup plate method previously described [4] was used for the different estimations with the first four Lactobacilli, and the method of Capps, Hobbs and Fox[5], modified for the cup plate technique by replacing the sodium acetate by sodium citrate and incorporating 1.6 per cent of bacteriological agar in the single strength medium, was used for the *S. faecalis* test.

Crystalline vitamin B_{12} (Cobemin, Merck), a synthetic citrovorum factor (folinic acid, Leucovorin [6], kindly supplied by Lederle lab. through the courtesy of Chemical Interests Co., Stockholm) and sodium salt of folic acid (Folivite Solution, Lederle), were employed as standards. Known amounts of vitamin B_{12} were added to algal extracts in which the vitamin had been destroyed by autoclaving with alkali as described above. Three different concentrations of vitamin B_{12} in such algal extracts covering a satisfactory assay range (*i. e.* standards with both higher and lower concentration of vitamin B_{12} than that of the algal extracts not treated with alkali) were normally used and a dose-response curve was plotted. The exhibition rings of the test materials were then evaluated with the help of this curve. The results of vitamin B_{12} estimations with three strains of Lactobacilli are shown in Table 1. The results in each column are mean values obtained from 5 to 30 single estimations. Results difficult to evaluate are represented by plus signs.

All the values in Table 1 are calculated in micrograms of vitamin B_{12} standard per gram dry weight of the plant material. It is necessary to consider not only the values of the samples not treated with alkali but also the values of the alkali-treated samples to get a somewhat closer approach to the "true" vitamin B_{12} contents. A summary of the approximate vitamin B_{12} values is presented in the first column of Table 2. By calculating the figures in Table 2 the weighted mean values of Table 1 have been used. The second column of Table 2 gives the values of the apparent desoxyriboside contents expressed in vitamin B_{12}. It should be realized that the values are not directly proportional to the real quantities of desoxyribosides because the desoxyribosides promote the same growth for these Lactobacilli only at about four hundred times greater concentration than that of vitamin B_{12}. Columns 3 and 4 show the approximate amounts of citrovorum factor (CF) and folic acid (FA) activity liberated by the extractions.

The results obtained with these Lactobacilli in general use for the assay of vitamin B_{12} evidently show the presence of vitamin B_{12} in at least

Table 1.

Seaweed	Activity expressed as μg B_{12} per g dry weight tested with					
	Lb leichmannii 313		*Lb lactis* D 10697		*Lb lactis* D 800	
	total	alkali-treated	total	alkali-treated	total	alkali-treated
Sphacelaria a. (number of determ.)	+ (5)	+ (5)	+ (5)	+ (5)	0.3 (10)	0.3 (10)
Laminaria s. (number of determ.)	1 (15)	0.5 (10)	+ + (5)	+ (5)	2[a] (15)	1 (8)
Fucus v. (number of determ.)	b)	b)	b)	b)	+ (5)	+ (5)
Furcellaria f. (number of determ.)	1 (20)	0.5 (15)	0.5 (15)	+ + (15)	1 (10)	0.5 (10)
Polysiphonia n. (number of determ.)	1[c] (10)	+ (5)	+ + (5)	+ (5)	1[c] (30)	+ (15)
Rhodomela s. (number of determ.)	2 (15)	+ (5)	1 (20)	+ (8)	1 (20)	+ (15)

a) double zones indicating the presence of at least two substances possessing bacteriological activity,

b) rings of successive salt diffusion in the agar gel covering the rings of bacterial growth,

c) inhibition rings, due to bacteriostatic substances.

three of the six seaweeds investigated, at the concentration of 0.5—1 μg per g dried plant substance *i.e.* 0.5—1 parts per million of vitamin B_{12}. In general, animal organs and non-photosynthetic microorganisms are considered as sources for vitamin B_{12}, and only traces of this type of growth factor are found in higher plants[7,8]. Alfalfa has perhaps been investigated most thoroughly, but although it contains only 5o to 62 parts per billion of *Lb leichmannii* activity more than 85 % of this was found to be caused, not by vitamin B_{12}, but by other microbiologically active substances[9]. The level of vitamin B_{12} in these *Phaeophyceae* and *Rhodophyceae* is remarkably high.

Table 2.

Seaweed	*Activity expressed as μg per g dry weight*			
	Approximate values obtained from Table 1		*Leuconostoc citrovorum* 8 081 activity compared with Leucovorin standard CF	*Streptococcus faecalis* 8 043 activity compared with Folivite standard FA
	"true" vitamin B_{12} amounts	desoxyribosides expressed in B_{12}		
	1	2	3	4
Sphacelaria a. (number of determ.)	no	0.3	0.5 (15)	0.6 (5)
Laminaria s. (number of determ.)	0.5[b]	0.5	0.3 (8)	0.2 (5)
Fucus v. (number of determ.)	no	traces	0.2 (2)	0.2 (2)
Furcellaria f. (number of determ.)	c)	0.5	0.2[a] (15)	0.2 (5)
Polysiphonia n. (number of determ.)	1	traces	0.2 (12)	0.5 (5)
Rhodomela s. (number of determ.)	1	traces	0.2 (15)	0.3 (5)

a) double zones indicating the presence of at least two substances possessing bacteriological activity,

b) due to the disturbing influence of desoxyribosides (column 2) this figure is very uncertain. A double growth zone indicates the presence of vitamin B_{12},

c) due to the disturbing influence of desoxyribosides (column 2) no estimation of the vitamin B_{12} content could be done. No double growth zones observed.

In *Fucus vesiculosus* and *Sphacelaria arctica* the concentration of vitamin B_{12} may be too low for direct estimation by the cup plate method.

The occurrence of FA and CF was more expected in this kind of plant material than that of vitamin B_{12}. It should be pointed out that the growth of *L. citrovorum* can also be accounted for by thymidine (thymine desoxyriboside), but not by other desoxyribosides [10].

The corresponding values of columns 3 and 4 show the existence of CF or high levels of thymidine. In those cases where the desoxyriboside effect measured with *Lb lactis* Dorner after alkali treatment is low or lacking (column 2) the values in column 3 and 4 may be due mainly to CF. Values in column 4 higher than those in 3 as for example in the case of *Polysiphonia* indicate the presence of FA in this material [11] at least. It is known that thymidine has quantitatively similar growth promoting effect for both *L. citrovorum* and *S. faecalis* but that FA gives a response with *L. citrovorum* only in concentrations about a thousand times as high as with *S. faecalis* and therefore the results with *L. citrovorum* could not be caused by FA. It would be quite unexpected to find only CF and no FA because of the close relationship between these factors and because of the general occurrence of FA in plant materials. Both CF and FA have been reported to occur in fresh natural materials of higher plants as well as in liver [12].

When *Furcellaria* was tested with *L. citrovorum* a double zone of stimulation appeared (Table 2, column 3) indicating the presence of at least two different growth factors. One of these factors was destroyed by the treatment with alkali described above.

Discussing the results it can be stated that vitamin B_{12} and desoxyribosides (sometimes in high concentrations) as well as folic acid and folinic acid occur in certain brown and red algae. The inhibition of the growth of the lactobacilli caused by *Polysiphonia* as shown in Table 1 is of interest as it might be due to some kind of antibiotic.

SUMMARY

Aqueous extracts of three brown and three red marine algae have been investigated with respect to their contents of vitamin B_{12}, folinic acid and folic acid. Bioassays using three different Lactobacilli showed vitamin B_{12} to be present in one brown, *Laminaria saccharina*, and two red algae, *Polysiphonia nigrescens* and *Rhodomela subfusca*, in a concentration of 0.5—1.0 μg per gram dry weight (due consideration having been taken to the influence of desoxyribosides). Folinic acid and folic acid were also detected.

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Contribution to the Hydrodynamic-Osmotic Theory of Sedimentation and Diffusion of an Incompressible Two-Component System

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A theory for the sedimentation diffusion process of a binary system at optional concentration is developed. The procedure and result is analogous to the familiar treatment of the dilute solution in that the diffusion coefficient and the sedimentation coefficients here defined, are expressed in terms of friction and of magnitudes determining the forces. The equations deduced serve the purpose to improve the accuracy of the extrapolation to zero concentration (which is in common use in ultracentrifuge work, e. g. for the determination of sedimentation constants), and to show the nature of the approximations which are introduced when using the "limiting" laws. The result may especially provide a base for experimental work with the ultracentrifuge in non-dilute systems. — The general formulation of the equilibrium condition, being thermodynamical in nature, is familiar to everybody working in this field. However, using the (symmetrical) differential osmotic pressure factor, suggests a formulation of the equilibrium condition which, in certain cases, is of advantage over the traditional formulation using the (unsymmetrical) activity concept.

Since the symmetric hydrodynamic treatment of pure diffusion gave a simple result[1–7], it was clear that the same must be true for the sedimentation diffusion process, the theory of which is especially of interest in connection with ultracentrifugal measurements. Such a general theory is necessary for the treatment of non dilute systems and for the understanding of the approximations made for instance in the case of dilute solutions. It is also beleived to be a point of departure for a refined treatment. The variation of partial volumes in

the cell during the process and the effects of ionic charges have not been considered. The validity of the theory is in general with good approximation unaffected by such effects as dissociation of or association between the components. The inner equilibrium of the solution is supposed to be rapidly adjusted. With the limitations already indicated, we may put the question so: What is revealed by measurements of the sedimentation diffusion process in the general case of a two-component system, for the time dependent as well as in the equilibrium case?

I. *Symbols.*

M	molecular weight
n	concentration in mole/cm^3
m	» » g/cm^3
N	mole fraction
Y	volume fraction
f	activity coefficient on the mole fraction basis
V	partial specific volume
$\bar{v}$	» molar »
$\bar{v}_{12}$	molar volume of a binary mixture
ϱ	density of the solution
C	linear velocity
x	force direction
ω	angular velocity
D	diffusion coefficient
s	sedimentation coefficient
φ	frictional coefficient/cm^3
Φ	» » /mole of a component
B_{12}	thermodynamic factor
Q_{12}	differential osmotic factor.

II. *Reference equations.*

1) $$\varrho = \frac{M_1N_1 + M_2N_2}{M_1N_1V_1 + M_2N_2V_2}$$

2) $$\frac{\partial N_1}{\partial x} = \frac{\bar{v}_{12}^2}{\bar{v}_2}\frac{\partial n_1}{\partial x}$$

3) $$\bar{v}_{12} = \frac{1}{n_1 + n_2} = N_1\bar{v}_1 + N_2\bar{v}_2$$

4) $$\bar{v}_1 dn_1 + \bar{v}_2 dn_2 = 0$$

5) $$\bar{v}_1 n_1 + \bar{v}_2 n_2 = 1$$

6) $$V_1 m_1 + V_2 m_2 = 1$$

III. *Theory.* We consider a non-compressible two component liquid system of mechanically normal properties. This is exposed to a centrifugal field in a sector shaped cell, as in the ultracentrifuge. The sedimentation diffusion process is convection-free because of the sector shape of the cell and of the control of temperature.

The sum of the (pressure-) centrifugal force K'_{12}/cm^3 and the diffusion force K''_{12}/cm^3 on component 1 equals the mutual friction force. The latter is the difference of the component velocities in the direction of the force, C_1-C_2 cm/sec, multiplied by the frictional coefficient φ_{12} of the solution/unit volume *. We obtain

$$K'_{12} + K''_{12} = (C_1-C_2)\ \varphi_{12} \tag{1}$$

$$K'_{12} = (1-V_1\varrho)\omega^2 x \cdot M_1 n_1 \tag{2}$$

$$K''_{12} = -RTB_{12}\frac{\partial \ln N_1}{\partial x}\cdot n_1 \tag{3}$$

B_{12} is the well-known factor $\frac{\partial \ln a_1}{\partial \ln N_1} \equiv 1 + \frac{\partial \ln f_1}{\partial \ln N_1} \equiv 1 + \frac{\partial \ln f_2}{\partial \ln N_2}$. The force/gram is the gradient in "centrifuging potential" (*cf.* Tiselius [8]) $\frac{\partial \pi}{\partial x} = (1-V_1\varrho)\omega^2 x$ and $M_1 n_1$ the number of grams/cm^3. K''_{12} equals the diffusion force/mole, multiplied by n_1**. Further, the linear velocity of the column in bulk in the direction of force will be written

$$x' = C_1 n_1 \bar{v}_1 + C_2 n_2 \bar{v}_2 \tag{4}$$

---

* The (component) friction for diffusion and for sedimentation are not exactly equal, compare Lamm [4, p. 8] for the special case of associated components.

** A more direct deduction of the total force is arrived at in the following way: The differential of the chemical potential at constant temperature is

$$\mathrm{d}\mu = RT\left(\frac{\partial \ln a_1}{\partial x}\right)_P \mathrm{d}x + \bar{v}_1 \frac{\partial P}{\partial x}\,\mathrm{d}x \ ; \quad \left(\bar{v}_1 = \frac{\partial \mu}{\partial_P}\right)$$

Presupposing that $d\mu$ causes a force/mole of the magnitude $-\left(\frac{\partial \mu}{\partial x}\right)_T$, we have to add to this the centrifugal force/mole, $M_1\omega^2 x$, in order to get the total force k_1/mole of component one. Inserting $\frac{\partial P}{\partial x} = \varrho\omega^2 x$ provides

$$k_1 = -\frac{\partial \mu}{\partial x} + M_1\omega^2 x = -RT\frac{\partial \ln a_1}{\partial x} + M_1(1-V_1\varrho)\omega^2 x$$

Multiplying by n_1 gives the force/cm^3, in agreement with equations (2) and (3).

Eliminating C_2 between (1) and (4) we obtain

$$C_1 = (K'_{12} + K''_{12}) \frac{n_2 \bar{v}_2}{\varphi_{12}} + x' \tag{5}$$

For parallel flow we have $\frac{\partial n_1}{\partial t} = -\frac{\partial (C_1 n_1)}{\partial x}$, an equation which in the case of cylindrical flow takes the form

$$\frac{\partial n_1}{\partial t} = -\frac{1}{x} \frac{\partial (x C_1 n_1)}{\partial x} \tag{6}$$

Using reference equations 1, 2 and 3, the forces (2) and (3) are transformed to

$$K'_{12} = M_1 M_2 n_1 n_2 \ (V_2 - V_1) \omega^2 x \tag{7}$$

$$K''_{12} = -\ RTB_{12} \ (n_1 + n_2) \frac{\partial N_1}{\partial x} \tag{8'}$$

or

$$K''_{12} = -RTB_{12}(n_1 + n_2) \frac{\bar{v}_{12}^2}{\bar{v}_2} \frac{\partial n_1}{\partial x} = -\ RTB_{12} \frac{1}{(n_1 + n_2)\bar{v}_2} \frac{\partial n_1}{\partial x} \tag{8''}$$

Equation (8') being written also with respect to component 2, by permutation of indices, it is easy to see that the sum of the diffusion forces is zero, using the Gibbs-Duhems relation. Naturally, the same is true of the centrifugal-pressure force according to (7). Specifying the forces in (5) according to (7) and (8''), and introducing C_1 in equation (6) gives

$$\frac{\partial n_1}{\partial t} = \frac{1}{x} \frac{\partial}{\partial x} \left[RTB_{12} \frac{n_1 n_2}{\varphi_{12}(n_1 + n_2)} x \frac{\partial n_1}{\partial x} - \right.$$

$$\left. - \frac{M_1 M_2 n_1^2 n_2^2 (V_2 - V_1) \bar{v}_2}{\varphi_{12}} \omega^2 x - n_1 x x' \right] \tag{9}$$

This equation is multiplied by $\bar{v}_1$ and the corresponding equation for component 2 by $\bar{v}_2$ and these magnitudes are supposed to be independent of x *. Addition of left and of right members of the resulting equations gives according to reference equations 4 and 5 $0 = \frac{1}{x} \frac{\partial}{\partial x} (-xx')$. Thus $x' = k/x$, which means a c o n s t a n t flow "in bulk" of the solution in the sectorshaped cell. As a flow of this kind is of no interest in our problem, we may put $k = 0$ and $x' = 0$, this leading to a simplification of equation (9):

* This assumption has to be specially observed in the case of sedimentation of high molecular substances, the concentration of which may become very high at the bottom of the cell.

$$\frac{\partial n_1}{\partial t} = \frac{1}{x}\frac{\partial}{\partial x}\left[RTB_{12}\frac{n_1 n_2}{\varphi_{12}(n_1+n_2)}\,x\,\frac{\partial n_1}{\partial x} - \frac{M_1 M_2 n_1^2 n_2^2 (V_2 - V_1)\bar{v}_2}{\varphi_{12}}\,\omega^2 x^2\right] \tag{10}$$

This is a fundamental equation for the sedimentation diffusion at constant partial volumes. In order to become independent of molecular weights (in the second term on the right), we introduce gram concentrations and partial specific volumes:

$$\frac{\partial m_1}{\partial t} = \frac{1}{x}\frac{\partial}{\partial x}\left[RTB_{12}\frac{n_1 n_2}{\varphi_{12}(n_1+n_2)}\,x\,\frac{\partial m_1}{\partial x} - \frac{m_1^2 m_2^2 (V_2 - V_1)V_2}{\varphi_{12}}\,\omega^2 x^2\right] \tag{11}$$

Measuring the process, we determine coefficients of this equation. It has to be compared with an equation

$$\frac{\partial m_1}{\partial t} = \frac{1}{x}\frac{\partial}{\partial x}\left[D_1 x\frac{\partial m_1}{\partial x} - s_1 m_1 \omega^2 x^2\right] \tag{12}$$

which is analogous to the second diffusion equation of Fick (with variable diffusion coefficient D_1 and sedimentation coefficient s_1), and which easily follows as a generalization of a previously deduced equation [9] (with constant coefficients). It requires the same restriction as equation (11) regarding absence of total (bulk) velocity. $s_1\omega^2 x$ is the sedimentation velocity in cm/sec. The comparison gives

$$D_1 \equiv D_2 \equiv D_{12} = RTB_{12}\frac{n_1 n_2}{\varphi_{12}(n_1+n_2)} \tag{13}$$

which is the generalized Sutherland-Einstein relation, and

$$s_1 = \frac{m_1 m_2^2 (V_2 - V_1)V_2}{\varphi_{12}} \tag{14'}$$

and by permutation

$$s_2 = \frac{m_1^2 m_2 (V_1 - V_2)V_1}{\varphi_{12}} \tag{14''}$$

From these equations one obtains

$$s_1 m_1 V_1 + s_2 m_2 V_2 \equiv s_1 n_1 \bar{v}_1 + s_2 n_2 \bar{v}_2 \equiv s_1 Y_1 + s_2 Y_2 = 0 \tag{15}$$

which gives the sedimentation coefficient of one component if that of the other one is known. $Y = mV = n\bar{v}$ is the volume fraction. As $s\,\omega^2\,x$ represent sedimentation velocities we see from (15) that the condition $\Sigma\ Cn\bar{v} = 0$ (*cf.* equation (4)) is valid for sedimentation and for diffusion independently.

As in a previous work [7] we introduce a factor Q_{12} based upon the osmotic pressures p_1 and p_2

$$-Q_{12} = n_2 \frac{\partial p_1}{\partial n_2} \equiv n_1 \frac{\partial p_2}{\partial n_1} \tag{16}$$

Unlike B_{12} this is independent of a choice of molecular weights. These magnitudes are related to one another by the equation

$$Q_{12} = \frac{RTB_{12}}{(n_1 + n_2)\bar{v}_1\bar{v}_2} \equiv \frac{RT}{M_1V_1}\,\frac{\partial \ln a_1}{\partial \ln m_1} \tag{17}$$

Introducing Q_{12} in the expression (13) provides

$$D_{12} = \frac{Q_{12}m_1m_2V_1V_2}{\varphi_{12}} \equiv \frac{Q_{12}Y_1Y_2}{\varphi_{12}} \tag{18}$$

Eliminating the friction using equation (14″) gives

$$Q_{12} = \frac{D_{12}m_1V_1\left(\frac{1}{V_2} - \frac{1}{V_1}\right)}{s_2} \tag{19}$$

Equations (19) and (17) show the possibility of measuring activity, from the sedimentation and diffusion processes, by integration.

We will now proceed to discuss the determination of molecular weights by the sedimentation diffusion process. For this purpose φ_{12} is eliminated between (13) and (14″)

$$m_1M_2 + m_2M_1 = \frac{RTB_{12}s_2}{D_{12}(V_1 - V_2)Y_1} \tag{20}$$

If, now, component 2 is d i l u t e and m a c r o m o l e c u l a r, $m_1M_2 \gg m_2M_1$ is an extremely advantageous approximation, so we may in this case write

$$M_2 = \frac{RTB_{12}s_2}{D_{12}(1 - V_2/V_1)Y_1^2} \tag{21}$$

Further $(1-V_2/V_1)Y_1 \equiv 1-V_2\,\varrho$. Formula (21) represents a generalization of Svedberg's equation $M_2 = RTs/D\ (1-V\varrho)$ to finite concentration and non-ideal solution. As has been discussed at some length in an earlier paper [7] B_{12}

is not defined unless the molecular weights of the components are known or values of these have been assumed. B_{12} being a correction factor in (21), the practical use of this factor for molecular weight determinations should be of the character of a successive approximation. The molecular theory is then introduced in other ways, *e.g.* by using the vapour pressure of the solvent as an activity measure.

Equation (20) may be transformed into the symmetrical formula

$$m_1 M_2 + m_2 M_1 = \frac{RTB_{12}\,[(-s_1) + s_2]}{D_{12}(V_1 - V_2)} \tag{22}$$

This corresponds to writing (19)

$$Q_{12}[(-s_1) + s_2] = D_{12}\left(\frac{1}{V_2} - \frac{1}{V_1}\right) \tag{23}$$

The equations (19), (20), (22) and (23) are of identical meaning. (19) and (23) are independent of molecular weights. Thus, the other two do not express anything regarding the molecular weights either, if not through the introduction of the molecular theory by special assumptions.

Analogous to (21) we obtain from (22):

$$M_2 = \frac{RTB_{12}\,[(-s_1) + s_2]}{D_{12}(1 - V_2/V_1)Y_1} \tag{24}$$

For the ideal solution activity and mole fraction are, by definition, identical so we obtain $B_{12} = 1$ and

$$M_2 = \frac{RT[(-s_1) + s_2]}{D_{12}(1 - V_2\,\varrho)} \tag{25}$$

From this is seen that Svedberg's molecular weight expression, which is the fundamental limiting law for a dilute solution, may be regarded to hold for finite concentrations if the r e l a t i v e sedimentation of the components is introduced.

IV. *Solvation.* The definition of components is always arbitrary in the sence that these may be A, B or $A(B)_n$, B etc. In the latter case, AB_n may express the solvation of the substance A. In our equations, magnitudes such as the sedimentation coefficients and the partial volumes and others depend on how the components are defined. When applying Stokes' law or any other hydrodynamic friction formula, it may be advantageous to define a component in the way indicated, and to use the r e l a t i v e sedimentation velocity $s\omega^2 x$ between the components AB_n and B, where $s = (-s_1) + s_2 = s_2/Y_1 =$

$-s_1/Y_2$. Such a procedure bears a close connection to a theory of Enoksson [13] on the influence of solvent movement on the sedimentation. Enoksson's equation (4) is, from the practical point of view, identical with our expression $s_1 = -sY_2$, if AB_n represents the solvated, macromolecular particle A and $s_1\omega^2x$ is the directly observed sedimentation velocity of this relative to the cell. — There is in Enoksson's work evidence for the view that, for certain protein solutions, s is independent of concentration (in the measured region). This would mean that the solution is both thermodynamically and hydrodynamically ideal, indicating a fixed degree of solvation.

V. *Sedimentation equilibrium.* Returning to equation (11), the sedimentation equilibrium is characterized by $\frac{\partial m_1}{\partial t} = 0$ and the condition $\frac{\partial m_1}{\partial x} = 0$ for $x = 0$ (the axis of rotation). This gives, using equation (17), the equilibrium condition

$$Q_{12}\frac{\partial \ln m_1}{\partial x} = \left(\frac{1}{V_1} - \frac{1}{V_2}\right) Y_2\,\omega^2 x \tag{26}$$

This is suited for the determination of Q_{12}, which is needed in connection with diffusion work on binary mixtures, compare equation (18). The equation is applicable without the use of molecular weights.

On the other side, we may ask if the present treatment gives something new regarding the determination of molecular weights. Equations (26) and (17) give, after permutation of indices

$$M_2 = \frac{RT\frac{\partial \ln a_2}{\partial x}}{(1 - V_2/V_1)\,(1 - m_2V_2)\omega^2x} \tag{27}$$

This is a thermodynamic relation, the literature concerning which has been especially completely given by Drucker [15] *cf.* Tiselius [8] and Pedersen [14]. According to (12) we may also use the equilibrium condition

$$D_{12}\frac{\partial \ln m_2}{\partial x} = s_2\omega^2x \tag{28}$$

and combine this with the approximate equation (21) in order to get

$$M_2 = \frac{RTB_{12}\frac{\partial \ln m_2}{\partial x}}{(1 - V_2/V_1)\,(1 - m_2V_2)^2\omega^2x};\quad 1-m_2V_2 \equiv Y_1 \tag{29}$$

This expression is easy to obtain also from equation (27), putting $a_2 = N_2f_2$ and using $m_1M_2 \gg m_2M_1$ (after derivation), just as we proceeded in deducing

equation (21). — It is seen that we again are met with a factor Y_1 which the older treatments of dilute solutions ($B_{12} = 1$) have not observed. Although it is here arrived at in a rather trivial way, the method of correct approximation is not quite simple, as is seen from an earlier, unsuccessful attempt [6].

The sedimentation diffusion equilibrium is most conveniently measured by the refractive index $\bar{n}$ of the mixture. Let $\bar{n}_1^0$ and $\bar{n}_2^0$ be the refractive indices of the pure components, then the following expressions, based on the Dale-Gladstone formula, may prove to be sufficiently good approximations

$$\bar{n} = \bar{n}_1^0 m_1 V_1^0 + \bar{n}_2^0 m_2 V_2^0 \ *$$

$$\frac{\partial \bar{n}}{\partial x} = \bar{n}_1^0 V_1^0 \frac{\partial m_1}{\partial x} + \bar{n}_2^0 V_2^0 \frac{\partial m_2}{\partial x} \tag{30}$$

Index ^{0} denotes the pure component. Taking the concentration derivatives from equation (26) and its analogue after permutation, we obtain the approximate formula

$$Q_{12} \frac{\partial \bar{n}}{\partial x} = (\bar{n}_1^0 - \bar{n}_2^0) \left(\frac{1}{V_1} - \frac{1}{V_2} \right) m_1 m_2 V_1^0 V_2^0 \omega^2 x \tag{31}$$

demonstrating the determination of Q_{12} by the ultracentrifuge. As the relative change in concentration will be small over the centrifuge cell (for low molecular solutions), this equation means that $\partial \bar{n}/\partial x$ is approximately proportional to x. Such a sedimentation equilibrium is well measureable already in moderate fields, at least in non-dilute solutions and if the components are not unfavourably chosen. This is fortunate, as the methods of determining the activity or osmotic properties of ordinary mixtures are quite limited. In addition to equation (17) we quote the following formulation of Q_{12}

$$Q_{12} = RT \left(\frac{N_1}{\bar{v}_2} + \frac{N_2}{\bar{v}_1} \right) \frac{\partial \ln a_1}{\partial \ln N_1} \tag{32}$$

which suggests how activity may be determined from equilibrium measurements by integration of the functions $Q_{12}(N_1)$ or $Q_{12}(m_1)$. Concerning the question of activity measurements by the ultracentrifuge, compare the articles by K. O. Pedersen [14] and C. Drucker [15]. The present theory has, on principles, nothing to add to their thermodynamic treatment of the sedimentation equilibrium.

VI. *Isotopically labelled components.* Although a selfsedimentation analogous to selfdiffusion does not exist, it may be appropriate to give here the

* This formula gives good result also for a non ideal mixture such as ethanol — water.

equations, valid for the process at optional concentrations if the only difference between the components are their molecular weights. For this purpose, the formulas are expressed in molar quantities, as the molar volumes are identical, $\bar{v}_1 = \bar{v}_2 = v$. The friction per cm^3 φ_{12} is replaced by the "characteristic friction sum" $\Sigma\Phi_{12}$ according to the relation $\Sigma\Phi_{12} = \varphi_{12}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)$, the reason being that this sum is independent of the concentration in the case of selfdiffusion, according to (compare ref. [7] and [16])

$$D_{12} = \frac{RTB_{12}}{\Sigma\Phi_{12}} \qquad (B_{12} = 1 \text{ for selfdiffusion}) \tag{33}$$

From equation (14') is obtained

$$s_1 = \frac{(M_1 - M_2)Y_2}{\Sigma\Phi_{12}} = \text{const. } Y_2 \tag{34}$$

From this is seen that, unlike the diffusion coefficient, the sedimentation coefficient is fundamentally concentration dependent.

For the sedimentation equilibrium the same assumptions give, as is seen from equations (26) and (32) ($Q_{12} = RT/v$)

$$RT\frac{\partial \ln Y_1}{\partial x} = (M_1 - M_2)Y_2\omega^2 x \; ; \; Y_1 + Y_2 = 1 \tag{35}$$

which is directly integrable.

The fundamental equation (11) for the sedimentation diffusion process cannot be integrated in the general case, as B_{12} and φ_{12} are unknown functions according to (13) and (14'). In the special case under consideration, the equation should be integrable, as we may write

$$\frac{\partial n_1}{\partial t} = \frac{1}{x}\frac{\partial}{\partial x}\left[D_{12}x\frac{\partial n_1}{\partial x} - K_0' n_1(1 - n_1 v)x^2\right]$$
$$K_0' = (M_1 - M_2)\omega^2/\Sigma\Phi_{12} \tag{36}$$

where all the parameters (D_{12}, K_0' and v) are constants. (When permutating indices $1 \rightarrow 2$, consider $K' \rightarrow -K'$, $D_{12} \rightarrow D_{12}$ and $n_1 + n_2 = 1/v$).

For the corresponding process in a parallel-sided cell in a homogeneous gravitational field g one obtains

$$\frac{\partial n_1}{\partial t} = \frac{\partial}{\partial x}\left[D_{12}\frac{\partial n_1}{\partial x} - K_0'' n_1(1 - n_1 v)\right] \tag{37}$$
$$K_0'' = (M_1 - M_2)g/\Sigma\Phi_{12}$$

The boundary condition at the top and the bottom of the cell, expressing their impermeability, coincides with the equilibrium condition as given by equation (35) for the centrifugal field, and for the gravitational field as well ($\omega^2 x = g$). This means that at the boundaries the concentration gradients have values which make the diffusion force equal and opposite to the centrifugal — pressure force. Compare the paper of Archibald [22].

It is of interest to discuss our problems in the cases in which a complete description is obtainable by mathematical analysis of the differential equations (36) and (37). This must be reserved for later publication.

The properties of the differential equation for the sedimentation diffusion process in the case of a d i l u t e solution were investigated by Faxén [10], Oka [17,18] and Archibald [19–22].

VII. *Conclusions.* The general signification of measuring the three processes of diffusion, sedimentation velocity, and sedimentation equilibrium may be expressed in terms of the differential osmotic pressure factor Q_{12} and the friction coefficient/cm^3 φ_{12} of the two-component system according to the following scheme:

1. Diffusion measurement gives Q_{12}/φ_{12}, equation (18).
2. Sedimentation velocity measurement gives φ_{12}, equation (14) *.
3. Sedimentation equilibrium method gives Q_{12}, equation (26) (compare (31)).

The theory of these processes does not contain molecular weights of the components in such a form that these can be experimentally determined unless approximations are introduced. Equation (27) may, at first sight, seem to contradict this. The activity is, however, not defined unless the molecular weight is known or, at least, an agreement has been made regarding this.

Reviewing the molecular weight relations which are used in ultracentrifugal work, from the point of view of clarifying the approximations introduced, shows that a factor of the magnitude of the volume fraction of the solvent may be considered in order to increase the accuracy of the relations in question at finite concentrations.

The symmetrical treatment of the two components and the resulting formulae makes it natural to speak of sedimentation coefficients in analogy with the diffusion coefficient. The former are concentration dependent also if the components differ only by (ideal) isotopic labelling. A simple formula (15) connects the sedimentation coefficients of "solvent" and "solute".

The special case of components, only differing in their molecular weights, was treated because it represents a rare instance in which the sedimentation

* Combining 1. and 2. measures Q_{12} according to equation (19).

diffusion process of a non-dilute system can be described by equations with c o n s t a n t coefficients, with the result that these are completely integrable.

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Succinylcholine Iodide (Celocurin)

A Synthetic Drug with a Curare-like Effect

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The di-choline esters of the aliphatic dicarboxylic acids show a pronounced curariform action and Bovet [1,2] *et al.* and Fusco [3] in particular have studied an extremely large number of such compounds. These authors have tested clinically the di-(N,N-dimethyl-N-ethylammoniumethyl) ester of succinic acid. In Austria, co-operation between Die Österreichische Stickstoffwerke, Linz, and Ginzel *et al.*[4–6] and Brücke [7] in Vienna resulted in the close study of O,O-adipylcholine and this compound also was given clinical trials.

Löw and Tammelin [8] described a pure preparation of *O,O-succinylcholine iodide* and preliminary reports were given of its synthesis, pharmacology and biochemistry. Shortly afterwards Thesleff [9] and Holmberg and Thesleff [10] were able to claim that the succinylcholine, as prepared by Tammelin and Löw [11] was useful clinically. The preparation has since been subjected to close study in over two thousand cases, and no harmful side-reactions have been observed.

In 1951, simultaneously with but independently of the Swedish group, the Viennese research team published the results of clinical tests with succinylcholine chloride (Arnold [12]) and as far as can be judged from their limited material their results are largely in agreement with those obtained in Sweden.

Synthesis of Dicholine Esters of Dicarboxylic Acids

For the synthesis of compounds of this type three main methods have found favour. The acid chloride may be coupled with choline chloride directly, or with di-methylaminoethanol, followed by quarternisation with methyl iodide. A third method is to start from the diethyl ester of the carboxylic

acid, bring about the exchange reaction with dimethylaminoethanol, and quarternise with methyl iodide.

A brief description of the synthesis of succinylcholine has been given by Löw and Tammelin [8]. This substance is now produced on a large scale and sold for medical use under the name of CELOCURIN.

Phenylsuccinylcholine has been synthesized by the method below, which is better suited to laboratory conditions. As far as the author is aware, the substance has not been described before.

Phenylsuccinyl chloride: 1 Mole (194.2 g) of phenylsuccinic acid [13] is mixed with 2 moles (417 g) of PCl_3 in a flask fitted with a reflux condenser. One hundred ml of $POCl_3$ is added as solvent. When the reaction, initially vigorous, slows down, the mixture is heated at 60° C for 12 hours, then fractionally distilled giving phenylsuccinyl chloride b.p. 132—134° C/4 mm in 50 % yield.

Phenylsuccinylcholine Chloride. 1 Mole (231.2 g) of phenylsuccinyl chloride and 2 moles (278 g) of choline chloride are heated together in dioxan at 90° C in a flask equipped with a reflux condenser. After 18 hours a very viscous phase develops under the dioxan consisting largely of phenylsuccinylcholine chloride. This material was not purified.

Phenylsuccinylcholine Iodide. Phenylsuccinylcholine chloride, obtained as described above from 1 mole of phenylsuccinyl chloride is dissolved in a minimal quantity of water. 2.2 moles of potassium iodide as a saturated aqueous solution are then added, and the phenylsuccinylcholine iodide which precipitates is purified by dissolution in water and precipitation with ethanol. Yield: 10 % based on phenylsuccinic acid. Melting point 250° (decomp.). Found: C 39.0; H 5.6; N 4.5. Calc. for $C_{20}H_{34}O_4N_2I_2$ (620.2): C 38.7; H 5.5; N 4.5.

Pharmacodynamic Effect of Succinylcholine and Phenylsuccinylcholine.

Succinylcholine is remarkable for its relatively short-lived curare-like action. Like *d*-tubocurarine it paralyses the skeletal muscles; and, as with the former drug, the effect starts with the eyes and pharyngeal muscles, and ends with the diaphragm. The smallest dose that can paralyse the neck muscles in a rabbit (the headdrop dose) has been found to be between 0.20 and 0.25 mg/kg. Phenylsuccinylcholine, though it can produce the same effect in the rabbit, is considerably less powerful in this respect, the head-drop dose being 8 mg/kg. Typical of this substance, at a moderate dose level, is its power to produce a severe paralysis of the extremities, unaccompanied, however, by spontaneous cessation of respiration in rabbits.

Hydrolysis of Succinylcholine

Succinylcholine (SCh) hydrolyses in aqueous solutions. This property is of significance, where both the stability of the injection solution and the breakdown of the substance in the body are being considered. According to Whit-

taker [14] SCh on hydrolysis gives rise, in the first place, to a monoester of succinic acid (SmCh) and choline as follows.

$$\text{SCh} = \text{SmCh} + \text{Choline};\ \text{SmCh} = \text{Succinic acid} + \text{Choline}.$$

Thus the following equations may be derived for the hydrolysis of SCh:

$$\begin{aligned}(\text{SCh}) &= a-x\\ (\text{SmCh}) &= x-y\\ \text{succinic acid} &= \quad y\end{aligned}$$

$\frac{-\mathrm{d}(a-x)}{\mathrm{d}t} = k_1(a-x); \frac{\mathrm{d}y}{\mathrm{d}t} = k_2(x-y)$. Solution of the differential equations gives:

$$x = a(1-e^{-kt_1}) \text{ and } y = \frac{a}{k_1-k_2}(k_2e^{-k_1t}-k_1e^{-k_2t} + k_1-k_2)$$

If k_1 and k_2 are known, it is possible to follow the breakdown and formation of the three substances.

To investigate the tendency to hydrolyse, the present author has made use of, and closely adhered to, Hestrin's [14] method for the photometric determination of choline esters. This method is based on the formation of hydroxamic acid, which in its turn forms a coloured complex with the ferric ion. This complex exhibits maximum absorption at about 5 400 Å. A "Lumetron" photoelectric colourimeter with a filter for 5 300 Å and a 1 cm cell was used for the determinations.

The extinction (E) obtained is composite. That is:

$$E = E_1\ (\text{SCh}) + E_2\ (\text{SmCh}) \qquad (1)$$

Though this function is composite, it is of interest to express the extinction of SCh solutions as a function of time (t) under different conditions. The author has determined E as a function of (t) at a constant pH with different temperatures (a); with different pH (b); with different concentrations of SCh (c); and in a solution containing the cholinesterase from cobra venom (d).

Within the range of measurements a linear relationship between concentration and extinction is obtained, and E_2 is thereby found to be 230. E_2 from corresponding experiments with phenylsuccinylcholine is 205. These values were noted before any appreciable hydrolysis could take place.

a) 0.00266 M SCh-solutions in a buffer of the following composition were prepared: 45.2 ml of 0.1 M NaOH and 50 ml of 0.1 M KH_2PO_4 plus 4.47 g of KCl, the whole being diluted to 100 ml. Potassium chloride was added to make the experiment with cobra venom possible. The pH of the buffer following dilution was found to be 7.3. The results are presented in Fig. 1.

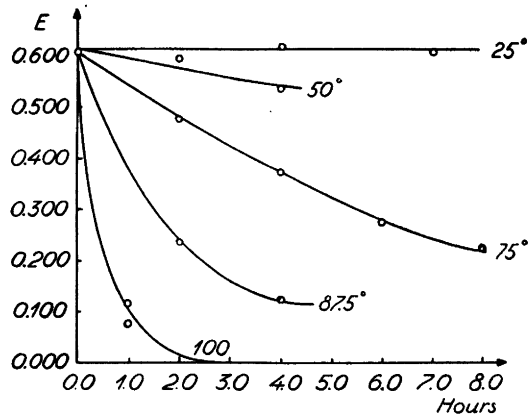

Fig. 1. Photometric determination of the rates of hydrolysis in a 0.00266 M succinylcholine solution at pH 7.3. The various curves represent the rates of hydrolysis at the temperatures given in the figure.

b) 0.00266 *M* solutions of succinylcholine were prepared in the buffer mixtures employed by Clark and Lub (Kolthoff [16]) with pH = 1, 4, 5, 6, and 8 respectively. The solutions were maintained thermostatically at a temperature of 75° C, since at this temperature the hydrolysis reaction proceeds at a convenient rate. At pH 4 or 5, no decrease in the extinction was observed, even after 6 hours. The rate of the breakdown reaction at the other pH values is shown in Fig. 2.

A sensitive test for a substance with curare-like action is the earlier described head-drop dose (H.D.) in rabbits. This test is very suitable for following the hydrolysis of SCh, since the H.D. of SCh is 0.22 mg/kg, and the H.D. for the monoester, according to Bovet *et al.*[2] 10 mg/kg. In three experiments with the same rabbit, it was shown that the dose in ml must be doubled if the solution has been at a pH of 7.3 for half an hour at 75° C. The half-life of SCh under the above-mentioned conditions is thus 30 minutes, and so $k_1 = 1.4$. The determination of this constant is admittedly not particularly accurate, but the error should not exceed 15 %. The value for k_1 indicates that the concentration of SCh may be neglected after 4 hours (see Fig. 3); and k_2 can be directly calculated from the extinction curve (Fig. 1) after that time. From the graph it is seen, that at pH 7.3, k_2 is 0.16

The vessels in which the reaction took place were fitted with reflux condensers which had been in use immediately beforehand. For this reason, the error due to "hold up" could be neglected. The vessels were placed in a water thermostat.

Results

a) and b) Results are given in Fig. 1 and Fig. 2.

c) Estimations of the concentrations of ester linkages in 0.00266 *M*, 0.0266 *M* and 0.0399 *M* solutions of SCh, in a buffer with pH 7.3, after 2, 4 and 6

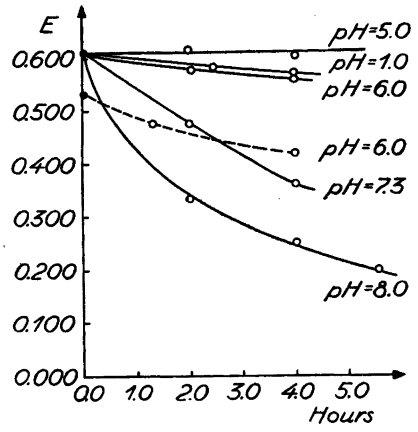

Fig. 2. Photometric determination of the rates of hydrolysis in solutions of succinylcholine (0.00266 M) and of phenylsuccinylcholine at 75° C. Continuous lines are for the hydrolysis of succinylcholine at the various pH values shown in the diagram. The dashed line is for the hydrolysis of phenylsuccinylcholine.

hours at 75° C show that the rate of breakdown is independent of the concentration.

d) The extinction of a 2.66×10^{-3} M solution of SCh in the buffer with pH 7.3 is reduced from 0.610 to 0.477 in 42 hours by the addition of cobra venom. A blank test without cobra venom reveals no such changes. The hydrolysis of SCh is therefore catalysed by the esterase in cobra venom.

By substitution of the value 0.0026 for a, and also the values for k_1 and k_2 in the kinetic equations, the curves in Fig. 3 are obtained.

Succinylcholine and Cholinesterases

An important clinical advantage possessed by SCh is its relatively rapid inactivation in the body, due mainly to its susceptibility to enzymatic breakdown and loss of curarising power. Glick [17] and Bovet-Nitti [18] found that SCh is broken down by bovine plasma at about one twentieth the speed with which acetylcholine is broken down by the same concentration of enzyme. The breakdown of SCh is prevented by cholinesterase inhibitors, such as prostigmine and eserine (shown by Bovet-Nitti [18]) and tetra-ethyl pyrophosphate (demonstrated by Löw and Tammelin [8]). The low rate of hydrolysis means that the results from all of the determinations described above must necessarily be approximate. For this reason it was considered to be impossible to identify with the aid of inhibition experiments the actual enzymes responsible. This held true, whether the Warburg or electrometric method (described by Tammelin and Löw [19] and Tammelin and Strindberg [20]) was used. The latter method was chosen for the investigations to be described here.

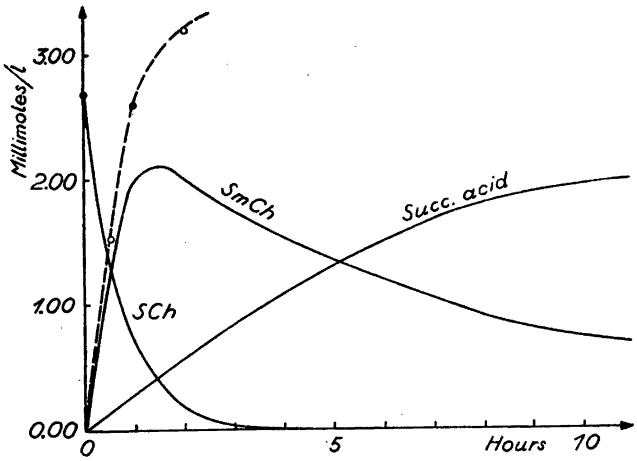

Fig. 3. Curves calculated from the rate constants determined for the hydrolysis of succinylcholine and succinylmonocholine. The continuous lines are for the breakdown of succinylcholine at pH 7.3 and 75° C. The dashed line gives the rate of liberation of equivalents of acid.

Apparatus.

The electrometric method makes use of the fact that acid formation due to the hydrolysis of the ester leads to a measurable change of pH in the buffer solution. Each of the six pairs of electrodes is connected successively to the pH-meter by means of an electrically driven switch of the drive shaft type. The pH is registered over a period of about 7 minutes and is represented as a short line for each of the six solutions. After about twenty minutes, three short lines are obtained from each solution, and when joined together give six lines, the slopes of which represent the rates of hydrolysis in the six solutions. The switch placed between the glass electrodes and the pH-meter must be well shielded, and the whole switch mechanism is therefore housed in an earthed copper chassis. Because of the high internal resistance between the glass and calomel electrodes the insulation between the incoming and outgoing leads, on the one hand, and the earth, on the other, has to be particularly effective. The desired result is achieved by ensuring that all leads entering the copper plate are at least 2 cm from the nearest metal by being sheathed in plexiglass. The rotating axle is made of glass and the cam wheel is of plexiglass and has a radius of 2 cm.

Cholinesterase-Inhibiting Effect of Succinylcholine and Phenylsuccinylcholine

By making use of Augustinsson's [21] graph (see Fig. 4), it is possible to determine the quantity of SCh which reduces the activity of the cholinesterases by half (I_{50}). Experiments have been made using plasma and erythrocyte haemolysate. Moreover, the effect of phenylsuccinylcholine as an inhibitor of the esterases in erythrocytes has been studied.

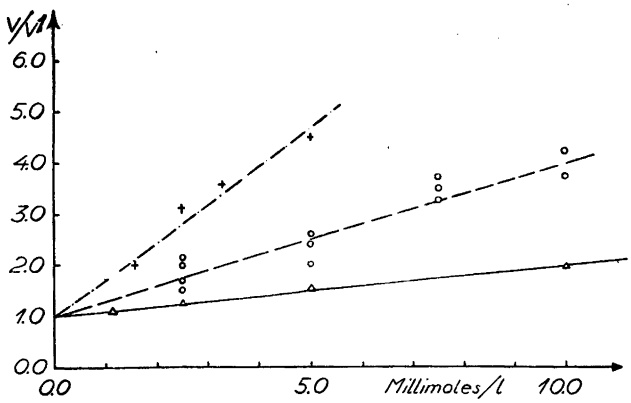

Fig. 4. Inhibition of cholinesterase as a function of the succinyl- and phenylsuccinylcholine concentration. v = reaction rate with uninhibited enzyme. v' = reaction rate with inhibited enzyme. The curve for the erythrocytes and phenylsuccinylcholine is represented by the continuous line; that for the erythrocytes and succinylcholine by the dashed line; and that for the plasma and succinylcholine by the dashed and dotted line.

The esterase was incubated for one hour in a solution consisting of 3 ml of Michel's buffer, plus 0.66 ml of erythrocyte haemolysate, diluted to thrice the volume of blood first used or plasma diluted to twice its volume plus 2.34 ml of a solution containing SCh. To this mixture was added 0.60 ml of an acetylcholine solution, prepared by dissolving 0.110 g of ACh iodide in 5 ml of water. The amount of acid formed was now determined at 25° C, with the help of the electrometric apparatus described above. As SCh and phenylsuccinylcholine gives rise to the formation of acid, a blank test was also done with SCh alone. The activities determined in this way were substracted from the results obtained in the inhibition experiments.

I_{50} is obtained from the diagram by finding the concentration which corresponds to $\frac{v}{v^1} = 2$. Thus the I_{50} value for SCh in plasma is 0.00135 and in erythrocytes 0.00335. I_{50} for phenylsuccinylcholine in erythrocytes is 0.01.

The enzymatic Breakdown of Succinylcholine

To ascertain if any particular organ plays a predominant role in the breakdown of SCh in the body, experiments were first of all performed on enzyme extracts made from various organs of the rabbit, as well as from the electric organ of the electric ray *Torpedo*.

The organs were extracted by grinding with kieselguhr in a mortar containing solution (A) to obtain a suspension as fine and homogeneous as possible. Solution (A) was prepared by adding 6 ml of Michel's buffer to 7.2 ml of water. One part by weight of the

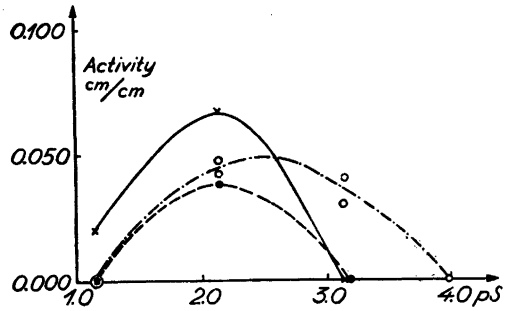

Fig. 5. Enzyme activity as a function of the negative logarithm of succinylcholine concentration during breakdown of succinylcholine. Dashed line for erythrocyte haemolysate. Dashed and dotted line for plasma. Continuous line for cobra venom.

organ was extracted with 5 parts by weight of solution A. The electrometric method was used in the investigation. The reaction vessel contained 3 ml of solution A, 3 ml of the enzyme extract, and 0.6 ml of a solution containing 0.219 g of SCh iodide per 5 ml of water. For purposes of comparison, the power of the enzyme extract to break down acetylcholine in an equimolar solution was determined, and 0.6 ml of a solution containing 0.110 g ACh iodide in 5 ml of water was used to assess this activity.

The results of these determinations are given in tabular form below.

Table 1.

Species Rabbit	Activities represented as	△ pH/min.
	SCh	ACh
Whole blood	0.0012	0.013
Liver	0.0012	0.015
Tibialis Anterior	0.0012	0.008
Heart	0.0030	0.008
Elect. Org. *(Torpedo)*	0.0030	0.019

The values given in the table for SCh are so low that the degree of accuracy of the method permits only a qualitative interpretation of results. It is clear, however, that SCh is broken down considerably more slowly than acetylcholine. Since the breakdown figures from the various organs differ only slightly, it would appear that the decomposition occurs at approximately the same rate in blood, liver and voluntary and smooth muscle.

The hydrolysis of SCh has also been studied by mixing it with the haemolysate from human erythrocytes, human plasma and cobra venom. In order to

ascertain if some optimal substrate concentration could be found, the quantity of the latter used was varied.

The reaction mixture in these experiments contained the following: 3 ml of Michel's buffer, 2.94 ml of the solution of SCh iodide, and 0.66 ml of undiluted plasma or erythrocyte haemolysate. In experiments with cobra venom the latter was dissolved in Michel's buffer (0.33 mg/ml). The composition of the mixture was 3 ml of Michel's buffer containing cobra venom, 2.94 ml of SCh solution and 0.66 ml of redistilled water.

In Fig. 5 the activity has been plotted against the negative logarithm of the substrate concentration. The curves obtained in this way link low, and, consequently only approximate values, but they do show that the optimal values are obtained at about the same pS as those found in the corresponding acetylcholine experiments (Tammelin and Strindberg [20]).

Discussion

The picture of SCh hydrolysis given by Fig. 3 shows that it is not at all certain that the values of the enzyme activities obtained by means of the Warburg or electrometric methods give a direct measure of the quantity of SCh broken down per unit time. The amount of carbon dioxide formed, and the change in the pH both depend on the number of equivalents of acid set free. For monoesters, such as acetylcholine, the number of acid equivalents parallels the amount of ester hydrolysed but with the diester the relationship is complicated by the formation of the dibasic acid. In this case the acid equivalents form more rapidly than the diester breaks down by spontaneous hydrolysis. See Fig. 3. By comparing the activities determined at the onset of hydrolysis for different enzyme preparations the error due to this may at times be neglected, and the figures obtained for the activities can always be accepted as a measure of the number of ester linkages hydrolysed.

As no particular organ has turned out to have a marked effect on the hydrolysis of SCh, it seems reasonable to suppose that the cholinesterases found in the blood and the neuromuscular end-plate, where the action of the drug is realised, are chiefly responsible for its inactivation. The rapid breakdown at the end-plates must therefore be of considerable significance. Experiments with enzyme extracts of the electric organ, which can be regarded as a huge end-plate, suggest that the cholinesterases in end-plates should strongly effect the rate of SCh hydrolysis.

The I_{50} determinations indicate that cholinesterase is not inhibited by SCh to an extent that can be of any importance for its physiological actions. However, the determinations indicate that cholinesterase has about the same affinity for SCh as for acetylcholine. Moreover, the enzymatic hydrolysis of SCh proceeds at a rate, which is 5—40 %

of the rate for acetylcholine. Both these observations prove that combination of cholinesterase with SCh must take place in the body. The complex once formed exists for a longer time than the corresponding acetylcholine complex. The physiological significance of such a complex is admittedly uncertain, but it is more than likely that the SCh-esterase complex formed in the blood must affect significantly the distribution of SCh in the body. It is furthermore, by no means inconcievable that the formation of such a complex may lead to inactivation of the curarising properties of SCh, if the affinity of the latter for the enzyme overshadows its tendency to accumulate at the end-plates, causing neuromuscular block and consequent paralysis.

SUMMARY

The synthesis of phenylsuccinylcholine has been described.

The hydrolysis of succinylcholine (Celocurin) and phenylsuccinylcholine have been followed using Hestrin's method for photometric determinations. The effect of succinylcholine concentration as well as pH and temperature on the stability of the ester linkages have been studied by this method. It has also been shown that the hydrolysis of succinylcholine is catalysed by cobra venom.

An electrometric apparatus for the determination of the esterase activity was modified to allow six readings in rapid succession.

The cholinesterase-inhibiting actions of succinylcholine and phenylsuccinylcholine have been determined and evaluated as I_{50}.

The hydrolysis of succinylcholine by enzyme preparations from different organs has been studied.

The enzymatic breakdown of succinylcholine has been studied as a function of the substrate concentration.

The relationship between the amount of succinylcholine hydrolysed and the number of equivalents of acid set free in this reaction, and the role played by the cholinesterases in changing the clinical picture after the injection of succinylcholine 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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The Isolation of Phosphothreonine from Bovine Casein

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Lipmann [1] showed that at least part of the phosphoric acid present in casein is bound by an ester linkage to serine. This made it also conceivable that a part of the phosphoric acid might be coupled to threonine, although the phosphoric acid ester of this β-hydroxy acid had never been isolated from a protein hydrolysate. Moreover, since Plimmer [2] showed that synthetic phosphothreonine has about the same stability toward acid hydrolysis as phosphoserine, it seemed that it might be possible to isolate phosphothreonine from partial, acid hydrolysates of casein. A preliminary report describing efforts to isolate phosphothreonine and phosphopeptides from partial hydrolysates of casein has been given elsewhere [3]. The present paper describes the isolation of phosphothreonine.

EXPERIMENTAL

Preparation. The procedure used by Lipmann for the isolation of phosphoserine has been partially followed here. Commercial bovine casein was hydrolysed with 2 *N* hydrochloric acid for 20 h at 100° C, according to Lipmann. The hydrochloric acid was neutralized with barium carbonate and conc. ammonia, and the resulting barium complex was then precipitated by adding an equal volume of 96 % ethanol. The precipitate was collected, dried with ethanol and ether, and was thus obtained as a greyish-brown powder.

200 g of this powder was suspended in 500 ml of distilled water, and most of it was dissolved by adding 340 ml of 4 *N* hydrochloric acid. The solution was filtered and subsequently run through a 32 × 8 cm column of a sulphonated polystyrene resin (Dowex 50) of technical grade. By this procedure, most of the barium was removed. To get suitable particles, the ion exchange resin was milled in a ball mill. The larger particles were removed by passing the resin through a sieve (9 mesh per cm), and the smaller particles were separated off by sedimentation in water. The resin was used in its acid form. The rate of flow of solution through the column was about 100 ml per hour. 3 l of distilled water were then used to wash the column. The resin was regenerated with 4 *N* hydrochloric acid.

The combined effluent volumes from three experiments (corresponding to 600 g of the greyish-brown powder) were concentrated to 2 l. The solution was heated twice with charcoal to decolorize it and finally concentrated to a small volume. At this stage a crystalline precipitate appeared which was shown to consist mostly of barium phosphate. This precipitate was filtered off, and the solution was once more run through the Dowex 50 column to remove the last traces of barium.

After evaporation to a concentration of 10.9 mg total nitrogen/ml the solution was analysed using two-dimensional paper chromatography (phenol-cupron-NH_3/pyridine-isoamyl alcohol)[4]. The resulting chromatogram showed a large spot corresponding to phosphoserine and fainter spots corresponding to glutamic acid, aspartic acid, leucine, tyrosine and serine. 100 ml of the solution corresponded to about 500 g casein.

To remove the free amino acids and free phosphoric acid present, displacement chromatography according to Partridge and Brimley [5] was used. The resin Dowex 2 was milled similary to Dowex 50 and used as the free base. A column consisting of three parts was set up. The dimensions of the three resin beds were 24 × 3.5, 12 × 2.2, and 7.5 × 1.3 cm. The resin was regenerated according to Partridge and Brimley.

100 ml of the above mentioned solution containing 10.9 mg total nitrogen/ml was allowed to run slowly into the bed. The solution was subsequently displaced with 0.2 *N* acetic acid and finally 0.2 *N* hydrochloric acid. By this method it was possible to obtain three separate fractions, as checked by paper chromatography. The first fraction was displaced with acetic acid and contained the ordinary amino acids. The second fraction was eluted with acetic acid and contained acid peptides. After about 1 400 ml had run through the column, the solvent was changed to hydrochloric acid, which displaced the third fraction consisting mainly of phosphoserine. Following this fraction, free phosphoric acid was eluted from the column.

After some of the phosphoserine had been crystallized out of solution the third fraction, was concentrated to about 20 ml and applied to another column for the final separation. This column (115 × 3.6 cm) was packed with Dowex 50 (analytical grade, 250—500 mesh, 8 % crosslinking) and fitted with a waterjacket to keep the temperature at 37° C. A separation was obtained by elution with 0.01 *N* hydrochloric acid. The effluent fluid was separated into 10 ml fractions, and each fraction was tested for ninhydrin-reacting substances. By this method it was possible to obtain seven different fractions, some of which could be easily crystallized. The fourth fraction to emerge from the column, after 1 500 ml, was phosphoserine. The fifth fraction to emerge was a crystalline substance, which yielded threonine on hydrolysis, suggesting that the original substance might be phosphothreonine. The yield was about 20 mg. A study of the remaining fractions will soon be published.

Synthesis of DL-*phosphothreonine.* The barium salt of DL-phosphothreonine was synthesised according to Plimmer [2]. Barium was removed by electrodialysis and the substance further purified by running through a small Dowex 50 column (30 × 0.9 cm) at room temperature, otherwise the procedure described above was used. DL-phosphothreonine crystallized out of a water-methanol mixture in lustrous, stout prisms. The yield was low, about 5 % of the theoretical. M. p. 194° (decomp.), (Found: C 24.2; H 5.16; N 6.89; P 13.4; Calc. for $C_4H_{10}O_6NP$ (199.1): C 24.1; H 5.06; N 7.04; P 15.6).

Phosphorus was determined according to Teorell [6] with a combustion time of 4 days. As in the case of phosphoserine [7] it seems to be difficult to completely split off all the phosphorus. Further hydrolysis did not give higher values. As expected, the substance was optically inactive.

Fig. 1. Photographs (negative) of paper chromatograms of a hydrolysate of phosphothreonin (right) compared with threonine (left). Left chromatogram run with pyridine + isoamyl alcohol, the right one with phenol + cupron. The faint spot corresponds to unhydrolysed phosphothreonin.

Identification of L-*phosphothreonine.* One fraction from the Dowex 50 column gave after hydrolysis (20 h, 120° C, in sealed tubes) a ninhydrin-reacting substance with the same R_F-values as threonine. It was tested with one dimensional paper chromatography, run both with phenol + cupron and pyridine + isoamyl alcohol [4] (Fig. 1). On a one-dimensional chromatogram the fraction before hydrolysis gave a spot in the same position as synthetic phosphothreonine. In this case the chromatogram was developed with phenol + cupron.

The X-ray powder diffraction patterns from synthetic phosphothreonine and the isolated fraction were identical (Fig. 2). M. p. 194° (decomp.), (Found: C 24.4; H 5.19; N 7.03; P 14.0; Calc. for $C_4H_{10}O_6NP$ (199.1): C 24.1; H 5.06; N 7.04; P 15.6). The phosphorus value is low also in this case. Optical rotation: α_D^{24} −0.19° (water; *l*,1; *c*, 2.58); $[\alpha]_D^{24}$ −7.37°.

X-ray crystallographic investigation. The synthetic DL-phosphothreonine and the prepared L-compound were studied with single crystal methods. Rotation-crystal diagrams and Weissenberg photographs from rotation about the *c* axis gave identical pictures for the two compounds. The crystals belonged to the orthorhombic system with the cell dimensions: $a = 7.74$ Å; $b = 9.28$ Å; $c = 10.74$ Å. The density was 1.72 (flotation method). A formula weight of 199.1 gives four molecules per cell.

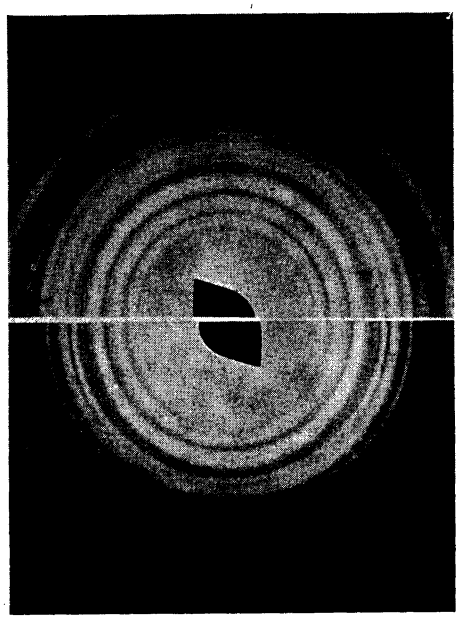

Fig. 2. X-ray diffraction patterns of synthetic DL*-phosphothreonine (above) and the isolated* L*-phosphothreonine (below).*

DISCUSSION

From the results it seems to be proved that the phosphorus in casein is not only bound to serine but also to threonine. Casein has about the same content of serine and threonine (6.3 % and 4.9 %, respectively [8]) but it is possible to isolate much greater amounts of phosphoserine than phosphothreonine. Two explanations of this fact seem possible, either a difference in the phosphorylation affinity or a difference in the stability toward acid hydrolysis. Which is the case is still a problem to be solved.

The optical rotation shows about the same values as phosphoserine, but with different sign. Phosphothreonine: $[\alpha]_D^{24}$ −7.4°; $[M]_D^{24}$ −14.6°. Phosphoserine: $[\alpha]_D^{23}$ + 7.2° *; $[M]_D^{23}$ +13.5°. The free amino acids threonine and serine also rotate the polarized light in opposite directions.

The identical X-ray diagrams of the optically active and the racemic form of phosphothreonine might indicate that the DL-form is a conglomerate. This is the case for the unphosphorylated amino acid [9].

* The value earlier reported [7], referred to the specific rotation. Owing to an error in proofreading, the parentheses around the letter *a* are missing.

SUMMARY

L-phosphothreonine has been prepared from an acid hydrolysate of bovine casein. It has been characterized by its position on paper chromatograms, by elementary analysis, melting point, optical rotation and the dimensions of the unit cell.

The author is indebted to prof. G. Ågren for valuable help and criticism. The investigation was supported by a grant from the Swedish Medical Research Council. The author is furthermore grateful to Mr. W. Kirsten and Mr. E. Lindberg for the elementary analyses.

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Chromatographic Separation and Quantitative Determination of Monosaccharides

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The identification of the single monosaccharide components occurring in polysaccharides and their quantitative determination used in the past to be a quite difficult task. The introduction of partition chromatography for carbohydrate analysis completely changed the situation. The single monosaccharides can now easily be separated, and minute amounts of them determined quantitatively by accurate colorimetric methods.

Prior to the introduction of partition chromatography many attempts were made to separate different sugar derivatives by means of adsorption and elution (see Binkley and Wolfrom [1]). The procedures used, however, were far from satisfactory.

Partridge [2] in 1946 first used paper chromatography for the qualitative analysis of sugar mixtures and Flood, Hirst and Jones [3] in 1948 developed a paper chromatographic method for the quantitative determination of single monosaccharides. A number of drops of the solution to be analyzed were placed along the starting line on the filter paper, one of them some distance apart from the others. This last drop was used for the qualitative identification of the various components. These being identified, strips containing the various monosaccharides were cut from the dried filter paper parallel to the starting line. The strips were eluted and the amount of sugar determined quantitatively. The technique had its limitations as some pairs of sugars such as glucose and galactose, ribose and lyxose as well as ribose and fucose migrate with almost equal speeds, thus making separation difficult.

The first separation of unsubstituted monosaccharides on a column of powdered cellulose was performed by Hough, Jones and Wadman [4] in 1948. After further development their technique [5] permitted the isolation of sufficient amount of sugar for qualitative identification of the components as well.

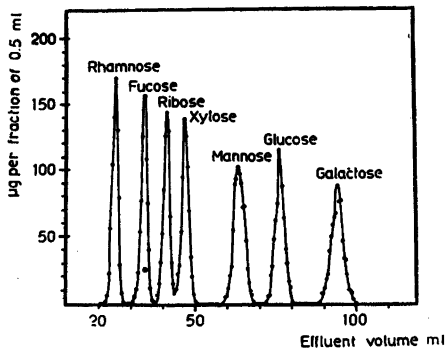

Fig. 1. Separation of seven monosaccharides on a 20 × 0.9 cm starch column. Solvent butanol : propanol : water 4 : 1 : 1.

Khym and Zill [6] separated several monosaccharides on columns of ion exchange resins using a borate buffer as eluent.

The present author has elaborated a technique for the separation and quantitative colorimetric estimation of the ordinary pentoses and aldohexoses. A starch column was used with a *n*-butanol : *n*-propanol : water mixture as the moving phase. Equal fractions of the effluent were collected and their content of sugar was determined colorimetrically directly on the solvent mixture as described by Gardell [7]. The separation could be visualized by constructing concentration-effluent curves (see Fig. 1). By summing the quantities found in the separate fractions the total amount of any individual sugar could be obtained, and the effluent volume for each peak in the curve indicated the nature of the individual sugar. If a column of a definite length is used the effluent volume for a monosaccharide is fairly constant. In Fig. 1 the separation of seven monosaccharides on a 20 × 0.9 cm starch column, and their quantitative analysis are demonstrated. (Run no. 2 in Table 1.)

Table 1. Recovery

Running no.	Rhamnose			Fucose			Ribose			Xylose			Mannose			Glucose			Galactose		
	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery
1	338	358	105.9	1056	1084	102.7	175	170	97.1	861	925	107.4	372	355	95.4	1061	1111	104.7	368	360	97.8
2	720	746	103.6	734	690	94.0	745	698	93.7	726	708	97.5	802	834	104.0	702	703	100.1	746	746	100.0
3	674	728	108.0	104	97	93.3	336	347	103.3	384	390	101.6	890	848	95.3	365	359	98.4	346	324	93.6

EXPERIMENTAL

The *starch* and the *solvents* were purified routinely as described by Åqvist [8 p. 1035].

Preparation of the column: About 9 g of starch to be used in the 20 × 0.9 cm column was suspended in 25 ml of dry butanol to which had been added sufficient water to bring the total water present to 30 per cent of the weight of the anhydrous starch.

The suspension was filled into the glass tube, 50 × 0.9 cm, and left to settle under an air pressure of 5 cm Hg. When all the butanol had passed through the column, 200 ml of the butanol : propanol : water mixture (4 : 1 : 1) was run through under an air pressure of 10 cm Hg.

The hydrolysate of the polysaccharide was concentrated to a syrup *in vacuo* and excess hydrochloric acid was removed by repeated evaporation to dryness *in vacuo* after adding portions of water. Any insoluble residue was removed by centrifugation prior to the last evaporation to dryness.

Amino sugars and other ions occurring in quantities give rise to droplets of water in the solvent mixture to be applied and disturb the chromatographic separation. They were therefore removed by passing the hydrolysate before concentration to dryness through columns of cation and anion exchange resins.

The dry residue from the hydrolysate was dissolved in one volume of water (usually 1 ml), followed by one volume of dry propanol and four volumes of dry butanol. If the solution was not clear 0.4 ml of ethyl alcohol was added and the solution was made up to volume (10 ml) with the same solvent mixture. One ml of solution should contain 0.5—3 mg of each sugar component.

0.2—0.4 ml of the solution containing 100—1 200 μg of each component was run on to the top of the column and allowed to run down the column under an air pressure of 7—8 cm Hg, followed by 2 × 0.1 ml of the pure solvent mixture. Solvent mixture was then allowed to run through the column at a speed of 2 ml per hour, this requiring an air pressure of 10 cm Hg if the packing of the column was correct.

Collecting the effluent: Fractions of 0.5 ml of the solution leaving the column were collected. Since the colorimetric analysis to follow demands a definite composition of the solvent mixture the moving parts of the fraction collector were housed in a chamber saturated with the vapour of the solvent mixture (see Carlander and Gardell [9]). The fraction collector was started at the moment when the hydrolysate was applied to the column.

For quantitative determination of the amount of sugar present in the different fractions the method elaborated by Gardell [7] using the aniline-trichloracetate reagent was used. In this case, when 0.5 ml of effluent had been collected in each fraction, 0.5 ml of 96 per cent alcohol was added before adding the reagent. This was done with all the test solutions, standards, blanks and reference solutions.

Different sugars give different colour intensities with the reagent used. Therefore the nature of the sugar in each fraction was deduced from the effluent volume, which is fairly constant for columns of a definite length and width. The effluent volume was determined empirically with samples of pure monosaccharides. The columns to be compared were prepared with starch from the same batch.

All the readings of the tests as well as of the standards and of the blanks were made against a non-boiled reference solution consisting of 0.5 ml of the solvent mixture to which 0.5 ml of alcohol, 1 ml of the reagent and 2 ml of alcohol had been added. The optical density of the blank was subtracted from all the other values. The blank for the standards

had a composition similar to the reference solution but had been boiled, together with the standards.

The blank for the test solutions had been treated similarly but the 0.5 ml of the solvent mixture used had passed through the starch column, as described by Gardell [7, p. 1013].

DISCUSSION

When analyzing small amounts of sugars it is necessary to get sharp peaks in the effluent volume-concentration curves so as to be able to identify the single components. Preliminary experiments showed that this goal cannot be reached with the cellulose column which Hough, Jones and Wadman[4] used for separating larger amounts of the monosaccharides. Stein and Moore [10] showed very convincingly that starch columns give well reproducible chromatograms. We found the starch column still more advantageous, because, provided it is prepared exactly as described by Stein and Moore, it gives an improved separation of ribose from fucose, of xylose from ribose and of glucose from galactose. However different kinds of starch demand different amounts of water for saturation. When adequately packed, a starch column of 20 cm length allowed 1.5—2 ml of the butanol : propanol : water mixture to run through per hour under an air pressure of 10 cm Hg. The starch columns should be used only once. On repeated use the separation proved less satisfactory.

The composition of the solvent mixture, butanol : propanol : water (4 : 1 : 1) and the length of the column used allowed a good and quick separation of the monosaccharides occurring in the animal mucopolysaccharides. The water in the solvent mixture did not disturb the colorimetric analysis.

On such a column, 20 × 0.9 cm, the seven sugars rhamnose, fucose, ribose, xylose, mannose, glucose and galactose were completely separated. If arabinose was present, it preceded the mannose but was not completely separated from it. Lyxose came between ribose and xylose partly mixed with them. If only hexoses are present they can be separated in half the time on a shorter column of *e.g.* 10 cm length. If a component is not present in its expected place in the chromatogram, its presence can be excluded within the sensitivity limits of the colorimetric analysis. The identification of a component depends of course upon the accuracy with which its effluent volume can be determined. To be certain, a pure specimen of any component should be quantitatively recovered, if added to the hydrolysate, without any change in the symmetry of the concentration-effluent curve.

Ketoses cannot be analyzed with the aniline-trichloracetate method. On a column of this size 100—1 200 μg of each of the single monosaccharides

can be analyzed. For those components which are eluted most slowly the lower limit is higher due to the flatter shape of the curves at the end of the run. For the same reason a larger quantity of the hydrolysate than that given above cannot be applied as the components appearing first in the chromatogram would be found in concentrations so high as to be outside the range of the colorimetric method.

By using larger columns, larger fractions of the effluent can be collected, and this permits duplicate analyses on the single fractions and even isolation of material for chemical identification. For large scale preparative work cellulose columns are to be preferred for in spite of their poor resolving power they have a greater capacity and yield less impurities.

RESULTS

The yield in some experiments with pure specimens of monosaccharides are given in Table 1.

SUMMARY

A chromatographic technique has been elaborated for separating methylpentoses, pentoses and aldohexoses in amounts of 100 to 1 200 μg of each component as they occur in hydrolysates of polysaccharides and mucopolysaccharides. The separation was performed on a starch column using butanol: propanol : water (4 : 1 : 1) as the moving phase.

The effluent was collected in 0.5 ml portions with an automatic fraction collector, and the amount of sugar present in each fraction was determined quantitatively colorimetrically directly on the solvent mixture leaving the column.

Concentration-effluent curves were constructed allowing a qualitative identification of the components in the hydrolysate. By summing the amounts in each fraction containing a single sugar the amounts of the various components could be determined quantitatively.

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Separation on Dowex 50 Ion Exchange Resin of Glucosamine and Galactosamine and their Quantitative Determination

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Glucosamine (chitosamine) and galactosamine (chondrosamine) occur simultaneously as components of a number of natural polysaccharide mixtures. Morgan *et al.*[1] *e.g.* demonstrated that both of them are found in the blood group A substance isolated from the pseudomucin of ovarian cysts.

The amino sugars are usually identified as their hydrochlorides which crystallize from methanol on the addition of acetone or ether. Glucosamine can be identified fairly easily in this way, even when it is present in small amounts, but galactosamine hydrochloride is difficult to crystallize because of its solubility. Other methods have therefore been suggested, such as the isolation of the carbobenzoxy derivative [2] and isolation of various Schiff bases, such as those formed with 2.4-dihydroxybenzaldehyde [3] and with 2-hydroxynaphtaldehyde [4]. For the identification of small amounts of amino sugar Blix, Svennerholm and Werner [5] used X-ray spectrography.

Paper chromatography has been used by Partridge [6], Aminoff and Morgan [7], Gardell, Heijkenskjöld and Roch-Norlund [8], and also by Kent, Lawson and Senior [9]. A chromatographic method for the isolation and identification of small amounts of amino sugars in mixtures, based on separation of their N-dinitrophenyl derivatives has been worked out by Annison, James and Morgan [10].

Quantitative methods for the determination of amino sugars were elaborated by Elson and Morgan [11], Dische [12] and Tracy [13]. With none of these methods, however, is it possible to differentiate between glucosamine and galactosamine.

Flood, Hirst and Jones [14] used paper chromatography for quantitative determination of single monosaccharides occurring in mixtures. Morgan *et al.*[1] used the same principle for the determination of mixed amino sugars. After separation in a collidine water mixture the different components were eluted

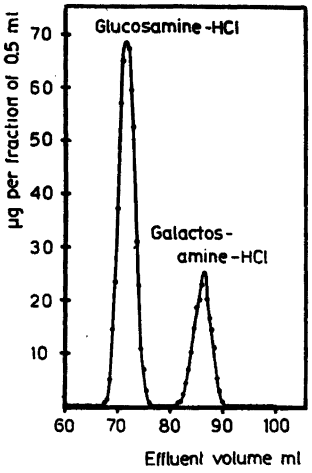

Fig. 1. Separation of 500 µg glucosamine-HCl and 225 µg galactosamine-HCl. Column 0.7 × 39.0 cm.

separately and the amount of amino sugar determined colorimetrically by the Elson and Morgan method. With this technique, not more than 30 per cent of the amino sugar was recovered, possibly due to destruction caused by the alkaline collidine.

The present author has endeavoured to avoid this source of error in the paper chromatographic method by separating the components in acid solvents. In none of the solvent systems tried (butanol, propanol and hydrochloric acid; propanol and hydrochloric acid; acetone, methanol and hydrochloric acid; methanol and hydrochloric acid, and others) was it possible to achieve a good separation. Separation in phenol is possible [6], but only if the chromatography is done with ammonia in the atmosphere, when the same source of error appears as with collidine.

Moore and Stein [15] and Partridge [16], in connection with their work on the separation of the amino acids, demonstrated that glucosamine migrates on columns of ion exchange resins.

In the present paper a chromatographic method is described for the separation of glucosamine and galactosamine hydrochloride on Dowex 50 ion exchange resin with 0.3 *N* hydrochloric acid as solvent. The liquid-chromatogram principle was applied and the effluent was collected in fractions of equal size, which were analyzed essentially by the Elson and Morgan method as modified by Blix [17]. By constructing concentration-effluent curves the resolving power of the column can be demonstrated directly. The effluent volumes corresponding to the peaks of the two amino sugars are so different when 0.3 *N* hydrochloric acid is used, that the separation of the components will be complete, thus making a quantitative determination of each of them possible. It is even pos-

sible to get sufficient amounts for crystallization. Fig. 1 shows a chromatogram made on a column 39.5 cm long and having a diameter of 0.7 cm.

PROCEDURE

Preparation of the column. Dowex 50, 250—400 mesh *, is washed with 4 *N* hydrochloric acid until the filtrate is colourless. The mass is then suspended in one volume of 4 *N* hydrochloric acid and is stored in this form. In preparing a column the mass is resuspended and an amount sufficient for a column of 35—40 cm length with a diameter of 0.6—0.7 cm is poured into a chromatographic tube (45 × 0.6—0.7 cm) fitted with a glass filter plate at the bottom and dilated at the top (2 × 5 cm). The tube is filled to a point about 0.5 cm from the upper edge. With the aid of a rubber stopper, the upper part of the tube is connected with a separatory funnel. A pressure of 10 cm of mercury is exerted on the funnel with the aid of compressed air. When the mass has formed a deposit of constant length the pressure is released and the funnel is filled with 0.3 *N* hydrochloric acid. A pressure of 7 cm of mercury is now applied and the column is washed with 0.3 *N* hydrochloric acid until the concentrations of the inflowing and outflowing acid are identical. This takes about 10 hours. The rubber stopper is now removed and the dilated upper part including the uppermost 1—4 cm of the tube are emptied of exchange resin and hydrochloric acid. The tube is once more connected to the funnel and a pressure of 10 cm applied until the upper surface of the column becomes dry without any air entering the column. The column is now ready for use. After use, the resin is removed, suspended in 4 *N* hydrochloric acid and washed with several volumes of 4 *N* hydrochloric acid.

Hydrolysis of the polysaccharide. The polysaccharide is hydrolysed with hydrochloric acid. After the hydrolysis the mixture is placed in a small beaker and evaporated to dryness in a desiccator over sodium hydroxide. The residue is then taken up in a suitable volume of 0.3 *N* hydrochloric acid, centrifuged to remove insoluble particles and chromatographed.

Application of the amino sugar mixture. 0.1—0.4 ml of amino sugar solution in 0.3 *N*, or stronger (up to 1 N), hydrochloric acid containing 60—600 μg of each component is added to the column and allowed to run into the column by gravity. When the amino sugar mixture is being added, care is taken to see that the upper surface of the column is not stirred up. The walls above the column are washed three times with 0.1 ml of 0.3 *N* hydrochloric acid without extra pressure, and the space in the tube, above the column, is filled with 0.3 *N* hydrochloric acid. By means of a tightly fitting rubber stopper and a piece of tubing the chromatographic tube is connected with a 500 ml flask containing the same acid and the position of the flask is regulated so that the rate of flow will be 1.5—2 ml an hour.

Collection of the effluent. The column is connected to a fraction collector ** and the effluent is collected in thin-walled test tubes, 15.4—16 × 120 mm, in 0.5 ml fractions. The volume of the effluent is measured from the moment when the amino sugar mixture is applied to the column.

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* From Microchemical Specialities, Berkeley 3. California.

** A modification of the apparatus described by Stein and Moore [18] was used. The apparatus was made by Svenska Mätapparater Fabriks Aktiebolag, Stockholm, Sweden, and is described in a paper by Carlander and Gardell [19].

Analysis. Reagents. Standard solutions of glucosamine and galactosamine hydrochloride in 0.3 *N* hydrochloric acid containing 10, 20, 50, 75 and 100 μg/0.5 ml. These solutions keep for about one month if stored in a refrigerator at + 4° C.

Acetylacetone solution. 1.5 ml of pure, colourless acetylacetone is diluted to 50 ml with 1.25 *N* sodium carbonate. The solution can only be kept at room temperature for three hours at the most. The stability is not appreciably increased in the cold.

The Ehrlich reagent. 1.6 g of p-dimethylaminobenzaldehyde, A. R., is dissolved in 30 ml of concentrated hydrochloric acid and 30 ml of 96 per cent alcohol is then added. The solution is pale yellow in colour and can be used for about two months if stored in the cold.

Technique. To each test tube containing a whole fraction in a volume of 0.5 ml, 1 ml of acetylacetone solution is added. The tubes are thoroughly shaken and placed in a rack equipped with a cooling system as described in a previous publication [20]. The openings of the tubes are covered with aluminium caps and the rack is heated in a water bath at 96° C for 60 minutes. A standard series with the above-mentioned five concentrations, 0.5 ml of each, and two blanks (0.5 ml of 0.3 *N* hydrochloric acid), all containing acetylacetone, are heated simultaneously. After heating, the tubes are placed in a cooling bath (tap water) for 5 minutes. The tubes are then removed from the bath, and 10 ml of 96 per cent alcohol followed by 1 ml of Ehrlich's reagent are added. The contents of the tubes are mixed carefully by blowing a stream of air through a capillary into the solution. After one hour, readings are made in a Beckman model B spectrophotometer or other similar apparatus with a 1 cm cell at 535 mμ. The standard series is read off first against the blank. As a correct blank for the amino sugar fractions is necessary for quantitative determinations, and as this blank does not necessarily need to be the same as that of the standard series, a few fractions of the effluent not containing amino sugar are included in each analysis. These fractions are read off against the blank of the standard series and the fraction giving a value most closely corresponding to the mean of these readings is used as blank in analyzing the amino sugar fractions. This value should not diverge from the blank of the standard series by more than ± 0.020 with a 1 cm cuvette.

Calculation. A standard curve is compiled for each amino sugar. In order to know which component corresponds to the respective amino sugar, it is necessary to know the effluent volume for both of the amino sugars. As this varies slightly for different batches of the Dowex resin it must be determined with the pure amino sugars. For one and the same batch of resin used in the same chromatographic tube, the effluent volume is constant and proportional to the length of the column. For columns of different diameters it is also proportional to the area of the cross section.

When the peaks have been identified each fraction belonging to the same peak is read off against the standard curve for the corresponding amino sugar. The total amount is obtained by adding the amounts of the different fractions belonging to the same peak. The separation can be illustrated by constructing concentration-effluent curves, the amount of amino sugar in each fraction being given on the ordinate and the total amount of ml of effluent on the abscissa.

Identification of the components. This can be done with the aid of the effluent volume for each peak. When there is any uncertainty as to the identity of the amino sugar, especially if there is only one peak, the presumed component is added to the hydrolysate in a new test and the mixture is chromatographed. The presence of only one peak in the concentration-effluent curve indicates with a high degree of certainty the identity of

the amino sugar. If large amounts of material are present the hydrolysate can be chromatographed on a column of larger dimensions and the components crystallized (see below).

RESULTS

Recovery. The recovery from a series of chromatograms in which varying amounts of the two components were chromatographed is demonstrated in Table 1.

Table 1. Recovery

Glucosamine hydrochloride				Galactosamine hydrochloride		
Running no.	μg	Recovered in the first component μg	Percentage recovery	μg	Recovered in the second component μg	Percentage recovery
1	200	201.8	100.9	400	380.0	95.0
2	300	286.0	95.3	—	—	—
3	200	225.0	112.5	400	427.0	106.8
4	400	415.0	103.8	400	426.0	106.5
5	400	397.9	99.5	400	397.7	99.4
6	250	279.0	111.6	500	499.4	99.9
7	600	547.5	91.3	60	64.8	108.0

Identification of the components by crystallization. In order to determine which amino sugar corresponded to the respective peaks a mixture of 50 mg of each component was chromatographed. A column having the dimensions 3.9 × 48 cm was used. This was prepared in the same way as those of smaller dimensions described above. The substances were dissolved in 2 ml of 0.3 N hydrochloric acid and the chromatography was performed in the usual manner with a rate of flow of about 5 ml an hour. The effluent was collected in 7.5 ml fractions. An amount of 0.5 ml was pipetted off from every third fraction and analyzed as described above. Fractions belonging to the same component were pooled and the hydrochloric acid was removed by repeated evaporation *in vacuo*. It was finally concentrated to a volume of 2 ml. The solution was mixed with 2 ml of methanol and was placed in a refrigerator at −10° C. Cold acetone was added dropwise to opalescence and the solution was set aside in the cold overnight and more acetone was then added. When crystallization was complete the crystals were collected and washed first with methanol : acetone : 1 *N* hydrochloric acid (2 : 4 : 1) and then with pure acetone. After

drying *in vacuo* the substance was weighed and dissolved in 1 ml of water. Optical rotation was determined in a 0.5 dm tube. The results are set out in Table 2.

Table 2. Identification of the components

Component I Glucosamine-HCl		Component II Galactosamine-HCl	
Found	Theoretical	Found	Theoretical
$(\alpha)_D^{20}$ final + 71.8°	+ 72.5°	+ 96.2°	+ 96.4° (Levene)

As may be seen from the table, the first component is glucosamine hydrochloride and the second galactosamine hydrochloride.

DISCUSSION

Size of the fractions. By collecting fractions of 0.5 ml pipetting is avoided. Larger fractions do not allow the resolving power of the column to be fully utilized.

Length and diameter of the column. As the method is intended to be a micromethod, the dimensions of the column was chosen to comply with this. However, the method can be scaled up for larger amounts.

Use of the column for several experiments. As the amino sugars are quantitatively recovered in the effluent the same column can be used again when dealing with pure substances. When hydrolysates are analyzed there is a possibility that other substances may be present which can disturb the quantitative analysis.

Amount of amino sugar. Amounts of 800 μg of each component have been separated on columns of the dimensions 0.6 × 40 cm but the yield tends to be less satisfactory as a number of fractions are too concentrated to permit accurate quantitative analysis by the colorimetric procedure. If amounts larger than 600 μg of each component are to be separated, larger columns should be used and the effluent must be collected in larger fractions and an aliquot pipetted off for analysis.

Relationship between the components. A ratio of 1 : 5 between the amounts of the components makes no difference to the analytical results as compared with the ratio 1 : 1. If the differences are larger, the determination of the smaller component will involve relatively large errors. It is then necessary to make two chromatograms with different amounts of the hydrolysate. By reducing the rate of flow, ratios slightly greater than 5 : 1 can be analyzed.

Rate of flow. An increase in this means that the concentration-effluent curve will be flatter and broader; a larger number of fractions will then have concentrations lower than desirable for analysis by the colorimetric method, and the errors will be larger. For a column of the dimensions mentioned, a rate of flow of 1.5—2 ml an hour has been found to be optimal for good separation. As a reduction in the rate of flow gives a slightly sharper separation it is sometimes of value to reduce the rate, especially if one of the components is present in small amounts, this making it possible to determine the smaller component with greater accuracy.

Hydrochloric acid concentration. If the hydrochloric acid concentration is increased the effluent volume for the peaks will be smaller while the separation will be less satisfactory. A decrease in the concentration of hydrochloric acid produces an increase in the effluent volume. The difference between the maxima for glucosamine and galactosamine will be greater but the curves will be flatter and broader, with the above-mentioned disadvantages as the result.

Technique of analysis. Blix [17] has demonstrated that an acetylation time of 60 minutes is necessary for concentrations of more than 0.09 mg hexosamine hydrochloride and is applicable up to 0.14 mg in 26 ml. The upper limit for the technique described in this paper seems to lie at 0.1 mg hexosamine hydrochloride in the final volume of 12.5 ml. The portions should not be neutralized before the addition of acetylacetone solution since the pH of the mixture before boiling is 9.7 and after boiling 9.4, an alkalinity which according to Sørensen [21] is optimal for the colour development. The volume of the fractions is not allowed to vary more than $\pm$ 5 per cent.

Influence of other sugars and amino acids. The common hexoses, methylpentoses and pentoses do not interfere with the separation and the analysis as their effluent volume is very small. Amounts of up to 600 μg of each of the common amino acids have no effect on the separation of the amino sugars.

Analysis of a polysaccharide fraction from the cornea. A polysaccharide fraction obtained from cornea after digestion with proteolytic enzymes and removal of the proteolytic split products by means of Lloyd's reagent, was analyzed.

Preliminary paper chromatographic experiments had shown that both amino sugars were present as found also by Woodin [22].

114.5 mg of the polysaccharide dissolved in 5 ml of 6 *N* hydrochloric acid were heated under reflux. After 2, 8 and 24 hours 1 ml samples were pipetted off, placed in a small beaker and cooled. They were then evaporated to dryness *in vacuo* over sodium hydroxide. The residue was taken up in 3 ml of 0.3 *N* hydrochloric acid and 0.4 ml samples were analyzed with the technique described.

The results are given in Table 3.

Table 3. Glucosamine and galactosamine in the corneal polysaccharide

Time of hydrolysis h	Percentage of amino sugar expressed as hydrochloride				
	Component				Total
	I	II	III (glucosamine)	IV (galactosamine)	
2	2.0	1.8	13.7	7.9	25.4
8	1.4	—	14.9	9.6	25.9
24	Trace	—	15.1	9.4	24.5

It is evident from the table that besides the components III and IV identified by means of their effluent volumes as glucosamine and galactosamine hydrochloride respectively there are other components giving the Elson-Morgan reaction. After 2 hours of hydrolysis there are two of them and after 8 hours one is still left. The identity of these components has not been further investigated. They, however, diminish in quantity with time of hydrolysis. They are probably products of partial hydrolysis. The value for the total amount of hexosamine corresponds fairly well to the amount of 25 per cent of glucosamine hydrochloride as found by direct determination after hydrolysis of the polysaccharide.

SUMMARY

A chromatographic technique is described for the separation of glucosamine hydrochloride and galactosamine hydrochloride when they occur together in hydrolysates.

The separation is carried out on a column of Dowex 50 cation exchange resin with 0.3 *N* hydrochloric acid as the developing solvent.

The effluent is collected in 0.5 ml fractions which are analyzed quantitatively by the Elson and Morgan method as modified by Blix. A concentration-effluent curve demonstrated the resolving power of the column.

Using this technique the individual amino sugars could be analyzed quantitatively in amounts between 60 and 600 μg. The technique can be modified for the separation of larger quantities. A polysaccharide from the cornea has been analyzed by the procedure developed.

The Author is greatly indebted to the head of the institute, Professor Erik Jorpes, for invaluable advice and criticism during the performance of the work and also to Miss Janina Patmalnieks for technical assistance.

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Uptake of Sulphates in Mucopolysaccharides Esterified with Sulphuric Acid in the Skin of Adult Rats after Intraperitoneal Injection of S^{35}-labelled Sodium Sulphate

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Meyer and Chaffee [1] reported the occurrence of chondroitin sulphate in the skin of swine, and Pearch and Watson [2] showed it to be present in human skin.

Dziewiatkowski *et al.*[3] found that articular cartilage of 7 days old rats showed a high uptake of S^{35} after injection of labelled sodium sulphate. The major portion of the labelled sulphur in cartilage is present in the sulphate group of the chondroitin sulphate (Dziewiatkowski [4], Boström [5]) from which it is slowly eliminated (Boström [5]).

In the present investigation the exchange of the ester sulphate group in the chondroitin sulphate of the skin has been studied. A mucopolysaccharide fraction containing sulphate esters was isolated from the skin of adult rats at different times during a period of 16 days after intraperitoneal injection of sodium sulphate labelled with S^{35}.

EXPERIMENTAL

Nine groups of adult white rats *, each group containing 20 animals weighing 250 to 300 g, were given sodium sulphate ** labelled with S^{35} intraperitoneally, 1.5 mg of sodium sulphate dissolved in 0.5 ml of water to each animal (3.5×10^6 counts per minute per square centimetre measured as barium sulphate at infinite thickness). The animals were killed at intervals, those in the first group after two hours, those in the last group after 16 days, and the animals within the different groups were pooled. The skin was removed and washed thoroughly with cold water, then finely ground in a mechanical meat grinder.

* The same animals as used for previous experiments (Boström [5]).

** Obtained from A. E. R. E., Harwell, England.

The material was immediately suspended in 20 parts of acetone, filtered with suction and dried with acetone. The dry powder from each group of 20 animals weighed 200 to 250 g and was divided into three portions which were treated as follows.

I. Twenty g of the dry powder was hydrolyzed with 100 ml of 6 *N* HCl on a boiling water bath for four hours. The hydrolysate was diluted to 600 ml and the sulphate was precipitated with barium chloride. The precipitate was collected on a Gooch porcelain crucible, ignited and weighed. The combined free and ester sulphates in the skin corresponded to 0.02 to 0.03 per cent of sulphur in the dry powder.

II. Five g of dry powder was hydrolyzed with 20 ml of 10 per cent sodium hydroxide on a boiling water bath overnight. The solution was transferred to a nickel crucible and evaporated to approximately 5 ml. Five g of anhydrous sodium carbonate was added and the solution was evaporated in an oven at 110—120° C overnight. The material was then oxidized with sodium peroxide, as recommended by Bailey [6], and the sulphate was precipitated as barium sulphate. The total sulphur content of the dry powder was found to be approximately 0.5 per cent.

III. The remainder of the dry powder, about 175 to 225 g, was added in small portions to 1 l. of boiling water. When addition was completed, the suspension was boiled for 15 minutes, then allowed to cool to about 40° C, and the pH was adjusted to 7.5—8 with 2 *N* sodium hydroxide. Five ml of glycerol extract of pancreas dry powder was added and the mass was digested in the presence of toluene at 40° C. The pH of the mass, which during the digestion turned slightly acid was tested at frequent intervals and when necessary adjusted to 7.5—8. The digestion was followed by determining the amount of amino nitrogen liberated.

The glycerol extract had been obtained by shaking 1 part of pancreas dry powder for four hours with 10 parts of 87 per cent glycerol; the mixture was then diluted with an equal volume of water and filtered.

Five ml portions of glycerol extract were added during the course of the digestion after one, two and three weeks, respectively. As after this period of time the digestion did not seem to proceed further, it was interrupted and the mixture was concentrated to a thick syrup and dialyzed against running water in a cellophane bag for 48 hours. After further concentration, 10 volumes of alcohol were added and the precipitate was centrifuged and dried with alcohol and ether. It was again dissolved in about 250 ml of water and digested with 5 ml of a glycerol extract of pig's intestinal mucosa obtained in the following way: 1 part of the intestinal mucosa was shaken for four hours with 10 parts of 87 per cent glycerol, diluted with 2 volumes of water and centrifuged. After digestion for about two weeks the mixture was concentrated by evaporation, dialyzed, concentrated again, and treated with 10 volumes of alcohol. The precipitate was suspended in 100 ml of water, made alkaline to litmus with 2 *N* sodium hydroxide and centrifuged. The clear centrifugate was precipitated with 10 volumes of alcohol and the precipitate formed was dissolved in a very small volume of water, and the solution centrifuged after acidification to Congo red with 2 *N* HCl. The centrifugate was precipitated with 10 volumes of alcohol. The precipitate formed was again dissolved in water, 5 gm of thoroughly washed Lloyd's reagent was added and the mixture made just acid to Congo red with HCl. The sample was shaken for 15 minutes and centrifuged, and the clear centrifugate was precipitated with 10 volumes of alcohol. The precipitate was dried with alcohol and ether. Yield, 250 to 800 mg.

The analyses of the various preparations are given in Table 1.

Table 1. Analysis of the mucopolysaccharide fractions.

	Per cent of air dry substance		
	Mean value	Maximum	Minimum
Nitrogen (Kjeldahl)	4.76	6.73	3.42
Sulphur in ester sulphates	1.51	2.04	1.17
Uronic acid (Tracy [11])	23.3	26.1	19.2

CHARACTERIZATION OF THE SKIN MUCOPOLYSACCHARIDE

In order to characterize the polysaccharide more precisely, the following experiment was carried out. When the amount of material necessary for the analyses and radioactivity measurements had been taken, the nine preparations were combined. The polysaccharide was then subjected to electrophoresis in Hyflo Supercel as described by Gardell, Gordon and Åqvist [7]. The result of this investigation is shown in Fig. 1.

Two distinct components were present. Three runs were made and all the fractions containing the same component were combined. After evaporation of the solution *in vacuo* to a syrup the polysaccharide was precipitated with alcohol, dried and weighed. The substances were then hydrolyzed on a boiling water bath for 8 hours with 6 *N* hydrochloric acid. As the amount of substance was too small to permit measurement of radioactivity and determination of sulphur, sodium sulphate equivalent to 25 mg of barium sulphate was added to the hydrolysate and the sulphate was precipitated with a slight excess of barium chloride. The precipitate was collected for radioactivity measurements in the usual way and weighed. This gives the approximate amount of sulphur present in the polysaccharide. The supernatant was evaporated several times *in vacuo* to remove the hydrochloric acid and finally taken up in 2 ml of 0.3 *N* hydrochloric acid. The amino sugar in the hydrolysate was then determined qualitatively and quantitatively by the chromatographic method described by Gardell [8]. Since the polysaccharide was a mixture of the original nine preparations, no quantitative interpretation of the radioactivity measurement was made on this preparation. The slow component,

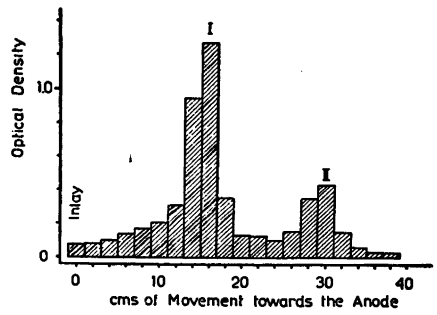

Fig. 1. Electrophoresis of rat skin polysaccharide in Hyflo Supercel.

however, showed no significant radioactivity while the fast one had about 1 000 counts/min./cm ^{2}. The results of the chemical analyses are given in Table 2.

Table 2. Analysis of the slow (I) and fast (II) moving fractions of the skin polysaccharide.

Component	Weight	Per cent of air dry substance		
		Sulphur in ester sulphates	Glucosamine-HCl	Galactosamine-HCl
I	31.6 mg	None	10.4	Trace
II	15.2 mg	4	Trace	19.8

The analysis of the polysaccharide from the rat skin thus indicated that the sulphur-containing portion was chondroitin sulphate. This is in agreement with the results obtained by Meyer and Chaffee [1] who found chondroitin sulphate and "isomers" of it as the only sulphur-containing polysaccharides in the skin of pig. The slow moving fraction contains hyaluronic acid.

RADIOACTIVITY MEASUREMENTS

In order to exclude the possibility that the samples of mucopolysaccharides obtained were contaminated with free sulphates labelled with S ^{35} the following experiment was undertaken.

100 mg of one of the samples was dissolved in 20 ml of distilled water, 200 mg of non-labelled sodium sulphate was added and the solution was dialyzed against running tap-water in a cellophane bag for 24 hours. After

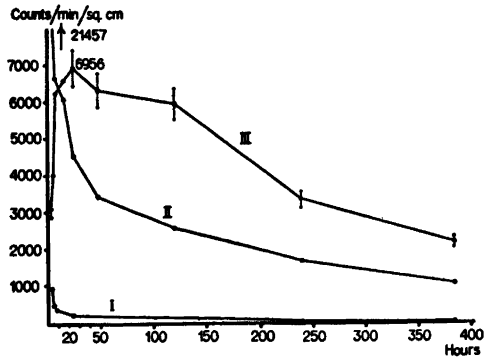

Fig. 2. Radioactivity (as counts/min./cm²) plotted against time in hours. Curve I relates to the total sulphur of the skin, curve II sulphur obtained after acid hydrolysis of the skin, and curve III ester-bound sulphate in the isolated mucopolysaccharide fraction of the skin. The possible errors in the measurements are marked on curve III.

concentration by evaporation the sample was precipitated with 4 volumes of alcohol and dried with alcohol and ether.

No significant difference in the radioactivity of the sample before and after this procedure could be demonstrated.

Approximately 250 mg of these mucopolysaccharide preparations were hydrolyzed with 20 ml of 6 *N* HCl, and the sulphate was precipitated as barium sulphate for radioactivity measurements.

The radioactivity of the various barium sulphate precipitates was determined as described in a previous communication (Boström [5]), using a Geiger-Müller counter with 2.1 mg per square centimetre mica end window tubes. After plating, the samples contained at least 25 mg of barium sulphate per square centimetre, corresponding roughly to infinite thickness. The results are expressed as counts per minute per square centimetre. The error in measuring the radioactivity of a sample amounted to $\pm$ 7 per cent.

RESULTS AND DISCUSSION

Fig. 2 shows the measured activities, expressed as counts per minute per square centimetre, plotted against the time in hours. The total sulphur in the skin of rats (curve I) shows marked activity merely during the first few hours (950 counts/min. after two hours), followed by a rapid decline (200 counts/min. after 24 hours and 20 counts/min. after 16 days). Evidently the administered sulphate labelled with S^{35} was highly diluted with sulphur-containing compounds with an extremely low or no demonstrable uptake of sulphur after injection of the labelled sulphate. According to Tarver and Smith [9] sulphate sulphur cannot be utilized for the synthesis of, for example, cystine in the rat.

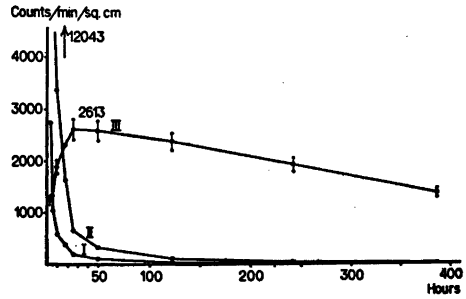

Fig. 3. Radioactivity (as counts/min./cm ^{2}) plotted against time in hours. Curve I relates to the total sulphur in the blood, curve II free sulphates in the costal muscles, and curve III the chondroitin sulphate. The possible errors in measurements are marked on curve III.

Curve II, which was obtained after acid hydrolysis of the dry powder prepared from rat's skin, shows a very high initial value (21 500 counts/min. after two hours), followed by a very rapid decline (3 500 counts/min. after 48 hours), and a considerably slower fall during the remaining part of the experimental period (1 020 counts/min. after 16 days). This curve is a summation of the curves for the free sulphates and the ester sulphates. The initial rapid decline in activity is due to the quick excretion of free sulphates (Dziewiatkowski [10], Boström [5]), while the more gently sloping portion of the curve depends mainly on radioactive sulphur in the ester sulphates.

Curve III shows the radioactivity of the sulphates liberated during acid hydrolysis of the skin mucopolysaccharide. This graph shows a 2 hour value of 3 400 counts/min. and a steep rise to a maximum value of 6 950 counts/min. after 24 hours, followed by a relatively slow decline to 1 020 counts/min. after 16 days. Nine to ten days after injection the content of isotope is half of the maximum.

If this graph is compared with the corresponding graph for chondroitin sulphate isolated from costal cartilage of rats in previous work (Boström [5], see Fig. 3), it is found that the curve for the mucopolysaccharides in the rat's skin reaches a considerably higher level and also shows a more rapid decline. Half of the radioactive sulphur which was introduced into the chondroitin sulphate of the costal cartilage was eliminated in about 17 days. The higher level reached in the skin polysaccharides is probably due to a more rapid exchange of fluid in the skin than in the cartilage. Both curves show a maximum uptake about 24 hours after injection.

SUMMARY

The exchange of the sulphate group in the mucopolysaccharides of the skin of the rat was studied by following the uptake and disappearance of S ^{35} during a period of 16 days after intraperitoneal injection of sodium sul-

phate labelled with S^{35}. Mucopolysaccharide fractions containing 1.2 to 2.0 per cent sulphur in ester sulphates were isolated from the skin of adult rats at various times after administration of S^{35}. The sulphur-containing fraction of skin polysaccharide was identified as chondroitin sulphate. The maximum uptake of radioactive sulphur was obtained 24 hours after the injection. The decline was relatively rapid, half of the maximum value being reached after nine to ten days.

The authors are greatly indebted to Professor Erik Jorpes for his valuable advice during the course of the work.

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Short Communications

The Importance of Alkali Chelates in the Alkylation of β-Ketoesters

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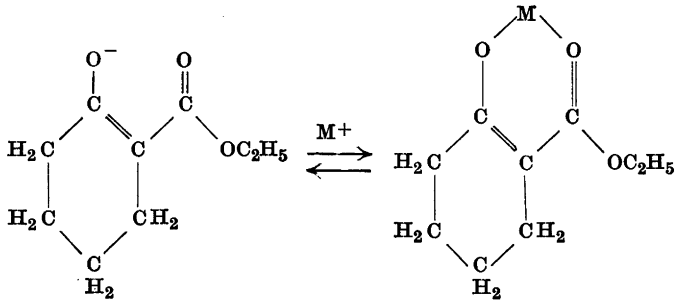
In a recent review of the chemistry of metal chelate compounds [1] Martell and Calvin gave an interesting example showing the difference in velocity of the alkylation of the sodium derivative of a β-ketoester from that of the corresponding potassium derivative. Thus the potassium compound of α-carbethoxy*cyclo*hexanone is alkylated about one hundred times as fast as the sodium compound in a toluene solution. In a tertiary butyl alcohol solution the reaction velocity of the potassium compound is nearly the same as that in the toluene solution. The potassium compound is alkylated twelve times as fast as the sodium compound when tertiary butyl alcohol is the solvent.

This remarkable difference in reactivity of the sodium and potassium derivatives was explained on the basis that the reaction was considered to take place with the enolate ion and that this ion was also engaged in a chelate formation reaction. See formulae below.

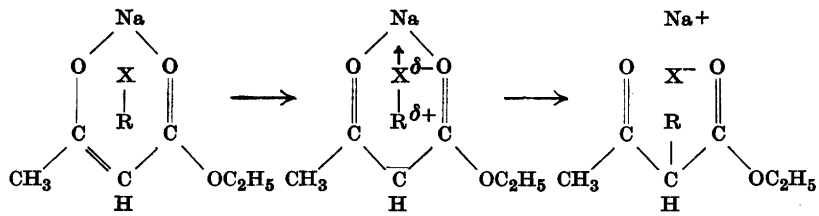
The tendency for K^+ to form chelates was considered much weaker than that of Na^+ with the effect that more enolate ions are available for alkylation when the potassium compound was used than when the sodium compound was employed. *The results are, however, better explained in the opposite manner.*

The statement, that the tendency for K^+ to form chelates is much weaker than that of Na^+, was based on the assumption that for chelate compounds of the primarily ionic type the strength of chelation should increase with increasing ionic charge of both metal ion and donor and increase with decreasing ionic radius of the metal ion. This assumption is, however, valid only when all other factors such as tendency for homopolar bonding, double-bond formation, and valence bond resonance are not important for the chelate formation. This is the case when, for example,



ethylenediamine tetraacetic acid is the chelation agent and the stability of the chelates formed with different alkali ions [2] is in this case in good agreement with those calculated on this purely ionic basis.

When the resonance effects are great, the above assumption is, however, not valid. This can be seen from the fact that no 2-covalent Li^+ compounds could be isolated from a great variety of β-ketoesters and related compounds by Sidgwick and Brewer [3]. Although many 2-covalent Na^+ compounds could be obtained, the K^+ had a still higher tendency to form such compounds. In the case under consideration (α-carbethoxy*cyclo*hexanone) the potassium compound is very soluble in toluene [4] but the sodium compound has only a limited solubility in the same solvent [5] indicating that *K^+ in this case has a stronger tendency than Na^+ to form a chelate compound.*



There are many indications which support the assumption, that the chelate compound and not the enolate ion is the one entering the C-alkylation reaction, but as this will be fully treated in a subsequent paper only one example will be given here.

The ammonium derivative of ethyl acetoacetate gives the O-alkylation compound with chloroacetone, whereas the sodium compound gives the C-alkylation compound [6]. This indicates that the enolate anion gives the O-alkylation product. (No chelate ammonium derivative is possible.) The fact that the sodium compound gives a C-alkylation product is good evidence that this reaction will not go *via* the enolate anion, as the sodium and the ammonium derivative should then give the same product which is not the case. The C-alkylation reaction will then very probably go *via* a chelate mechanism which is quite possible as chelate sodium compounds of ethyl acetoacetate have been isolated [3]. A chelate mechanism satisfactorily explains the fact that the sodium compound of ethyl acetoacetate gives C-alkylation compounds. This is best illustrated by the following formulae

The difference in the reaction rates of the Na^+ and K^+ compounds of α-carbethoxy*cyclo*hexanone can thus be explained in the following way:

The K^+ has a stronger tendency than Na^+ to give a chelate ring with the anion of α-carbethoxycyclohexanone, and thus, the C-alkylation reaction, which goes via the chelate compound, has a higher velocity with K^+ than with Na^+.

In tertiary butyl alcohol the alkali ions are more highly solvated and the difference in chelate formation of the K^+ and Na^+ ions is probably less in this medium than in toluene which can be seen from the fact that four covalent compounds are formed with Li^+, Na^+, and K^+ whereas two covalent ones are formed with Na^+ and K^+ only. The difference in reaction rates will hence be less in tertiary butyl alcohol than in toluene, which was also observed. In ethyl alcohol this difference is probably even less than in tertiary butyl

alcohol which can be seen from the fact that the relative reaction rates of the potassium and the sodium derivative of ethyl acetoacetate are almost equal [7].

In this connection it can be pointed out that a similar difference in reaction rates with different alkali derivatives of acetylacetone has been reported by the present author [8,9]. This was at first considered as a pure solubility effect [8], but further measurements showed that this is not the complete truth. Similar effects were observed in the methylation of ethyl α-cyanopropionate, ethyl α-carbethoxalylpropionate and ethyl acetoacetate with different alkali carbonates [10], but these kinetic results were not accurate enough to give any precise information about the mechanism.

1. Martell, A. E., and Calvin, M. *Chemistry of the Metal Chelate Compounds.* Prentice-Hall, Inc. New York 1952, p. 393.
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10. Brändström, A. Unpublished results.

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On the Structure of Nebularine

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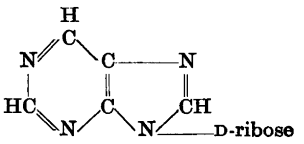
Nebularine, a principle active against *Mycobacteria* and present in the mushroom *Agaricus (Clitocybe) nebularis* Batsch., has been studied since 1945 by Löfgren *et al.*[1-3]. — We have now succeeded in isolating the compound in a pure state, m.p. 181—182° (corr.). The elementary analysis shows that the compound has the empirical formula $C_{10}H_{12}N_4O_4$. The hydrolysis gave the components purine and D-ribose. This is the first time purine has been demonstrated to be a component of a natural product. From spectroscopical data it can be concluded with high probability that the D-ribose is connected at position 9 of the purine nucleus. It is therefore possible to state that nebularine is 9-(D-ribosyl)purine:



Nebularine has a high activity against different types of tubercle bacilli *in vitro* (no experiments performed *in vivo*). Thus for instance, the bacteriostatic activity of nebularine on *Mycobacterium avium* in Dorset substrate is 1 : 3 000 000 after 12 days and 1 : 2 000 000 after 30 days *. — We found it to retard strongly the growth of barley seedlings and furthermore experiments on *Allium* roots show C-mitosis and chromosome breaks in a dilution of 1 : 100 000 **.

A full report will be published later.

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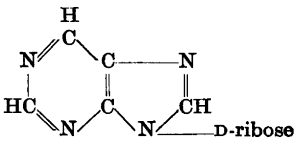

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Reactions of *Bis*(thiocarbamyl) Disulphides with Divalent Selenium and Tellurium Thiosulphonates, Thiosulphates and Xanthates

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The nucleophilic reactivities of thio anions, in displacements on divalent selenium and tellurium, increase in the order methanethiosulphonate, aromatic thiosulphonate, thiosulphate, xanthate and dithiocarbamate anions [1–6]. That is, the nucleophilic reactivities of the thio anions increase with decreasing oxidizing capacity of the corresponding thio pseudohalogens. Thus, dithiocarbamate anions react with divalent selenium and tellurium thiosulphonates, thiosulphates and xanthates, to displace the thio groups and giving divalent selenium and tellurium dithiocarbamates [1–6].

It has now been found that also *bis*(thiocarbamyl) disulphides react with these compounds, to give the same selenium and tellurium derivatives. In this case, the original thio groups are converted to the corresponding thio pseudohalogens, thus:

$$TeX_2 + (R_2NCS_2)_2 = Te(S_2CNR_2)_2 + X_2$$

Here, X represents methanethiosulphonate, aromatic thiosulphonate, thiosulphate and xanthate, and R_2N- is dimethylamino, diethylamino or piperidyl. In these reactions, the *bis*(thiocarbamyl) disulphides thus displace thio groups having higher oxidizing properties than themselves.

This behaviour must be due to a particularly high thermodynamic stability of the divalent selenium and tellurium dithiocarbamates.

The experiments were made with divalent selenium and tellurium methane- and benzenethiosulphonate and methylxanthate in chloroform solutions, by heating for a few minutes with a slight excess of the disulphide. In the case of the thiosulphates, the sodium salts were used, with methanol containing about 15 % of water and about 15 % of chloroform as a solvent.

Bis(xanthyl) disulphides do not react with the divalent selenium and tellurium thiosulphates or thiosulphonates, nor does tetrathionate react with the thiosulphonates. The difference in behaviour of dithiocarbamate and other thio compounds is further exemplified by the existence of tetravalent selenium [7] and tellurium [8,9] dithiocarbamates, whereas thiosulphate [1,2] and thiosulphonate [4,5] ions react with these tetravalent elements to give the divalent compounds together with the thio pseudohalogens. The tetravalent selenium and tellurium dithiocarbamates are, though, labile with respect to rearrangement into *bis*(thiocarbamyl) disulphide and the divalent dithiocarbamates. The selenium compounds "frequently, if not usually" [7] rearrange during the preparations, and the tellurium compounds do so on heating in solutions. The changes are not reversible; thus, *bis*(diethylthiocarbamyl) disulphide and divalent selenium and tellurium diethyldithiocarbamates do not react to give tetravalent diethyldithiocarbamates.

Bis(dimethylthiocarbamyl) disulphide, on heating with selenium and tellurium *bis*(diethyldithiocarbamate) in chloroform solutions, reacts to produce the corresponding divalent dimethyldithiocarbamates, which are less soluble and separate out. One may, by inference, conclude that exchanges probably take place also when the organic group of the *bis*(thiocarbamyl) disulphide is the same as that of the corresponding divalent selenium and tellurium derivative:

$$(Et_2NCS_2^*)_2 + Te(S_2CNEt_2)_2 \rightleftharpoons (Et_2NCS_2)_2 + Te(S_2^*CNEt_2)_2$$

and likewise with Se instead of Te.

It appears possible that such exchanges, and the reactions named in the title of this note, proceed over tetravalent selenium and tellurium compounds as intermediates.

1. Foss, O. *Acta Chem. Scand.* **3** (1949) 435.
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4. Foss, O. *Acta Chem. Scand.* **5** (1951) 115.
5. Foss, O. *Acta Chem. Scand.* **6** (1952) 521.
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Interactions of *Bis*(thiocarbamyl) Disulphides and *Bis*(*p*-anisyl) Ditelluride

OLAV FOSS

Universitetets Kjemiske Institutt, Blindern — Oslo, Norway

These reactions give tetravalent tellurium compounds, as follows:

$$(ArTe)_2 + 3\ (R_2NCS_2)_2 = 2\ ArTe(S_2CNR_2)_3$$

The *bis*(thiocarbamyl) disulphides here react with the ditelluride in the same way as do the halogens [1]. Disulphides show halogen-like properties also in other reactions [2, 3].

The products, *viz.*, *p*-anisyl telluritri-dithiocarbamates, represent a new type of organotellurium compounds. The same derivatives occur when *p*-anisyl telluritrichloride [1], in dioxane solutions, reacts with sodium dithiocarbamates:

$$ArTeCl_3 + 3\ R_2NCS_2^- = ArTe(S_2CNR_2)_3 + 3Cl^-$$

Thiosulphonate, thiosulphate and xanthate anions react with *p*-anisyl telluritrichloride in a different way, to give the corresponding thio pseudohalogens together with the tellurenyl compounds [4]:

$$ArTeCl_3 + 3\ X^- = ArTeX + X_2 + 3\ Cl^-,$$

The thio pseudohalogens, X_2 where X is methanethiosulphonate, aromatic thiosulphonate, thiosulphate and xanthate, do not react with *bis*(*p*-anisyl) ditelluride. The above equations thus illustrate, once more [5], a difference in the behaviour of dithiocarbamate and these other thio groups.

The *p*-anisyl telluritri-dithiocarbamates form stable, greenish yellow crystals, whereas ditellurides, and tellurenyl derivatives [4], have a red colour. The preparations were made by dissolving 2.5 millimoles of *bis*(*p*-anisyl) ditelluride and 7.5 millimoles of the disulphide in 10 ml of chloroform, heating, and addition of 15—20 ml of ethanol to the mixture. On continued heating, the products crystallized in about 90 % yields. The dimethylamino derivative is sparingly soluble in cold chloroform, the diethylamino and piperidyl derivatives are readily soluble. In pre-heated baths, these compounds melt and decompose at approx. 184°, 156° and 178° C, respectively.

Compound (Ar = *p*-anisyl)	% Te Calc.	Found
$ArTe(S_2CN(CH_3)_2)_3$	21.4	21.4
$ArTe(S_2CN(C_2H_5)_2)_3$	18.8	18.8
$ArTe(S_2CNC_5H_{10})_3$	17.8	17.7

In chloroform, tetrachloroethane and toluene solutions, the *p*-anisyl telluritri-dithiocarbamates possess pronounced thermochromic properties. On heating, the colour changes slowly and reversibly from greenish yellow to red. Addition of the parent *bis*(thiocarbamyl) disulphide to the hot red solutions causes the red colour to recede more rapidly on cooling. These phenomena are indications of a reversible

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dissociation, into the ditelluride and disulphide, or the corresponding radicals.

Some arylselenium tribromides, $ArSeBr_3$, dissociate into bromine and the selenenyl bromide [6]. For the *p*-anisyl telluritridithiocarbamates, the tellurenyl dithiocarbamate is, correspondingly, a possible dissociation product. However, these compounds appear to be unstable. Thus, they are not obtainable from *p*-methoxybenzenetellurenyl methanethiosulphonate [4] and sodium dithiocarbamates; instead, a mixture of *bis*(*p*-anisyl) ditelluride and the *p*-anisyl telluritri-dithiocarbamate results.

In ethylene dibromide at 20° C *bis*(dimethylthiocarbamyl) disulphide does not obey Beer's law, and thus perhaps dissociates into free radicals [7]. In view of this fact, and recent indications of radical dissociation of aromatic ditellurides [8], one may inquire whether the reactions of *bis*-(thiocarbamyl) disulphides with *bis*(*p*-anisyl) ditelluride take place by radical mechanisms. Qualitative observations have shown that *bis*(2-benzothiazyl) disulphide, for which radical dissociation in hot toluene solutions has been demonstrated by magnetic measurements [9], reacts with *bis*(*p*-anisyl) ditelluride in the same way as do the *bis*(thiocarbamyl) disulphides. Diphenyl disulphide, hot solutions of which do not obey Beer's law [10], is inactive.

Diphenyl ditelluride [8], according to preliminary experiments, reacts with *bis*(thiocarbamyl) disulphides to give compounds of the same type, and having analogous thermochromic properties, as does *bis*(*p*-anisyl) ditelluride.

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3. Schönberg, A., and Barakat, M. Z. *J. Chem. Soc.* **1949** 892.
4. Foss, O. *Acta Chem. Scand.* **6** (1952) 306.
5. Foss, O. *Acta Chem. Scand.* **7** (1953) 226.
6. Behaghel, O., and Müller, W. *Ber.* **68** (1935) 1540.
7. Bergem, N. *Contributions to the theory of vulcanization*, A/S Askim Gummivarefabrik, Norway, 1948, p. 136.
8. Farrar, W. V. *Research* **4** (1951) 177.
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α-N-Arylaminocarboxylic Acids as Plant Growth-Regulators

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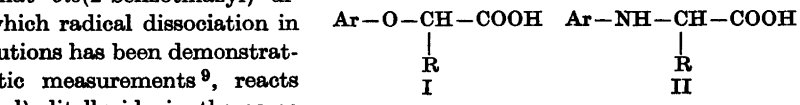
A considerable part of the work on the relations between chemical structure and biological activity of plant growth-regulators has been carried out on α-aryloxyalkylcarboxylic acids (I). A few α-N-arylamino-acids (II) have been investiga-



I II

ted in this respect and at least in one case such an acid was found to be highly active [1] while in another case it was stated that the replacement of the O atom by NH resulted in an inactive compound [2]. Thus our knowledge of the effect of replacing the oxygen bridge by an imino group is very unsatisfactory and this would be ample reason for a more thorough investigation of compounds of the type II. Also from a different point of view more notice should be paid to these amino-acid derivatives. Plant growth-regulators of the type I have not been found to occur in nature, which is very little surprising, as the active members of this series have structures which are far from "natural" looking. However, by replacing the oxygen bridge by an imino group, compounds are obtained,

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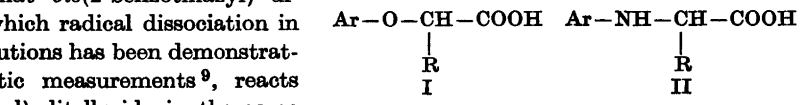

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which are closely related to the most important class of compounds occurring in nature, the α-amino-acids. These, with very few exceptions, belong to the L-series while optically active α-aryloxyalkylcarboxylic acids (I) with higher auxin activities than their respective antipodes most probably belong to the D-series [3–6]. A verification of the last statement would be of great value and might be carried out by an application of the method of quasi-racemates to optically active α-aryloxyalkylcarboxylic acids (I) and structurally similar α-N-arylamino-acids (II) prepared from optically active amino-acids with retention of the configuration. Another question which could be answered in connection with such an investigation is if the α-N-arylaminocarboxylic acids (II) show the same stereochemical specificity as do the corresponding oxy-acids of the type I.

Work along these lines is now in progress at this institute and a number of amino-acid derivatives have been prepared, some of which seem not to have been described in the literature before. The plant physiological properties of these compounds will be studied by Dr. B. Åberg who has kindly put some preliminary results at the author's disposal. According to Dr. Åberg α-(2.4-dichloroanilino)-propionic acid and α-(2-naphthylamino)-propionic acid possess auxin activities which are only a little lower than the activities of the corresponding α-aryloxyalkylcarboxylic acids.

Experimental. Optically active α-anilino-propionic acids. The strychnine salt of the racemic acid (m.p. 161—161.5°) was recrystallised several times from dilute ethanol. The pure salt on decomposition yielded the *(—)-acid* which was recrystallised from dilute ethanol; m.p. 149.5—150.2°. An acid with $[\alpha]_D = +50°$ (in ethanol) was obtained from the mother liquor from the first recrystallisation of the strychnine salt. This product with (+)-α-phenylethylamine yielded a crystalline salt which was recrystallised several times from acetone. From the pure salt the *(+)-acid* was obtained. After recrystallisation from dilute ethanol it melted at 149.5—150.4°.

0.1004 g (—)-acid in abs. ethanol to 10.00 ml: $\alpha_D^{25} = -0.627°$. $[\alpha]_D^{25} = -62.5°$; $[M]_D^{25} = -103.2°$.

0.1031 g (+)-acid in abs. ethanol to 10.00 ml: $\alpha_D^{25} = +0.643°$. $[\alpha]_D^{25} = +62.4°$; $[M]_D^{25} = +103.0°$.

Found:

Equiv. wt. 165.0 Calc. for (—)-$C_9H_{11}O_2N$ 165.2
» » 166.0 » » (+)- » 165.2

α-(2.4-Dichloroanilino)-propionic acid. 2.4-Dichloroaniline (8.1 g) and ethyl α-bromopropionate (4.5 g) were heated at 180° for one hour. The crystalline product was treated with water and the oil which separated was steam distilled with aqueous potassium hydroxide. The residue was acidified with dilute hydrochloric acid against methyl orange when the acid separated. The crude acid (2.5 g) was recrystallised from dilute ethanol yielding 2.2 g of pure acid; m.p. 148—149°.

Found: Equiv. wt. 233.4; N 5.96

Calc. for $C_9H_9O_2Cl_2N$: Equiv.wt. 234.1; N 5.985.

α-(2.5-Dichloroanilino)-propionic acid. 2.5-Dichloroaniline (8.1 g) and ethyl α-bromopropionate (4.5 g) were treated as above. 2.5 g of acid, recrystallised from dilute ethanol, were collected; m.p. 160.5—162°. Found: Equiv. wt. 234.7; N 5.93. Calc. for $C_9H_9O_2Cl_2N$: Equiv. wt. 234.1; N 5.985.

α-(3.4-Dichloroanilino)-propionic acid. 3.4-Dichloroaniline (16.2 g) and ethyl α-bromopropionate (9.1 g) were heated at 100° for two hours and at 130—140° for two hours and the product treated as above. After recrystallisation from dilute ethanol 7.8 g were collected; m.p. 145—146.5°. Found: Equiv. wt. 236.2; N 5.94. Calc. for $C_9H_9O_2Cl_2N$: Equiv. wt. 234.1; N 5.985.

This work is part of an investigation supported by *Statens Naturvetenskapliga Forskningsråd.*

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The Structure of Barium Tetrathionate

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The crystals of the salt, $BaS_4O_6 \cdot 2H_2O$, are monoclinic prismatic [1]; $a = 5.17$ Å, $b = 9.46$ Å, $c = 19.07$ Å, $\beta = 96°$. There are four molecules per unit cell; space group, $C_{2h}^5 - P2_1/c$. The present note gives preliminary results of a crystal structure analysis of the dihydrate.

Intensities of the reflections were estimated visually from Weissenberg photographs, taken with CuK radiation by means of the multiple film technique.

The y and z parameters of barium were found from a Patterson synthesis based on the $0kl$ data. A two-dimensional Fourier analysis, using signs of the reflections calculated from the barium contributions alone, gave a clear resolution of the four sulphur atoms. After successive refinements, the electron density map shown in Fig. 1 was obtained. The barium and sulphur parameters are:

	Ba	S_I	S_{II}	S_{III}	S_{IV}
y	0.093	0.190	0.101	0.090	0.283
z	0.136	0.285	0.382	0.446	0.502

The reliability factor $\Sigma||F|_{obs} - |F|_{calc}|/\Sigma|F|_{obs}$ for the $0kl$ reflections is 15.4 %.

Fig. 1 shows that the tetrathionate ion possesses an unbranched chain structure, the distances $S_I - S_{III}$ and $S_{II} - S_{IV}$ in the projection being 3.17 Å and 2.85 Å, respectively, *i.e.*, too large for bonds to exist between those atoms.

This is the first structure determination for a salt of tetrathionic acid.

1. Foss, O. *Acta Chem. Scand.* 6 (1952) 802.

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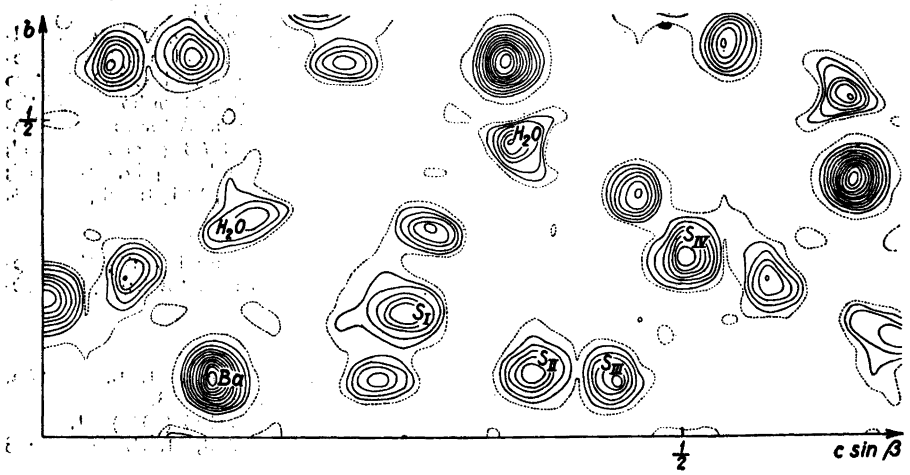

Fig. 1. Projection of $BaS_4O_6 \cdot 2H_2O$ along the a axis. The 4-electron line is dotted. Contour intervals: 10 e.Å^{-2} for the barium atom, 4 e.Å^{-2} for sulphur atoms, and 2 e.Å^{-2} for oxygen atoms and water molecules.

Piperazine Phosphate as Standard for the Neutral Range of the pH Scale Compared with Some Known Standard Solutions

K. V. GROVE-RASMUSSEN

Royal Danish School of Pharmacy, Department of Inorganic Chemistry, Copenhagen, and Bispebjerg Hospital, Pharmaceutical Department, Copenhagen, Denmark

When preparing standard solutions for calibration of glass electrodes it is advantageous to use a solution of a salt which forms spontaneously a well defined buffer solution when dissolved in water. The ideal properties required in such a salt are

1) Good buffer capacity,
2) Good crystallization conditions, *i.e.* on crystallization from an aqueous solution which contains an excess of acid or alkaline components, crystals of the proper composition must be formed,
3) Moderate solubility at room temperature so that easy purification of the salt can be obtained by recrystallization,
4) pH in the solution must show only slight dependence on concentration,
5) pH in the solution must show only slight dependence on temperature.

Hitherto three salts are known after thorough testing and examination to have proved satisfactory as standard solutions for the above-mentioned purpose. They are potassium hydrogen tartrate, potassium hydrogen phthalate and sodium borate, the former two representing the acid range of the pH scale, and the last one representing the alkaline range. Potassium hydrogen tartrate satisfies with reasonable tolerance all the above-mentioned conditions. Potassium hydrogen phthalate fails with respect to point (2), but a pure product is easily prepared when certain conditions are observed. Sodium borate fails with respect to point (5).

It is desired to obtain a buffer salt for the neutral range of the pH scale. The possibility has been chosen to find a secondary phosphate of a weak base which possesses as far as possible the mentioned properties. For this purpose piperazine phosphate meets in a very satisfactory way the first four of the mentioned conditions, whereas it fails with regard to temperature dependence, which is of the same direction and order of magnitude as that of sodium borate. As in calibration of glass electrodes a system is most frequently used which besides potassium hydrogen tartrate or potassium hydrogen phthalate, respectively, also includes sodium borate, the dependence on temperature of piperazine phosphate will not prevent its inclusion in such a system.

Piperazine phosphate ($C_4H_{12}N_2HPO_4$, H_2O; m.wt. 202.16) consists of fine white tabular or platy crystals. It is soluble in 80 parts water of room temperature and in 10 parts boiling water, insoluble in alcohol, ether and chloroform. A saturated solution at room temperature is about 0.065 molar. It may be prepared by crystallization from an aqueous solution of an equimolar mixture of piperazine and phosphoric acid. After one recrystallization it is fit for use. A comparison of pH in solutions of piperazine phosphate prepared in this way at five different laboratories showed an agreement of ± 0.001 unit.

pH in aqueous solutions depends equally on systems

$$C_4H_{12}N_2^{++}/C_4H_{11}N_2^{+} \text{ and}$$

$$H_2PO_4^{-}/HPO_4^{--}$$

with the acid dissociation exponents of the orders of magnitude of 5.5 and 7, respectively. The buffer capacity, which is almost linear in the pH 5—7.5 range, is 0.037 for an 0.05 *M* solution. For purposes of comparison it may be stated that the buffer capacity in a potassium hydrogen phthalate solution of the same molarity is 0.023.

pH in a piperazine phosphate solution is almost independent of concentration. At dilution 1 + 1 an 0.05 *M* solution showed no measurable change in pH. At dilution 1 + 7 pH was found to be 0.008 unit higher than in the undiluted solution. Thus it is possible to prepare an accurate pH standard by simply shaking an excess of piperazine phosphate with water. Lingane [1] has proposed the same method for potassium hydrogen tartrate.

For calibration of glass electrodes at ordinary room temperatures the values given in the following table are proposed:

pH Values for Calibration of Glass Electrodes

	14°C	16°C	18° C	20° C	22° C	24° C	26° C
0.025 *M* potassium hydrogen tartrate	(3.60)	(3.59)	3.59	3.58	3.58	3.58	3.58
0.05 *M* piperazine phosphate	6.38	6.36	6.34	6.31	6.29	6.27	6.25
0.05 *M* sodium borate	9.28	9.26	9.25	9.23	9.21	9.19	9.17

Parenthesized values are for a saturated solution at the pertinent temperature

The values stated for piperazine phosphate are obtained on the basis of glass electrode measurements by graphic interpolation between a total of 28 determinations in the interval 14° C—25° C. At 18° C hydrogen electrode measurements were made as well, and the value 6.337 was found, which is in agreement with the value of the table.

The values stated for potassium hydrogen tartrate are obtained by extrapolation or interpolation from those stated by Bates and others [2].

As points of departure for obtaining the borate values are used those stated by Hitchcock and Taylor [3] and accepted by British Standard [4], *viz.* pH at 25° C = 9.18 and pH at 38° C = 9.07. The values given in the table were obtained by graphic extrapolation from the two values, the temperature dependence being assumed to take a course parallel to those applying to lower ionic strengths, which are known from a paper by Manov, DeLollis and Acree [5]. The position of the two above-mentioned values at 25° C and 38° C in relation to the latter values justifies the assumption.

The experimental part of this investigation has been carried out in accordance with British Standard. Similarly the same principles — now accepted by British Standard — have been applied in the quoted literature [1–5]. With respect to potassium hydrogen phthalate these principles involve a pH value of 4.005 at 25° C in an 0.05 *M* solution. At calibration of glass electrodes of Corning 015 glass or glass of a corresponding composition on the basis of the above table, values are obtained in the interval pH 2—9 for diluted aqueous solutions which with good approximation represent the negative logarithm of the hydrogen ion activity. The present values are 0.02 to 0.04 units higher than those obtained on the basis of the original system stated by S. P. L. Sørensen [6].

The Pharmacopeia Laboratories in Norway, Sweden and Denmark have prepared samples of piperazine phosphate for which the author hereby brings his thanks. The author has received support towards carrying out the experiments from "*Danmarks Farmaceutiske Højskoles Jubilæumsfond*".

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A Quantitative Autoradiographic Study on the Uptake of Labelled Sulphate in the Aorta of the Rabbit

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The aorta is an elastic organ which is known to contain chondroitin sulphuric acid [1]. Nothing is as yet known about the rate of renewal of this substance in the aorta. It has, however, been shown by different workers [2-4] using tracer methods that a fairly high sulphate fixation takes place in the aorta, as in other tissues containing sulpho-mucopolysaccharides. This seems to be due to an exchange of the ester sulphate groups in these compounds. From earlier investigations [4-7] we have collected experience on the application of quantitative autoradiography with S^{35} in studies on the sulphate exchange of the mucopolysaccharides. It is fairly difficult to isolate sufficient amounts of mucopolysaccharides from the rabbit or rat aorta for metabolic studies. The autoradiographic technique was therefore chosen for the present investigation, in which an attempt was made to follow the incorporation and elimination of S^{35} in the adult rabbit aorta after a single injection of S^{35}-labelled sulphate.

27 adult female rabbits *, weighing on an average 2.9 kg, were given carrier-free S^{35}-labelled sodium sulphate ** intravenously, in doses of 0.65 mC per kg of body weight. The animals were divided into nine groups with three animals in each, and were sacrificed by air embolism 2, 4, 8, 16, 24, 48, 120, 240 and 384 hours, respectively, after injection of the radiosulphate. Part of the thoracic aorta was excised and fixed in pure methanol for 24 hours. It was then passed through *iso*-propanol (for 3 hours) and xylene (for 3 hours), embedded in paraffin and cut into 10 μ thick sections. These were mounted on metacrylate slides and deparaffinized in xylene and ethanol. They were then pressed against Gevaert Dentus rapid film for 24—27 days in an iron press for exposure [8] (exposure time noted). The films were developed in Kodak DK 20 with 0.2 per cent KBr for 30 minutes at + 18.3° C. The density of the autoradiographic images was then measured with a recording photometer [8]. After correction for the decay of the radioactive sulphur during the exposure time, the relative amounts of S^{35} in the aorta could be calculated.

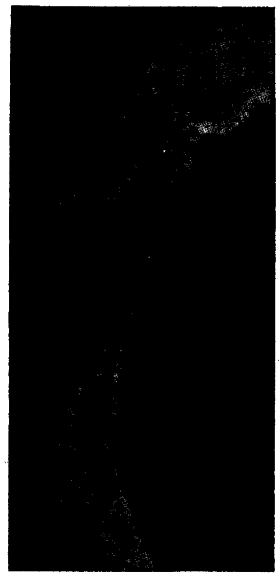

Fig. 1.

The autoradiographs obtained are exemplified in Fig. 1, which shows a fairly even distribution of S^{35} in all the layers of the aortic wall 48 hours after injection. The results of the quantitative estimations of the relative S^{35} content in the media are assembled in Fig. 2. As seen from the curve, a maximal accumulation of S^{35} seems to occur after about 2 days; about two weeks after the injection, half of this value could be noted.

Fixation with methanol results in retention of both inorganic sulphates and orga-

* The same animals as those used in a previous investigation [13].

** Obtained from A.E.R.E., Harwell, England.

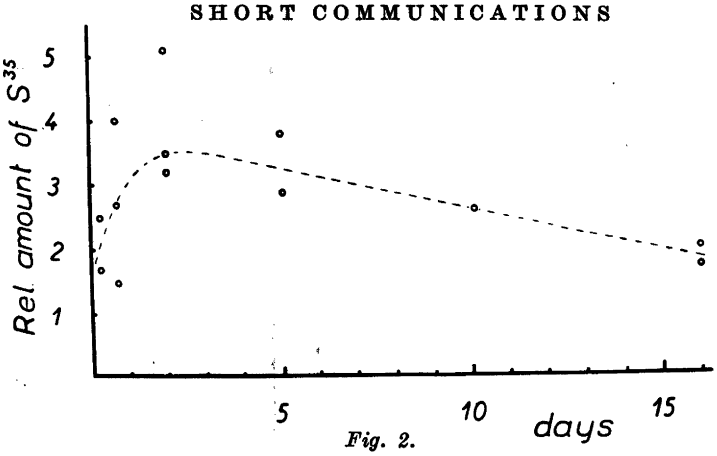

Fig. 2.

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For these reasons it appears probable that, in the aorta as well, the greater part of the S^{35} present more than 24—48 hours after the injection of sulphate is in the form of ester-bound sulphur in chondroitin sulphuric acid. Thus, the curve in Fig. 1 reflects the exchange of the sulphate group in the chondroitin sulphuric acid of the aorta, the rate of which exchange seems to be moderate.

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A Small Cell for Electrolytic Alkoxylation of Furans

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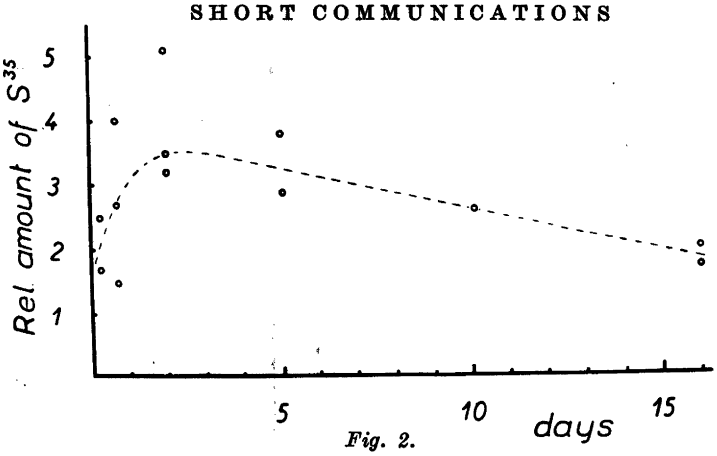
To date 14 different 2,5-dialkoxy-2,5-dihydrofurans have been prepared by electrolytic alkoxylation of furans [1-7]. These alkoxylations were carried out in a cell with an outer and an inner cathode, containing about 300 ml of electrolyte [1].



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The amounts of the various, analytically pure dihydrofurans, obtained in one run, varied from 11 to 96 g, the yields varied from 61 to 96 per cent. The dihydrofurans were all isolated by distillation.

We have now designed a small cell for alkoxylation of from 15 g down to less than 1 g of a furan. The cell, which only has one cathode, is filled with from 10 to 50 ml of electrolyte. The electrode distance is the same as in the larger cell (0.70 cm); but, since all the liquid in the small cell lies between the electrodes, the time for a single electrolytic preparation is reduced to about one third. The liquid is also cooled more efficiently than the liquid in the larger cell so that a larger current density can often be employed (about 4 instead of 2 ampere per dm^2). Hereby the time of electrolysis is further reduced.

The cell is especially useful for the preparation of small amounts of such dihydrofurans, as can be isolated by crystallization or be transformed without isolation into crystalline compounds.

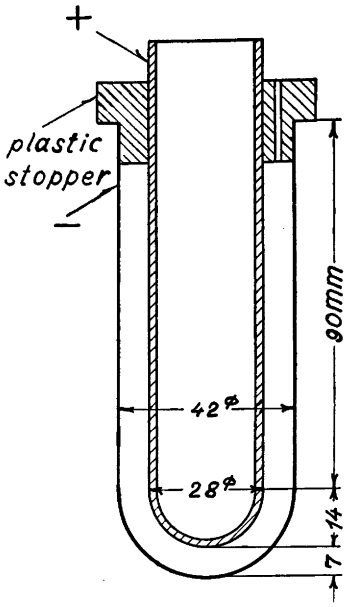

Fig. 1. Small cell for electrolytic alkoxylations.

Experimental. Fig. 1. gives a detailed design of the cell. The cathode consists of 0.5 mm sheet nickel, the anode is a glass tube covered with sheet platinum (welded on a metal form). The volume of the hemispherical part of the cell is 14 ml; the surface of the hemispherical part of the anode is 12 cm^2. During electrolysis the cell is placed in a bath of — 22° and connected with an ammeter and a coulometer of the ordinary domestic type.

In order to demonstrate the use of the cell for the preparation of small amounts of dihydrofurans a solution of 680 mg of furan (0.01 mole) and 150 mg of ammonium bromide in 10 ml of methanol was electrolyzed. 0.54 ampere hours (0.01 faraday) was passed through the cell (current 0.2—0.1 ampere, potential across the cell 4.2 volt, time of electrolysis 4.1 hours). After electrolysis 60 ml of *N*/10 hydrochloric acid was added and the mixture left standing overnight at room temperature. Hereby the dimethoxydihydrofuran formed was hydrolyzed to malealdehyde. 3.0 ml of phenylhydrazine and 5.0 ml of acetic acid in 40 ml of water were added. A yellow precipitate of malealdehyde bis-phenylhydrazone was immediately formed. After standing for 1 hour at room temperature the precipitate was filtered off, washed thoroughly with water and dried. Yield 1.84 g of crude hydrazone = 70 %; yield after crystallization from acetone-benzene 1.49 g = 56 %; m.p. 167—169° (Kofler stage, corr.); previously found [8] 171°. (Found: C 72.8; H 6.0; N 21.5. Calc. for $C_{16}H_{16}N_4$ (264.3): C 72.7; H 6.1; N 21.2.)

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Laboratory Preparation of the Fluorinating Agents SbF_3 and AgF

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I. ANTIMONY TRIFLUORIDE
(B. B. and A. H.)

Although SbF_3 is a compound which is necessary in carrying through the Swarts reaction in which chlorine may be replaced by fluorine, detailed instructions for its preparation on a laboratory scale is missing in the literature. Usually it is briefly reported that the substance has been prepared by the action of HF on Sb_2O_3, evaporation of excess HF and sublimation of the crude product. None of these operations can take place in glass vessels.

$$Sb_2O_3 + 6HF \rightarrow 2SbF_3 + 3H_2O$$

In the procedure given here it is described how great quantities of highly purified SbF_3 may be rapidly prepared in a platinum dish (or other dish resistent to HF and SbF_3) and purified in glass equipment without using the difficult and time-consuming sublimation.

Procedure. 100 g commercial Sb_2O_3 (0.34 mole) is gradually added to 200 g (4 mole) of freshly destilled hydrofluoric acid (37—40 %), placed in a platinum dish of known weight. The addition requires about 10 minutes. The dish is then placed on a sand-bath in a good hood and excess HF is evaporated. The heating is interrupted when the contents of the dish weigh 120 g (theoretical yield of SbF_3). After cooling to room temperature in a desiccator the crude SbF_3 is stirred with 120 ml methanol in the platinum dish for ten minutes. By rapid suction through filter-paper on a Büchner-funnel the methanolic solution of SbF_3 is freed from insoluble material and again placed in the platinum dish as fast as possible. The methanol is evaporated on a steambath, the process of evaporation being accelerated by the use of an upside-down funnel through which air is rapidly sucked. In twenty minutes the methanol is removed. The separated white crystals are crushed to a powder in the dish and treated with 100 ml anhydrous ethyl ether which dissolves the remaining traces of methanol. The SbF_3 crystals are separated by suction through filter-paper on a Büchner-funnel, washed twice with 50 ml anhydrous ethyl ether, dried for five minutes in the air and finally stored in a paraffined vessel *in vacuo* over concentrated sulfuric acid. Yield: 100 g (78 %).

Properties. SbF_3 is a white, crystalline compound. It attacks glass slowly and hydrolyzes in the air. 500 mg, placed on platinum in the atmosphere, gains 3 mg in 30 minutes at 22° C (relative humidity 60 %). If placed on glass 500 mg gains 6 mg in the same period. Thus, rapid operations in the air, involving the use of glass apparatus, are possible.

The purity of the preparation was controlled by determining the antimony and the fluorine equivalent weights. The former, found by titration with $KBrO_3$, was found to be 90.2 (theory 89.4), the latter, found by titration with $Th(NO_3)_4$ after precipitation of Sb^{3+} as Sb_2S_3 by H_2S, was 60.2 (theory 59.6). SbF_3 melts at 292° and boils at 319° C but these physical data are difficult to apply as criteria of purity in the ordinarily equipped laboratory.

SbF_3 is soluble in methanol (1 ml methanol dissolves 1.54 g of SbF_3 at 20° C) without chemical change as found and utilized by the present authors.

II. SILVER FLUORIDE.
(F.A.A. and B.B.)

AgF is of equal importance for fluorinating processes on a laboratory scale as SbF_3, but instructions for the preparation of this highly hygroscopic substance are not available in the literature.

$$Ag_2CO_3 + 2\ HF \rightarrow 2\ AgF + CO_2 + H_2O$$

Procedure. A solution of 38 g NaOH (0.95 mole) in 1 200 ml water is saturated with CO_2. The resulting solution of $NaHCO_3$ is slowly added to a solution of 136 g (0.80 mole) $AgNO_3$ in 400 ml water under vigorous shaking. This operation and the following must take place under exclusion of day-light (red light is permissable). The Ag_2CO_3 precipitate is separated from the $NaNO_3$-solution by decantation and afterwards washed four times with 500 ml water which is also removed by decantation. The remaining, moist Ag_2CO_3 is transferred to a platinum dish where 47 g 40 % HF (0.94 mole) is added in small portions under good stirring. When the initial, rapid evolution of CO_2 has ceased the reaction is finished by heating for thirty minutes on a steam-bath. The AgF-solution is separated from excess Ag_2CO_3 by filtering through ash-free filter-paper on a paraffined funnel into a paraffined beaker and afterwards placed in the (weighed) platinum dish again. The water is evaporated on a steam-bath under continued stirring until the weight of the sample is 115 g. The contents of the dish are now a mixture of about 90 g AgF (partly separated as crystals) and about 25 g water. In order to remove the water rapidly and completely 100 ml anhydrous methanol is added and, after thourough stirring, removed by decantation together with most of the water. The treatment with methanol is repeated twice and followed by a similar treatment with 100 ml anhydrous ethyl ether three times. The silver fluoride (moist with ether) is now quickly transferred to a round-bottomed glass flask with a side tube through which most of the ether is removed by a water aspirator. The last trace is evaporated by heating to 60—70° C in a water-bath and applying an oil pump. Yield: 75 g (70—80 g) of a light-brown powder.

The ether, used at the decantation, is added to the methanol together with 300 ml extra ether. A nice, yellow precipitate is separated from the mother liquor by decantation and washed twice with 25 ml anhydrous methanol and three times with 50 ml anhydrous ether. The last ether is removed as above. Yield: 15 g (14—16 g). The two crops are almost of the same quality although they differ in colour. Total yield: 90 g (0.72 mole) or 89 %.

Properties. AgF is highly hygroscopic and very sensitive to day-light. It attacks glass only very slowly at room temperature. It is stored in dark, paraffined vessels. The purity of the preparation was checked by determining the silver and the fluorine equivalent weights. For the brown and the yellow sample the silver equivalent weight was 126.4 and 127.3, respectively, (theory 126.9) while the fluorine determinations gave 124.4 and 122.0. 1 000 ml anhydrous methanol dissolves 14.5—15 g AgF at room temperature. In the dark and stored on a platinum container the solution is stable for at least 24 hours.

The method for the preparation of AgF here indicated is preferable to removing the water from an aqueous solution in a desiccator, a procedure which does not result in a well-defined product within a reasonable period.

Received January 8, 1953.

The Question of Furanosidic Bonds in Starch

BENGT LINDBERG

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In a recent study of the hydrolysis of starch with acids and with β-amylase, Blom and Schwartz [1] find that the change in specific rotation per mole hydrolysed bond, $[S_p]_D$, is 166—187° at the beginning of hydrolysis, the mean value for total hydrolysis 232° and the values for maltose and isomaltose 280° and 221°, respectively. They conclude that as the value for the first bonds hydrolysed is much lower than the value for maltose, these bonds must be of a quite different type, and assume, therefore, that they are furanosidic (1 : 4), and that the next glucose unit in the starch molecule is linked by a 1 : 5-bond.

The authors appear, however, to have overlooked the great differences between terminal and central bonds with respect to their contribution to the rotation. If, in a linear polysaccharide containing uniform bonds every second bond (n/2 bonds) is

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hydrolysed, n/2 molecules of the corresponding biose is obtained. If now the biose is hydrolysed, the net result is a total hydrolysis of the polysaccharide, and it is evident that the mean value of $[Sp]_D$ is the mean of $[Sp]_D$ for the central bond and that for the biose. For starch the $[Sp]_D$ for central bonds is thus calculated as 184°, in good agreement with the value found by Blom and Schwartz. In the series cellobiose to celloheptaose [2] and xylobiose to xylohexaose [3] there are also great differences between the rotation contributions of central and terminal bonds. The plot of $[M]_n/n$ against n-1/n, where $[M]_n$ is the molecular rotation and n the degree of polymerization, yields a straight line, however, which according to Freudenberg [4] means that the linkages in the series are uniform. In the maltose series only the next member, maltotriose, is known [5], but from the molecular rotation of this substance (+ 807°), of maltose (+ 470°), and of glucose (+ 95°), the $[Sp]_D$ for a central bond is calculated as 204°.

Thus Blom and Schwartz have merely confirmed experimentally the expected value of $[Sp]_D$ for central bonds in the starch molecule. They have not adduced any evidence supporting the occurrence of furanosidic bonds in starch. If the mere fact that the $[Sp]_D$ of a central bond is different from that of a terminal bond should constitute an argument for this assumption, it could equally well be applied to all tri- and higher saccharides.

1. Blom, J., and Schwartz, B. *Acta Chem. Scand.* **6** (1952) 697.
2. Wolfrom, M. L., and Dacons, J. C. *J. Am. Chem. Soc.* **74** (1952) 5331.
3. Whistler, R. L., and Chen-Chuan Tu. *J. Am. Chem. Soc.* **74** (1952) 4334.
4. Freudenberg, K. *"Tannin, Cellulose and Lignin"*, J. Springer, Berlin 1933, p. 104.
5. Sugihara, J. M., and Wolfrom, M. L. *J. Am. Chem. Soc.* **71** (1949) 3357.

Received January 13, 1953.

Crystalline Rhodanese

BO H. SÖRBO

Biokemiska Avdelningen, Medicinska Nobelinstitutet, Stockholm, Sweden

Recently we reported a method for the partial purification of rhodanese [1] from beef liver. The enzyme has now been further purified and obtained in the crystalline state.

The method of purification was as follows: Beef liver was extracted and the extract fractionated with ammonium sulfate at pH 3.8 and 7.8 as described before [1]. The enzyme was then dialyzed against 0.01 *M* sodium acetate and the pH of the dialyzed solution adjusted to 4.9. Fractionation with acetone was then carried out at −5° C and the precipitate appearing between 30 and 50 % by volume acetone was collected and dissolved in cold 0.01 *M* sodium acetate. The remaining acetone was removed by dialysis against 0.01 *M* sodium acetate. The pH of the solution was then adjusted to 4.5, the enzyme precipitated with ammonium sulfate at 40 % saturation and the precipitate dissolved in 0.01 *M* sodium acetate. When a 0.5 % solution of this preparation was examined in the "Spinco" ultracentrifuge of this department, only one homogeneous boundary with $S_{20} = 2.76 \cdot S$ was observed. The solubility of a similar preparation in ammonium sulfate of varying concentration was then studied at room temperature and pH 5. The logarithm of solubility for the enzyme was found to be proportional to the concentration of ammonium sulfate. An amorphous precipitate was obtained, but when those supernatants, which still contained enzymatic activity were brought to + 2° C, a pronounced prethixotropy slowly developed. No crystals were, however, visible in the microscope, but later experiments showed, that rhodanese could be crystallized from ammonium sulfate at

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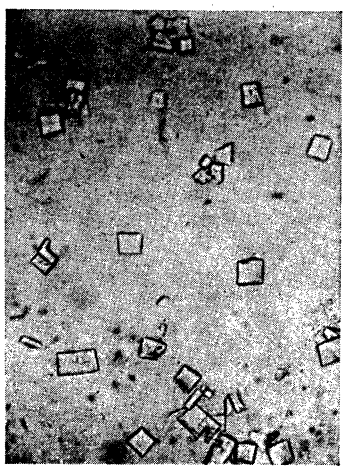

Fig. 1. Crystalline rhodanese from ammonium sulfate, pH 7.8. × 308.

pH 7.8 as small, strongly lightdispersing cubes or larger rectangular plates, see Fig. 1. The specific activity of the preparation was raised from 0.84 to 1.0 by crystallisation, the crystalline enzyme being purified 650-fold from the starting material.

1 μg of crystalline rhodanese was able to form 2.84 μ-equivalents of thiocyanate during 5 minutes incubation at 20° C. Assuming the molecular weight of the enzyme = 35 000 this corresponds to a turnover number of 20 000 min^{-1}. Our test system was of pH 8.75 and contained in a final volume of 2.5 ml 0.1 *M* thiosulfate, 0.05 *M* cyanide, 0.08 *M* phosphate and 0.125 mg bovine albumin. The albumin was found to be necessary in order to obtain full activity with the more purified enzyme preparations.

The crystalline enzyme gave a colorless solution with an ordinary protein absorption spectrum, which was not changed by the presence of 0.05 *M* thiosulfate at pH 7.4. No formation of an enzyme-substrate compound could thus be observed spectrophotometrically.

A detailed report will be published in a near future.

1. Sörbo, B. H. *Acta Chem. Scand.* **5** (1951) 724.

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On the Principle of Thermal Interaction

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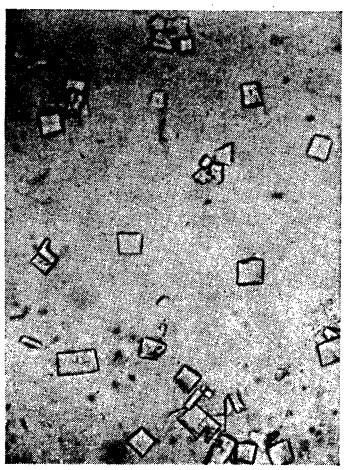
In a recent work Holtan [1] states a principle of thermal interaction: "In a non-isothermal process there is an interaction between the flow of heat and the transport phenomena in such a way that the loss of work (loss of potential energy) for the transport of entropy bound to chemical substance can be compensated partly or completely by the transport of free entropy contained in the flow of heat in the opposite direction."

This principle is embodied in the principle of least dissipation of energy. It is thus possible to prove the principle of thermal interaction for the cases where the principle of least dissipation of energy is valid. The restrictions and variations with respect to which the dissipation is a minimum, are stated by Wergeland [2].

For a process under consideration we can split the total rate of increase $\dot{S}$ in the entropy of the system in two parts:

$$\dot{S} = 2\Phi - \dot{S}^*$$

In the stationary state $\dot{S}$ will be zero, and the dissipation function Φ can be shown to be a minimum. Consequently the total flow of entropy across the surface of the system, $\dot{S}^*$, will be a minimum.



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In the stationary state $\dot{S}$ will be zero, and the dissipation function Φ can be shown to be a minimum. Consequently the total flow of entropy across the surface of the system, $\dot{S}^*$, will be a minimum.

This flow is given by the following expression (see for instance [3]):

$$\dot{S}^* = \int_F \left(\frac{\vec{J}_u}{T} - \sum \frac{\mu_i \vec{J}_i}{T}\right) \mathrm{d}\vec{F}$$

$\vec{J}_i$ is the flow, μ_i is the chemical potential of the i'th species of particles, and $\vec{J}_u$ is the flow of internal energy. F is the surface of the system.

If we now write, considering a one component system:

$$\vec{J}_i = \vec{J}, \mu_i = h - Ts$$

and

$$\vec{J}_u = \vec{q} + h\vec{J}$$

where $\vec{q}$ is the pure heat flow, we have:

$$\dot{S}^* = \int_F \left(\frac{\vec{q}}{T} + s\vec{J}\right) \mathrm{d}\vec{F}$$

If this integration is performed on the interface between two systems which, except for this interface, are isolated, we arrive at the following requirement:

$$S'_f + S'_b = \text{minimum},$$

where $S'_f = \int_F \frac{\vec{q}}{T} \vec{dF}$ is the entropy transport due to the pure heat flow and $S'_b = \int_F s\vec{J}\, \mathrm{d}\vec{F}$ is the entropy transport bound to the chemical substance. We therefore have:

$$\delta S'_f = -\delta S'_b$$

so that in the stationary case the two entropy flows tend to compensate each other, the compensation being complete in the reversible case where the dissipation is zero. This is exactly the principle of thermal interaction.

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The Swelling of Polymethylenes in Low Molecular Weight Alkane Vapours

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Physical Chemistry Institute, University of Copenhagen, Denmark

This paper presents a part of a general research on thermodynamic properties of paraffin mixtures carried out by the late professor J. N. Brønsted and collaborators.

By means of an especially designed electromagnetic balance the swelling of a thin polymethylene film was measured when suspended in an atmosphere of a volatile alkane **. The equilibrium vapour pressure in the system was observed on a mercury manometer against absolute vacuum.

Polymethylenes of average molecular weights approximately 5 000, 20 000, 35 000 and 40 000 were examined. As a volatile alkane mainly *n*-hexane was used. The sample was of a very high degree of purity ($n_D^{25} = 1.37251$; $\varrho^{25} = 0.65483$ g/ml; saturation vapour pressure $p^{22} = 13.25$ cm Hg). Preliminary experiments were carried out with *n*-heptane, *n*-octane, and 2,2,4-trimethylpentane. Vapour pressure isotherms were determined at 22° C, in a few cases also at 17° C and 27° C. The dependency of the swelling upon temperature, index (*i. e.* number of carbon atoms) of the two components and of the history of the individual film was investigated. The swelling was found to decrease with increasing temperature. The swelling of a given polymethylene film in low molecular alkanes at equal activity was found to decrease with increasing index. The effect of chain branching in the low molecular alkane seems to be to diminish the swelling relative to that of the normal compound.

The results in general proved consistent with current views on the structure of high polymer *n*-alkanes, a short survey of which is given. Owing to the peculiar mixed state of aggregation and the poorly defined molecular weights the data obtained for different polymethyl-

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** The name "polymethylene" for the high polymeric homologues of the formula $CH_3(CH_2)_nCH_3$ is preferred to the genetical "polyethylene" and the commercial "polythene".

enes were difficult to evaluate quantitatively. It is suggested that the observed differences in swelling are alone due to differences in the state of aggregation (degree of crystallinity) and that the swelling of amorphous polymethylene is in fact independent of its molecular weight.

It has been demonstrated that the expression $\frac{a}{c} = \alpha\, e^{\beta c}$ holds for the mixtures investigated, a being the activity of the volatile alkane, c its concentration in the unit of moles per gram polymethylene, and α and β being constants.

In 1946 J. N. Brønsted and J. Koefoed [1] proposed the concept of congruence as the basis for a general prediction of the thermodynamic properties of alkane mixtures. The index ν of a mixture was defined as

$$\nu = x_1\nu_1 + x_2\nu_2 + \cdots$$

where x_1, x_2 . . . are the mole fractions of the components in the mixture and ν_1, $\nu_2 \cdots$ the indices, which for pure compounds are identical with the number of carbon atoms in the molecule. It was postulated that certain thermodynamic properties of congruent mixtures of *n*-alkanes, *i.e.* mixtures having the same index, are identical irrespective of the individuality of the components. In other words the thermodynamic properties of an alkane mixture is determined by its index.

The hypothesis was experimentally tested on three sets of binary mixtures of liquid *n*-alkanes, $C_6 + C_{16}$, $C_7 + C_{16}$ and $C_6 + C_{12}$ at 20° C. From vapour pressure measurements a simple relation was derived between the activity coefficient f_1 of a component 1 in the mixture, the index ν_1 of the component and the index ν of the mixture. The relation, $\log f_1 = B\,(\nu - \nu_1)^2$ was found to hold for mixtures of *n*-alkanes containing from 6 to 16 carbon atoms. The numerical value of B for this range was -0.00048.

In order to test the hypothesis beyond these limits the scope has since been extended to straight chain alkanes of higher indices. Some of those in the range 16 to 36 have been investigated by other collaborators in this laboratory. The material has not yet been published. The present paper is devoted to binary mixtures of low molecular weight alkanes of index 6 to 8 and high polymeric alkanes of indices between 300 and 3 000.

Several difficulties are encountered when extending the scope from *n*-alkanes of short and medium chain length to long chain, high polymeric homologues. One is that the usual concepts of purity and identity grow more and more inapplicable. Up to about C_{80} it is still possible to obtain pure

chemical individuals characterized analytically and/or by way of unequivocal synthesis *e.g.* successive coupling of two identical alkyl groups. Pure alkanes of medium chain length can only be obtained synthetically from simple reaction mixtures containing few and widely different components, whereas an isolation of an individual alkane from a mixture of closely related homologues as *e.g.* natural products is extremely laborious if at all possible, even with the highly effective modern methods of separation.

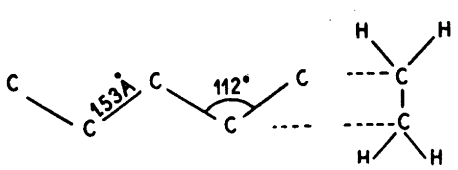
In the group of polymethylenes with several hundreds or thousands of carbon atoms it is no longer possible to synthesize or isolate chemical individuals with a definite number of carbon atoms. The syntheses, mainly polymerizations, inevitably yield a mixture of alkanes with different chain lengths, although the process may be conducted so as to give products of a certain desired average molecular weight. A further "purification" may be accomplished by fractional precipitation and by other methods. A somewhat better characterization of a product is at hand, when not only the average molecular weight but also a distribution curve, giving the relative amounts of the various chain lengths, is known. It is evident that the index, defined as above, is a very useful term for precise characterization of high polymer *n*-alkanes, since it takes into account not only the average molecular weight but also the distribution curve. Unfortunately the experimental data necessary for the calculation of the index cannot be obtained with an accuracy comparable to that in the low molecular region.

Although polymerisations of simple compounds as ethylene with only two reaction centres should lead to "one-dimensional" unbranched carbon chains, there is nevertheless evidence [2] that a certain degree of branching cannot always be avoided. It is not certain, therefore, that the material used in our investigations is entirely of normal paraffin structure, and this must be born in mind when evaluating the results. It is, however, always a matter of *small* side chains, probably methyl groups, and their relative number decreases with increasing chain length (Ref.[24, p. 126]).

In addition to the inaccessibility of individual well-defined long chain alkanes another difficulty also seriously interferes with the interpretation of some of the results, presented in this paper. That is the peculiar mixed state of aggregation of solid high polymers. A short summary will be offered below as a background for the discussion of results.

A. The structure of polymethylenes.

The molecular dimensions of polymethylene chains were established by C. W. Bunn in 1939 by means of X-ray methods [3] and are reproduced in Fig. 1.



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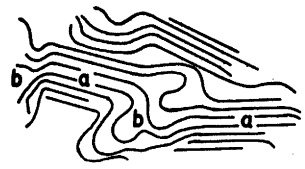

Fig. 2. Structure of solid polymethylene. a. crystalline parts. b. amorphous parts.

The dimensions are almost identical with those observed in alkanes of medium chain length *e.g.* $C_{29}H_{60}$ by A. Müller [4] in 1928. The figure shows the carbon chain stretched, with all carbon atoms coplanar. Rotation is possible, outer conditions permitting, about any single bond in the chain resulting in a variety of "curled up" shapes *cf.* Fig. 2.

At room temperature polymethylenes are known to be semicrystalline solids *i.e.* they consist of randomly oriented microcrystalline parts, crystallites, Fig. 2 a, imbedded in amorphous parts, Fig. 2 b. The crystallites are crystalline in the sense that they are composed of straight chains regularly arranged parallel to one another, but they have no flat crystal faces. A single carbon chain may well penetrate several crystalline regions and also several amorphous regions in which the chain may be randomly curled up. This mixed order-disorder texture is common to many linear high polymers and was first suggested by K. H. Meyer and H. Mark [5] in 1928 for natural fibres. In the case of polymethylene this view is strongly supported by X-ray crystallographic data, (Bunn and Alcock [6]), in that the crystallites have been shown to be considerably smaller than the average length of the carbon chains, and secondly the pattern display diffuse bands characteristic for amorphous solids and for liquids. The mechanical properties of polymethylene are easily explained on this basis. The substance is tough, often elastic, contrary to the typical crystalline alkanes containing up to about 80 carbon atoms. By stretching or "cold drawing" the chains in the amorphous regions are straightened and these as well as the crystallites are oriented parallel to the direction of stretching, thus increasing the overall orderliness of the material. This phenomenon has been proved by X-ray work [7] and is observed directly as an increased tensile strength of the fiber.

The relative crystallinity obviously depends upon temperature. On heating the crystallinity decreases gradually. There is no well-defined transition point corresponding to the melting point of typically crystalline solids. By X-ray diffraction Bunn and Alcock [6] observed a decrease of crystallinity, commencing at about 80° C. At about 100° C the value was *ca.* 50 %, at 120° it was zero,

and the substance appeared to be isotropic. The polymethylene sample used by these authors had an average m.w. of 17 000.

Hunter and Oakes [8] in another polymethylene observed a crystallinity of 55 % at room temperature. A detectable decrease began at 70° C. At 100—120° the crystallinity was zero. The authors found indication that a state of equilibrium is quickly attained at temperatures above 60° C, whereas the order-disorder state is „frozen" below 60° C. These results were obtained by density measurements.

Similar observations were made by Raine, Richards and Ryder [9] by investigation of the temperature dependency of the heat capacity. By this method changes in state could be observed from 50° C onwards. These authors found a relatively sharp transition point into nearly isotropic state at about 115° C. Even well above the "melting point" the molecules proved to be somewhat oriented, a fact suggested by Charlesby [10] a few years earlier.

Richards [11] has investigated the effect of chain branching and molecular weight distribution on the "melting point". Samples with the same average molecular weight, as indicated by intrinsic viscosities, were found to vary as much as 15° C in melting point, due to these reasons.

It is obvious from the above summary that the "melting point" of polymethylenes is of next to no value for the characterization of the products.

Polymethylenes are insoluble in low index alkanes at 20° C. On heating a homogeneous solution usually is formed at a certain temperature. For a mixture of polymethylene and *n*-heptane containing 5 % of the former the clarification temperature is 70—80° C, depending upon the molecular weight of the polymethylene. For *n*-hexane the corresponding temperature is above the boiling point [19, p. 22].

Whereas polymethylene does not dissolve in liquid low index alkanes at room temperature, these dissolve in solid polymethylene to a certain extent by swelling. This extreme form of the phase diagram and the fact that polymethylenes are completely non-volatile make the experimental approach particularly simple. The swelling in vapour phase is the subject for the following investigations.

EXPERIMENTAL

B. Apparatus.

The apparatus was designed by J. N. Brønsted and A. E. Lansner. It will be only briefly described here. It is shown diagrammatically in Fig. 3.

T is a water thermostat accurate to within ± 0.01° C. E is the all glass equilibration chamber with the built-in electromagnetic balance. To the left arm of the beam is attached a permanent magnet shaped as a rod and dipping into the stationary coil C.

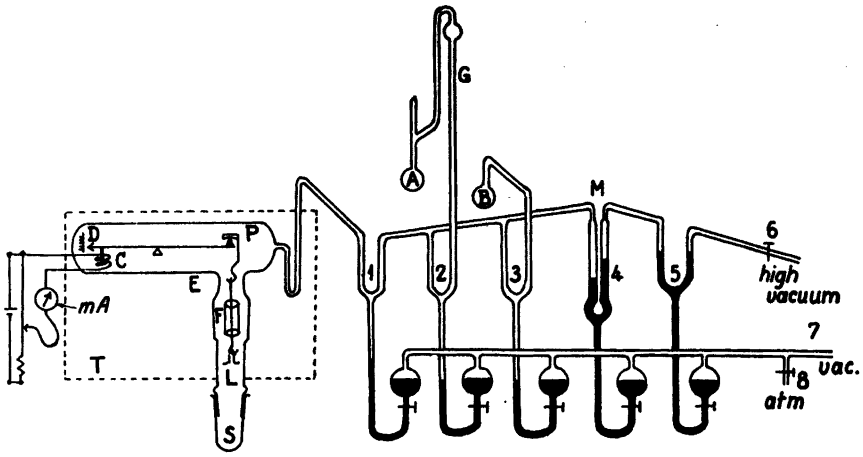

Fig. 3. Electromagnetic balance for weighing in an alkane atmosphere.

The latter is connected to the outer circuit by sealed-in platinum leads. The current through the balancing mechanism can be adjusted by means of the variable rheostat and read on the milliamperemeter mA with an accuracy ± 0.003 mA. All values were corrected according to a correction graph worked out by A. E. Lansner.

On the right hand knife of the beam a metal wire assembly is suspended, acting as a carrier for the film F and supplementary loads L. The plane P rested permanently on the knife but the lower parts of the carrier could be removed from and re-introduced into the chamber through the ground-glass-capped tube S emerging through the bottom of the thermostat.

To make a weighing the current was adjusted until an indicator mark on the left arm was brought to juxtaposition with the zero point of the indicator scale D. This adjustment was controlled visually through the glass wall of the thermostat and a special water-lens. With a total load of about 1.2 g, as in most experiments, the sensitivity of the balance was 0.03 mg, corresponding to 0.005 mA. When a standard procedure was followed, the readings were reproducible to within the sensitivity. As the absorbed weight of volatile alkanes usually have been between 1 and 15 mg, this means a relative accuracy of 3 to 0.2 %.

The zero current for the balance, even without film, was found to increase slightly with time, due to certain mechanical features of the beam. This phenomenon could, however, be completely eliminated by the following standard procedure. The current was adjusted to zero position of the balance. By means of a short-circuiting switch a "shock" of about 3 mA was applied to the coil, thus shaking the beam into a reproduceable position. After 5 such "shocks" 5 independent re-adjustments were made and the reading taken each time. Again 5 shocks were applied and five readings taken. The arithmetic mean of these ten readings was used in the calculations. The relation between current i (mA) and load m (mg) was determined by means of excess loads of known weight. Results are given in Fig. 4. They were determined at 22° C and *in vacuo*. The relation is linear and $\Delta m = 6{,}1\ \Delta i$. This value was used throughout this paper. It did

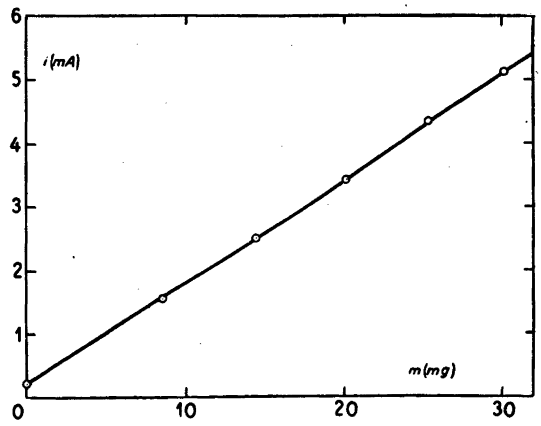

Fig. 4. Relation between current i *and load* m.

not alter preceptibly with temperature within the limits concerned. Buoyancy effects were found to be negligible under the given conditions.

The right hand parts of the apparatus in Fig. 3 serve the purpose of establishing and measuring the vapour pressure of the volatile alkane in the equilibration chamber. Communication between the various parts of the all-sealed system is controlled by the mercury valves 1—5 operated by means of a vacuum pump and the respective stopcocks. A and B are reservoirs for the volatile alkane. A, which also contains the drying agent (phosphorus pentoxide) is connected to the rest of the system by the tube G, which is of sufficient height to permit trapping of all the air-free alkane in A, when the atmosphere is admitted to the apparatus during exchange of films *etc.*

The equilibrium vapour pressure is read directly on the mercury manometer M against a high vacuum, *i.e.* effectively against the vapour pressure of mercury at 20° C, which amounts to 0.001 mm Hg. The difference in height of the two mercury menisci was read in the ordinary way using a cathetometer provided with a vernier. The accuracy was ± 0.005 cm. Obviously the temperature of the manometer, *i.e.* room temperature had to be kept approximately constant during measurements, and the manometer was protected from heat radiations. During evacuation of the equilibrium chamber and accessories, direct connection to the high vacuum pump was procured by emptying the manometer tubes by means of valve 4.

The effective volume during equilibration and measurements was, apart from the ground-glass-cap S, limited by either sealed glass or mercury surfaces, the involved parts of the glass tube system having been blown in one piece without stopcocks.

C. Materials.

n-Hexane. At the time, when the experimental work was carried out, no hexane of sufficient purity was commercially available. Fractionation of a technical grade product in a 60-plate column yielded a small quantity of about 99 % pure hexane, $n_D^{25} = 1.37300$, $\varrho^{25} = 0.65574$. The impurity was an isomer. This sample was used for the small series no. 5 given in Table 2. In all other series a product of very high purity was used. It was made synthetically from pure propanol-1, which was converted into 1-bromopropane.

The *n*-hexane was finally obtained in 70 % yield by a Wurtz synthesis. Only constant boiling fractions of the intermediates were used and the final product was subjected to careful fractionation according to A. Klit[13] in a 60-plate column. It had an absolutely constant boiling point of 68.9° C at 760 mm (Timmermans and Martin[14] give 68.80°). The refractive index was also constant $n_D^{25} = 1.37251$ over the whole fraction. The most reliable value extracted from the literature[15] seems to be 1.3751 at 20° C; using $dn/dt = -0.00056$ we get $n_D^{25} = 1.3723$. The density was found to $\varrho^{25} = 0.65483$ g/ml. Remeasurements on the hexane sample used for the investigations by Brønsted and Koefoed[1] showed complete identity.

n-Heptane. The preparation described in a previous paper[1] was used.

n-Octane was obtained by fractional distillation of a synthetical product. It had a constant boiling point 124.7° C at 760 mm.

2,2,4-trimethylpentane, "iso-octane". A commercial product holding 99 % purity was distilled. The intermediate fraction used had a boiling point 99.3° C at 760 mm. Ref.[16]: 99.3° C at 760 mm.

The last two alkanes of a less high purity were used only for some preliminary experiments.

The values of the saturation vapour pressures, used in the calculations, were determined experimentally in this laboratory and are given in Table 1. After the experimental part of this work was terminated, The National Bureau of Standards has published "*Selected Values of Properties of Hydrocarbons*" under "*The American Petroleum Project*". In the last column (N.B.S.) are given the values calculated from the Antoine equation $\log_{10} p = A - \frac{B}{C + t}$ using the values of A, B and C from the 1948-sheets of the said publication. They agree satisfactorily with our data.

Table 1. Saturation vapour pressures of the volatile components.

	t	p	p (N.B.S.)
n-Hexane	17° C	10.57	10.57 cm Hg.
	22° C	13.25	13.26
	27° C	16.47	16.48
n-Heptane	22° C	3.93	3.93
n-Octane	22° C	1.18	1.176
2,2,4-Trimethylpentane	22° C	4.29	4.268

Xylene was obtained by distillation of a commercial preparation. The fraction used had a boiling range of 137—144° C and consisted of a mixture of isomers of unknown but constant composition. It was only used as a solvent during preparations of polymethylene films. The content of non-volatile impurities (100° C) was less than 0.03 pro mille.

Polymethylenes. All the samples were supplied by H. Mark's laboratories, Polytechnic Institute of Brooklyn. The average molecular weights indicated by the supplier were 5 000, 20 000, 35 000 and 40 000. In the following the polymethylenes will be characterized by their indices, *i.e.* number of carbon atoms, 360, 1 430, 2 500 and 2 860 respectively. These values are calculated

from the above molecular weights by dividing by 14 (CH_2), and it should be clearly understood that they are approximate values.

All four polymethylenes were tested for presence of low molecular weight material. The substance was dissolved in xylene at 90° C and precipitated on standing at 20°. By evaporation at 100° of the mother liquor from repeated experiments it was shown, that all the polymethylenes were insoluble in xylene at 20° C and secondly, that they did not contain impurities soluble in xylene at 20° C, such as low molecular weight alkanes.

It was found that the polymethylenes did not loose in weight on heating to 130° C for 50 hours *in vacuo*. Consequently they did not contain volatile material, nor did any decomposition take place under the conditions at which the films were prepared (*vide infra*).

For the purpose of roughly checking the stated average molecular weights relatively to one another some viscosimetric measurements were undertaken. The time of efflux of solutions in xylene containing 0.5 g in 100 ml was determined in an Ostwald viscometer at 85° C. At this temperature all the polymethylenes formed homogeneous solutions. At 75° C, which was first tried, C_{1430} and C_{2500} dissolved clearly in the above concentration, whereas C_{360} and C_{2860} (!) did not.

In Table 2 τ is the time of efflux in sec. It was reproducible to within at least $\pm$ 2 sec. η is the viscosity in centipoise. Let ϱ be the density at 85° C, then it is well known that $\eta = C\varrho\tau$, where C is a constant characteristic of the apparatus. By calibration of the viscometer against water the value $C = 5.26 \cdot 10^{-5}$ was found at 85° C. According to Staudinger [22]

$$\frac{\eta_{sp}}{c} = \frac{\eta - \eta_0}{\eta_0 \cdot c}$$

where η_{sp} is the specific viscosity, subscript 0 refers to the solvent and c is the concentration of solute. For the present purpose we are justified in regarding the densities of the solutions equal to that of the solvent, hence

$$\frac{\eta_{sp}}{c} = \frac{\tau - \tau_0}{\tau_0 \cdot c}$$

The relation between the index and viscosity is according to W. Kuhn [23]

$$\lim_{c \to 0} \frac{\eta_{sp}}{c} = k \cdot \nu^{\alpha}$$

k and α are constants depending on the nature of the solute. For $\alpha = 1$ the expression is identical with the original Staudinger equation, for flexible molecules as polymethylene we have $\alpha < 1$ [22, p. 223]. For a rough comparison

we choose the concentration 0.5 %, without extrapolating to zero. Provided a is constant for the series of polymethylenes investigated the relation of log $\frac{\eta_{sp}}{c}$ to log ν should be linear.

Table 2. Viscosity of 0.5 % polymethylene in xylene at 85° C.

ν	τ (sec.)	η	η_{sp}/c	log ν	log $[\eta_{sp}/c]$
360	87	0.370	20	2.56	1.30
1430	128	0.545	124	3.16	2.09
2500	130	0.552	129	3.40	2.11
2860	151	0.642	182	3.46	2.26
xylene	$\tau_0 = 79$	0.336			

A plot of the values in the last two columns of the table shows this to be roughly true, at least the viscosity increases in the same order as the indices.

D. Experimental procedure.

The polymethylene films were generally prepared in the following way. A suitable quantity of polymethylene, usually 0.2 g was dissolved in *ca.* 5 ml pure xylene by heating to 90° C. The hot solution was poured into a rectangular brass pan 5 × 6 cm, carefully levelled. The solvent was slowly evaporated at 100° C, the last traces being removed in a vacuum drying oven. After removal from the mould and trimming, the dimensions of the film were approximately 4 × 5 × 0.005 cm. Thicker films were unsuitable owing to slow equilibration. A list of all the employed films and their data is given in Table 3.

Suitable quantities of the volatile alkane and drying agent (phosphorus pentoxide) were introduced in bulb A (Fig. 3). The bulb was cooled to −80° C in a mixture of solid carbon dioxide and acetone and then sealed off. The equilibration chamber was closed, the cap S being lubricated with high vacuum grease. All valves were opened and the entire system including the contents of bulb A, still being kept at −80° C, was evacuated with the high vacuum pump. In order to secure removal of all traces of air from the alkane sample, this was distilled several times between bulbs A and B and evacuated at −80° C after each distillation. During the distillations valves 1 and 4 were closed. The de-aeration was continued until the vapour pressure of the alkane

Table 3. Data of the polymethylene films.

Film no.	$\overline{M.W.}$	ν	Net weight in air (grams)	Surface (cm^2)	Average thickness (cm)	History of preparation
1	20 000	1 430	0.0955	38	0.005	Cylindrical film prepared by evaporation from a xylene solution at 100° C in a horizontal, rotating testtube. Dried *in vacuo* at 100° C to constant weight.
2	20 000	1 430	0.1020	4.5	0.05	A hot solution in xylene was cooled, the precipitated, swollen polymethylene was dried *in vacuo* at 20° C to constant weight.
3	20 000	1 430	0.1569	36	0.01	Plane film prepared by evaporation from a xylene solution at 100° C. Dried *in vacuo* at 100°C to constant weight.
5	20 000	1 430	0.1650	54	0.005	As film no. 3
6	35 000	2 500	0.1234	50	0.005	As film no. 3
7	35 000	2 500	0.1153	46	0.005	As film no. 3
8	5 000	360	0.1487	incoherent flakes		As film no. 3
10	40 000	2 860	0.1166	46	0.005	As film no. 3
10a	40 000	2 860	0.1151			Film no. 10 was soaked in liquid xylene at 50° C over night, dried *in vacuo* at *20°* C.
10b	40 000	2 860	0.1150			Film no. 10a was soaked in liquid xylene at 50° C for 48 h, dried *in vacuo* at *80°* C.
10c	40 000	2 860	0.1150			Film no. 10b was soaked in liquid xylene at 50° for 2 h, dried at 20° C *in vacuo*.
10d	40 000	2 860	0.1150			Film no. 10c was heated *in vacuo* to 100° C for 3 h.

sample at −80° C was less than the accuracy of measurements, 0.005 cm. For hexane, being the most volatile of the alkanes measured, the vapour pressure at this temperature should be 0.005 cm, for the other alkanes much less.

The dry and de-aerated alkane was stored in bulb A, while the atmosphere was again admitted to the high vacuum side. The film was cylindrically wound up on the carrier, the weight of which was 0.8202 g. The accurate weight of the film in air (approximately 0.1 g) was determined as a difference. By hanging on supplementary weights the total weight of the film + carrier was increased to approximately 1.17 g, which corresponded to a convenient zero current of 0.187 mA. The assembly was then suspended on the electromagnetic balance, the equilibration chamber closed and the "air reading" on the milliamperemeter taken. Then the system was evacuated for several hours, until the reading on the meter did not alter on further evacuation. This reading will be termed the "zero current". If the film had lost in weight, *i.e.* zero current lower than air reading, then the absolute net weight of the film was corrected accordingly before entering into the calculations. The corrections were very small, usually 0.1 to 0.2 mg (adsorbed air and moisture).

After the determination of the zero current and the corrected net weight of the film the pump was cut off by operating valve 5 and stopcock 6. Mercury was admitted into the manometer M, and by operating valve 2 and suitable heating and cooling of the bulb A, a sufficient quantity of volatile alkane was admitted to the evacuated system. In this way it was possible to establish approximately any desired vapour pressure, ranging from zero to the saturation vapour pressure of the alkane in question at the lowest temperature in the system, *i.e.* room temperature (manometer) or thermostat temperature, whichever was the lowest.

In the following the results of fifteen series of measurements will be discussed. All the relevant data are collected in Table 4. The number of the film in column 6 refer to Table 3. Column 7 contains the equilibrium vapour pressure p_{ν_1} of the volatile component in cm Hg. Column 8 contains the saturation fraction or vapour activity a_{ν_1}. The values of saturated vapour pressures $p_{\nu_1(\nu_1)}$, applied in the calculations, are given in Table 1. In column 9 g_ν denotes weight in grams of component ν in the mixture. Thus the column gives gram absorbed volatile alkane by 1 gram polymethylene. The tenth column contains the concentration c of the volatile component in millimoles per gram polymethylene. The values in the last column are calculated from those in column 8 and 10.

Table 4.

1 Series	2 t (°C)	3 Volatile component	4 v_1	5 Poly-methy-lene v_2	6 Film no.	7 p_{v_1}	8 $\frac{p_{v_1}}{p_{v_1(v_1)}} = a_{v_1}$	9 $\frac{g_{v_1}}{g_{v_2}}$	10 $\frac{g_{v_1} \cdot 10^3}{g_{v_2} \cdot M_{v_1}} = c_{v_1}$	11 $\log \frac{a_{v_1}}{c_{v_1}}$
2	17	*n*-hexane	6	1 430	1	2.100	0.1987	0.0137	0.159	0.097
						5.535	.5237	.0460	0.534	−0.009
						8.650	.8184	.0936	1.087	−0.123
						9.620	.9101	.1183	1.374	−0.179
						9.88	.9347	.1274	1.480	−0.199
						10.09	.9546	.1349	1.567	−0.215
						10.095	.9550	.1381	1.604	−0.225
						10.285	.9730	.1512	1.756	−0.256
						4.800	.4541	.0401	0.466	−0.011
						7.325	.6930	.0733	0.851	−0.089
3	17	*n*-hexane	6	1 430	2	3.850		.0278		
						9.640		.1733		
						4.885		.0431		
						5.805		.0548		
						7.170		.0782		
						9.040		.1382		
						1.150		.0991		
						0		.0028		
						1.850		.0141		
						8.550		.1191		
						8.160		.1057		
4	22	*n*-hexane	6	2 860	10	8.845	.6676	.0521	0.605	0.043
						9.985	.7536	.0625	0.725	0.017
						8.045	.6072	.0455	0.528	0.061
						3.625	.2736	.0191	0.222	0.091
					10a	6.570		.0368		
						11.820		.1001		
						9.220		.0640		
						10.980		.0860		
						8.060		.0516		
						6.140		.0356		
					10b	9.315	.7030	.0536	0.622	0.053
						11.990	.9049	.0851	0.988	−0.038
						5.240	.3955	.0258	0.299	0.122
					10c	7.455		.0436		
						12.020		.1063		
						9.815		.0694		
						11.070		.0876		

Table 4 continued.

					10d	11.580	.8740	.0762	0.885	−0.006
						8.595	.6487	.0476	0.553	0.069
						5.530	.4174	.0268	0.311	0.128
						2.185	.1649	.0100	0.116	0.152
						4.435	.3347	.0206	0.239	0.146
						3.615	.2728	.0161	0.187	0.164
						0.915	.0691	.0046	0.053	0.155
5	22	*n*-hexane	6	1 430	1	0.925	0.0698	0.0053	0.062	0.052
						2.037	.1537	.0122	0.142	0.034
						4.430	.3343	.0269	0.312	0.030
						9.040	.6823	.0740	0.859	−0.100
6	22	*n*-hexane	6	1 430	1	1.605	.1211	.0089	0.103	0.070
						3.695	.2789	.0217	0.252	0.044
						7.220	.5449	.0507	0.589	−0.034
						7.24	.5464	.0497	0.577	−0.024
						9.01	.6800	.0708	0.822	−0.082
						9.40	.7094	.0761	0.884	−0.095
						9.99	.7540	.0846	0.983	−0.115
						11.07	.8355	.1064	1.236	−0.170
						11.06	.8347	.1067	1.239	−0.171
						11.53	.8702	.1185	1.376	−0.199
						11.53	.8702	.1188	1.380	−0.221
						10.53	.7947	.0991	1.151	−0.161
						10.51	.7932	.0976	1.133	−0.155
						8.08	.6098	.0640	0.743	−0.086
						5.96	.4498	.0408	0.474	−0.023
						4.69	.3540	.0307	0.357	−0.004
						4.72	.3562	.0304	0.353	0.004
7	17	*n*-hexane	6	1 430	5	4.695	.4442	.0330		
						9.540	.9026	.1006		
						7.340	.6944	.0623		
						8.795	.8321	.0846		
						10.165	.9617	.1169		
						10.330	.9773	.1241		
8	22	*n*-hexane	6	1 430	5	11.190	.8445	.0924		
						11.925	.9000	.1042		
						7.975	.6019	.0534		
						9.655	.7287	.0704		
						5.765	.4351	.0348		
						2.875	.2170	.0155		
						12.235	.9234	.1090		

Table 4 continued.

9	27	*n*-hexane	6	1 430	1	4.590	.2787	.0216	0.251	0.045
						7.090	.4305	.0375	0.436	−0.006
						8.800	.5343	.0508	0.590	−0.043
						10.455	.6348	.0657	0.763	−0.080
						11.790	.7158	.0798	0.927	−0.112
						9.425	.5723	.0567	0.659	−0.061
						6.345	.3853	.0341	0.396	−0.012
10	17	*n*-hexane	6	1 430	3	1.885		.0106		
						9.000		.0856		
11	22	*n*-hexane	6	360	8	9.890	.7464	.0321	0.373	0.301
						7.995	.6034	.0220	0.255	0.374
						7.145	.5393	.0189	0.219	0.391
						3.340	.2521	.0053	0.062	0.609
						5.420	.4091	.0120	0.139	0.469
						11.495	.8676	.0425	0.493	0.246
						11.380	.8589	.0443	0.514	0.218
12	22	*n*-hexane	6	2 500	6	4.175	0.3151	0.0237	0.275	0.058
						7.045	.5317	.0478	0.555	−0.019
						9.825	.7415	.0864	1.003	−0.131
						11.790	.8898	.1323	1.535	−0.237
						11.180	.8438	.1195	1.387	−0.216
						6.545	.4940	.0455	0.528	−0.029
						8.650	.6528	.0695	0.807	−0.092
						10.725	.8094	.1054	1.223	−0.179
13	22	*n*-hexane	6	2 500	7	4.700	.3547	.0258	0.300	0.073
						10.580	.7985	.0885	1.027	−0.019
						11.845	.8940	.1159	1.345	−0.177
						8.525	.6434	.0615	0.714	−0.045
14	22	*n*-heptane	7	2 860	10d	2.520	.641	.0476	0.475	0.130
						3.265	.831	.0699	0.698	0.076
						2.295	.584	.0424	0.423	0.140
						0.500	.127	.0090	0.090	0.149
						1.565	.398	.0260	0.259	0.186
15	22	*n*-octane	8	2 860	10d	0.715	.606	.0450	0.394	0.187
						0.975	.826	.0697	0.610	0.131
						0.230	.195	.0125	0.109	0.253
						1.140	.966	.1000	0.876	0.042
						0.230	.195	.0127	0.111	0.245
						0.790	.670	.0517	0.453	0.170

Table 4 continued.

						0.850	.720	.0569	0.498	0.160
						0.595	.504	.0360	0.315	0.204
						1.030	.873	.0781	0.684	0.106
16	22	"iso-octane"	8	2 860	10d	3.180	.7421	.0505	0.442	0.225
						0.845	.1972	.0101	0.088	0.351
						2.025	.4726	.0277	0.243	0.289
						3.880	.9055	.0718	0.629	0.158
						4.180	.9755	.1077	0.882	0.044

E. Equilibration time.

The time elapsed from the establishment of a certain vapour pressure until an equilibrium is set up between the gaseous phase and the binary mixture represented by the swollen polymethylene film obviously depends on the dimensions of the film. Films about 0.005 cm thick were found suitable for measurements. Typical equilibration curves are shown in Fig. 5.

The quantity of hexane in milligrams absorbed by a polymethylene film (C_{1430}) is plotted against a time basis. The applied vapour pressure is for curve 1 0.925 cm Hg, the temperature 22° C. Equilibrium is attained after

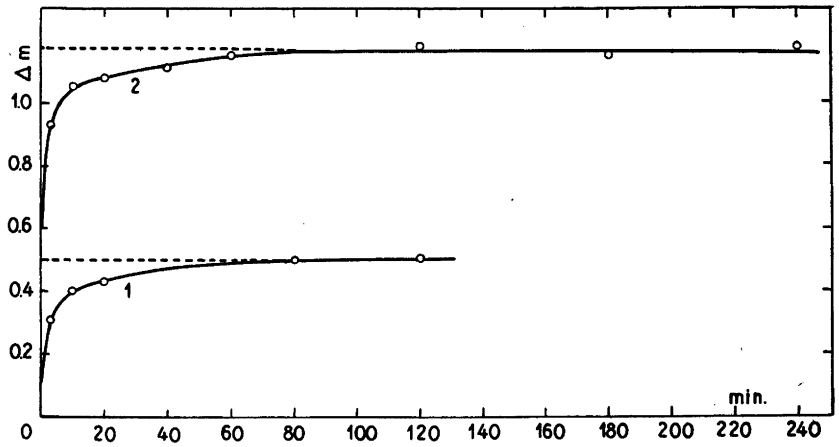

Fig. 5. Equilibration time. Film no. 1 (C_{1430}), swelling in n-*hexane at 22°. Absorbed* n-*hexane in milligrams against minutes after alteration of vapour pressure.*
1: Vapour pressure altered from 0 to 0.925 cm Hg.
2: Vapour pressure altered from 0.925 to 2.037 cm Hg.

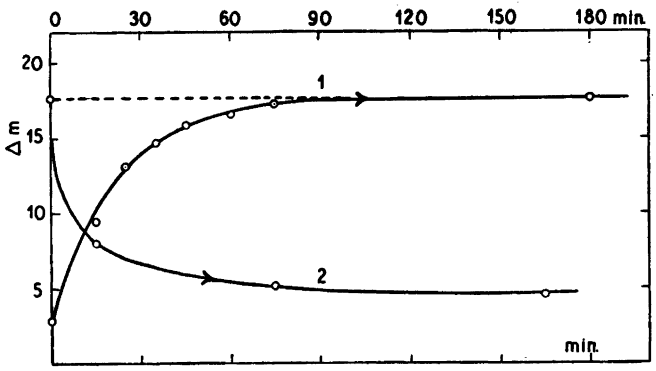

Fig. 6. Equilibration time. Film no. 2 (C_{1430}), thick film, swelling in n-*hexane at 17°. Absorbed* n-*hexane in milligrams against minutes after alteration of vapour pressure.*

1: Vapour pressure altered from 3.85 to 9.64 cm Hg.
2: Vapour pressure altered from 9.64 to 4.89 cm Hg.

1½ hour. Curve 2 represents the next step in the same series of measurements (Table 4, series no. 5). The vapour pressure has been altered to 2.037 cm by admitting more hexane into the system. Here, and in fact in all our measurements on thin films, the equilibration time was less than 2 h and practically independent of the sign and magnitude of the preceding pressure change.

Obviously complete equilibrium was secured in any single measurement by following the readings on the milliampere-meter and the manometer usually 20 min. after two subsequent readings having been identical.

Fig. 6 shows equilibration curves for a thicker film (no. 2; 0.05 cm) and somewhat higher hexane pressures. The curves are more flat, and safe equilibrium was only attained after 3—4 hours or more with films more than 0.005 cm thick.

F. Absorption or adsorption. Dependency of absorption upon the dimensions of the film.

Although it is not *a priori* very likely that the weight increase of a polymethylene film in an alkane atmosphere is due to adsorption on the surface, this possibility was tested by comparison of film no. 1 and 2 (Table 3), from the same batch of material, of approximately equal weight but differing in shape. No. 1 had a total surface of *ca.* 40 cm², no. 2 of only 4 cm². If due to *ad*sorption only, the weight increase of film no. 2 should be smaller than that of no. 1. Experimental results plotted in Fig. 7 show to the contrary. Here

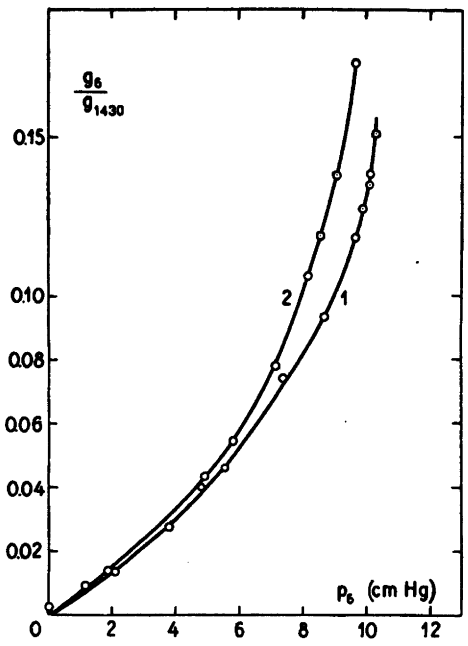

Fig. 7. Hexane absorption by polymethylene C_{1430} at 17°, dependency on the dimensions of the film. Grams absorbed hexane per gram polymethylene against equilibrium vapour pressure.

1: Film no 1, series 2 (thin film, large surface).

2: Film no 2, series 3 (thick film, small surface).

and in the following g signifies weight in grams, thus g_6/g_{1430}, the ordinate, is grams *n*-hexane absorbed per gram polymethylene of average index 1430. The abscissa p_6 is the equilibrium vapour pressure of the volatile component, *in casu* *n*-hexane, in cm Hg. It is evident, that for all pressures film no. 2, having the smaller surface, shows the larger weight increase per gram. The reason why the two curves do not coincide, as could be anticipated on grounds of *ab*sorption, is to be sought in the history of preparation of the two films, *vide* section H.

There are no other indications that adsorption phenomena seriously interfere with the results in this paper, except perhaps in those parts of the swelling curves that correspond to vapour pressures very close to the saturation pressure; in Fig. 7 this is $p_{6(6)} = 10.57$ cm. These extreme parts of the curves should therefore be treated with some reserve.

G. Reproducibility of absorption in an individual film at low temperatures. Hysteresis?

The equilibrium composition of an individual swollen film in the atmosphere of a given alkane only depends upon the temperature and the applied vapour pressure, but seems to be independent of the preceding pressure changes

in the series of measurements. Thus an absorption value could be reproduced without systematic deviations, when the corresponding vapour pressure was re-established later in the series. This means that the original structure, *e.g.* the degree of crystallinity, of the film is unaltered by the swelling, at least when using hexane vapours and not exceeding 27° C. To be sure, in any series of measurements, a number of readings were taken at increasing values of vapour pressure p, followed by a number of readings at decreasing values, or readings were taken going up and down the pressure scale several times. Fig. 10 (series 6, Table 4) gives an idea of the maximum error, the arrows indicating whether a point is a member of an ascending or a descending series. Series 6 was the very first measured, it is actually composed of three series of observations made by two different persons during a period of 10 days. It does therefore correspond to exceptionally unfavourable conditions. In all other series the reproducibility is much better, *vide e.g.* the points for film no. 5 in the same figure (squares). The sequence is as indicated in Table 4, series 8 from top to bottom.

Frequently the net weight of the film was re-determined at the end of a series after complete pumping out of the volatile component. The original value was always found.

H. Structural changes in the film at higher temperatures.

Some experiments were carried out in order to estimate, how far the properties of a polymethylene film could be altered by swelling at higher temperatures and drying again at different temperatures. The results are given in Fig. 8. The same film, no. 10, was measured, then soaked in liquid xylene at 50° C (polymethylene is insoluble at this temperature), carefully freed from xylene by drying *in vacuo* at *20°* C (no. 10 a), again soaked and dried at 80° C (no. 10 b), again soaked and dried at 20° C (no. 10 c) and finally without further soaking dried at 100° C (no. 10 d). These treatments did not appreciably lower the net weight of the film (*cf.* Table 3 column 4). A series of measurements of the hexane absorption was taken after each treatment. In Fig. 8 grams absorbed hexane per gram polymethylene is plotted against the equilibrium vapour pressure in cm Hg.

It is evident that the points group themselves about two curves, one of which represents "cold dried" films and the other "warm dried" films. It thus appears that two fairly reproducible states of aggregation of polymethylene are set up, corresponding to a lower and a higher temperature. None of these states could probably be thought of as representing a thermodynamic equi-

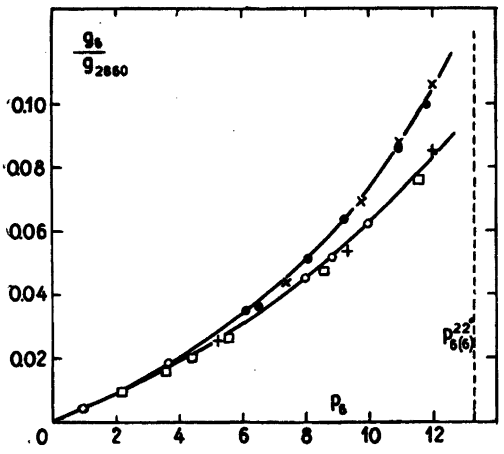

Fig. 8. Hexane absorption by polymethylene C_{2860} at 22°. Influence of the drying temperature upon the properties of the film. Series 4.

- ○ *film no 10, dried at 100°.*
- ● *10a, same film soaked in xylene at 50°, dried at 20°.*
- \+ *10b, again soaked in xylene 50°, dried at 80°.*
- × *10c, again soaked in xylene 50°, dried at 20°.*
- □ *10d, finally dried at 100°.*

librium between crystalline and amorphous structure, particularly not the "low temperature state", which is without doubt a more or less frozen metastable state.

The "high temperature state" shows the smaller absorption. This fact could be accounted for by assuming a larger degree of crystallinity in that state, which assumption is not *a priori* unlikely, as at the moderately high temperatures 80—100° C, well below the transition region, crystallization should occur more rapidly than at room temperature. Later work [17] has provided evidence of a gradual but very slow increase in crystallinity even at room temperature. It is not surprising either that the state of lower crystallinity can be restored by soaking the "warm dried" film in liquid xylene at 50° C, because the swelling, although taking place in the amorphous regions, might, to a certain degree, affect the interfaces of the crystallites forcing the parallel chains somewhat apart, and consequently reducing the overall relative crystallinity. This effect is not perceptible in our absorption measurements carried out at lower temperatures, $< 27°$ C.

The two curves in Fig. 8 probably give an idea of the maximum deviations to be expected, when two films are prepared from the same material and no special care is taken to standardize the operations.

Films no. 1 and 2 (Fig. 7) constitute such a pair of films, no. 1 having been dried at 100° C, no. 2 at 20°. It is interesting to notice, that here again the "cold dried" film shows the larger absorption.

Not only the drying temperature but also the rate of the drying and other conditions influence the properties of the film. Thus in Fig. 9 film no. 1 (circles) shows a larger absorption than films no. 3 and 5, although they were

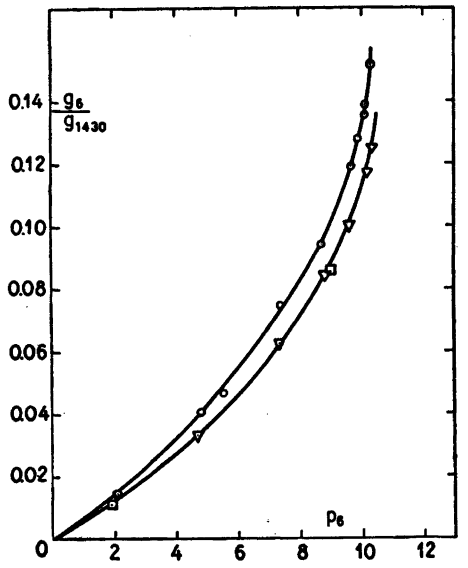

Fig. 9. Hexane absorption by polymethylene C_{1430} at 17°. Comparison of different films of the same material.

○ *film no 1, series 2.*
□ *3, series 10.*
▽ *5, series 7.*

made from the same material and dried at the same temperature, 100° C. No. 1, however, was prepared by evaporation of the solvent in a horizontal rotating testtube (*cf.* Table 3, last column) whereas no. 3 and 5 were plane films, left undisturbed during evaporation. As we should expect, the "undisturbed" crystallization leads to a higher degree of crystallinity, as indicated by the lower absorption of films no. 3 and 5. Fig. 10 giving results obtained with films no. 1 and 5, but at another temperature, shows similar features.

On the other hand, when a standard procedure of preparation is strictly adhered to, it seems possible to obtain two films of equal properties. Thus in

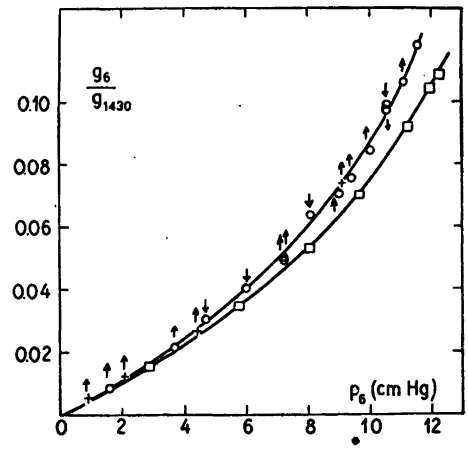

Fig. 10. Hexane absorption by polymethylene C_{1430} at 22°. Comparison of two films of same material.

○ *film no 1, series 6*
□ *5, series 8*
\+ *1, series 5*
↑ *observation in a series of increasing vapour pressures.*
↓ *observation in a series of decreasing vapour pressures.*

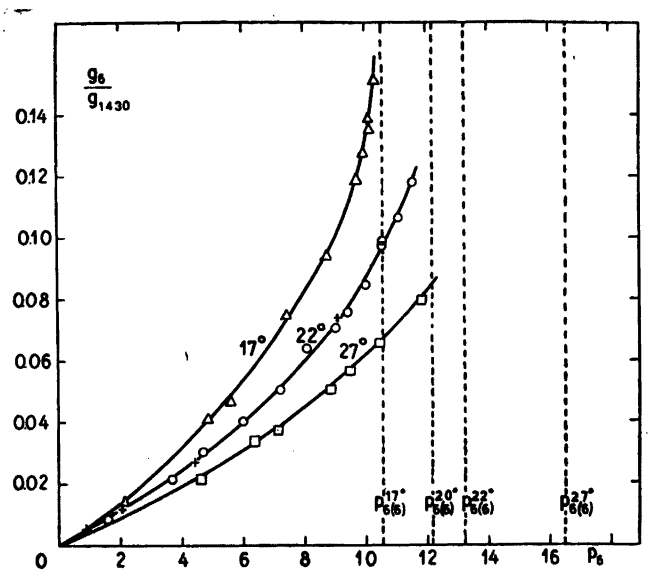

Fig. 11. Hexane absorption by polymethylene C_{1430} at different temperatures. Film no 1. Grams absorbed pr. gram polymethylene against equilibrium vapour pressure.

△ *17°, series 2*
○ *22°, series 6*
\+ *22°, series 5*
□ *27°, series 9*
Saturation pressures indicated by the dotted lines.

Fig. 9 the data of film no. 3 (squares) and film no. 5 (triangles) fit the same curve. The material however is scarce, and the consistency may be incidental rather than significant, so much the more as another pair of films, no. 6 and 7, prepared from C_{2500} under identical conditions shows quite considerable deviations, *vide* Fig. 15, triangles.

I. Hexane absorption by polymethylene C_{1430} at different temperatures.

Fig. 11 illustrates the temperature dependency of the swelling of polymethylene in hexane vapours. Film no. 1 was used for all measurements. Grams hexane absorbed by one gram polymethylene is plotted against the equilibrium vapour pressure p_6. The swelling at any given vapour pressure decreases with increasing temperature. As the applied vapour pressure approaches the saturation pressures $p_{6(6)}$ at the relevant temperature, indicated

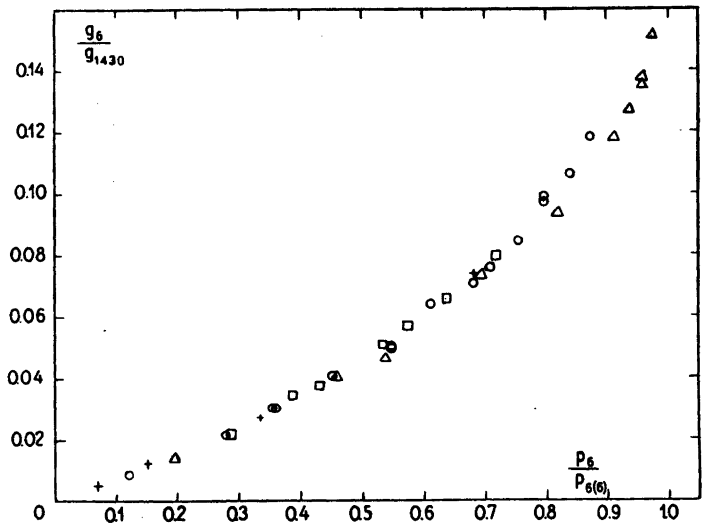

Fig. 12. Hexane absorption by polymethylene C_{1430} at different temperatures. Film no 1. Grams absorbed per gram polymethylene against saturation fraction.

△ *17°, series 2*
○ *22°, series 6*
\+ *22°, series 5*
□ *27°, series 9*

by the vertical dotted lines, measurements become impossible because of condensation on the film and on the balance. For the two series measured at 22° and 27° C the saturation pressure at room temperature (20° C) is the limiting value, at which condensation takes place in the manometer system. The values of saturation pressures are those given in Table 1. For $p_6 = p_{6(6)}$ the absorption probably reaches a finite value, *i.e.* that valid for swelling in liquid hexane. Then a new phase is formed, consisting of pure liquid hexane, since polymethylene C_{1430} is insoluble in hexane in the temperature range covered by the experiments. Similar behaviour is well known for other binary mixtures of a high polymer and a low molecular compound [18].

When the absorption is plotted against the saturation fraction (vapour activity) $p_6/p_{6(6)}$ as shown in Fig. 12 the three curves valid for 17°, 22° and 27° C respectively, very nearly coincide, showing very little effect of temperature changes. The tendency is toward a small increase of absorption with increase of temperature, which is also demonstrated in Fig. 14 (see below) but this point needs further confirmation by measurements at higher temper-

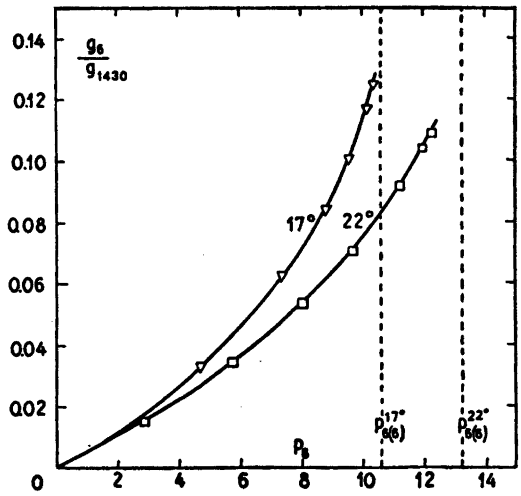

Fig. 13. Hexane absorption by polymethylene C_{1430} at different temperatures. Film no 5.

▽ *17°, series 7*
□ *22°, series 8*

atures. A positive temperature coefficient has been found for the swelling of other high polymers, see for instance [18].

A rough estimate of the solubility of liquid *n*-hexane in polymethylene C_{1430} can be obtained by extrapolating the graph in Fig. 12 to $p_6/p_{6(6)} = 1$. The value probably is between 0.15 and 0.20 gram per gram polymethylene at room temperatures.

Fig. 13 and 14 illustrates the data obtained with another film (no. 5) of the same material at 17 and 22° C. The results are concordant with the previous ones, but the absolute values of absorption differ, because the two films have a different degree of crystallinity.

J. The absorption of *n*-hexane by polymethylenes of different indices.

Fig. 15 shows the vapour pressure isotherms at 22° C for films prepared similarly from polymethylenes with the indices indicated at the end of the curves. The volatile alkane is *n*-hexane.

The results do not on the whole permit definite conclusions. For comparison the isotherm for crystalline $C_{36}H_{74}$ is also given. This substance does not absorb hexane under similar conditions and the isotherm coincides with the

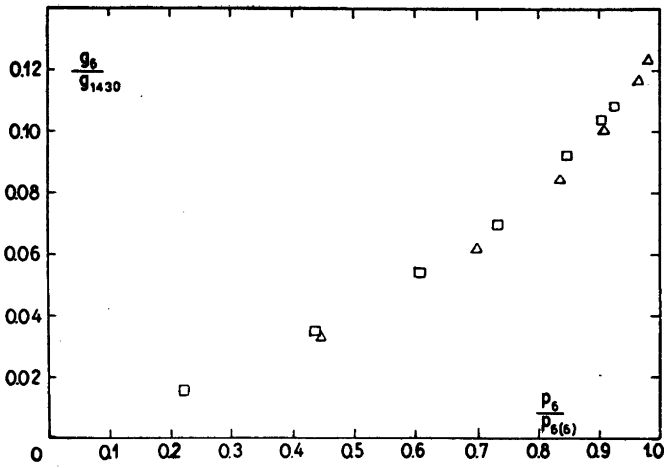

Fig. 14. Hexane absorption by polymethylene C_{1430} at different temperatures. Film no 5. Grams absorbed per gram polymethylene against saturation fraction.

△ *17°, series 7*
□ *22°, series 8*

abscissa. This is in accordance with the view that only the amorphous parts of polymethylene absorb hexane. If C_{36} is heated above the melting point it too will absorb.

Judging from the hexane absorption the degree of crystallinity or orderliness seems to decrease from 100 % in the typical low molecular solid *n*-alkanes to a considerably lower value in C_{360}, which has still retained the brittle character. It is still lower in the tough elastical and strongly absorbing high polymers*. This is not surprising as the probability that the molecules orient themselves parallel to one another on solidification must decrease with increasing chain length. For the high polymers proper in Fig. 15 the observed absorption does not depend on the index in any simple way, obviously because the influence of chance deviations in crystallinity is of the same order of magnitude

* In a quite recent paper by Ueberreiter and Orthmann [24], this view is in principle supported by measurements of specific volumes of *n*-alkanes. The following "Ordnungsgrade" were observed at 20° C.

C_{28}	100 %
C_{35}	93 »
C_{45}	90 »
C_{65}	87 »
C_{110}	89 »
C_{1430}	88 »

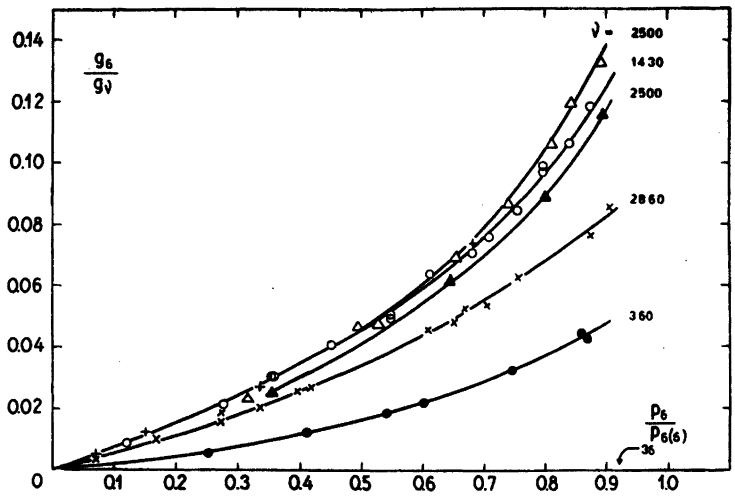

Fig. 15. Absorption of n*-hexane by polymethylenes of different indices at 22°. Grams absorbed hexane per gram polymethylene against saturation fraction.*

	ν	*film no.*	*series*
●	*360*	*8*	*11*
○	*1 430*	*1*	*6*
+	*1 430*	*1*	*5*
△	*2 500*	*6*	*12*
▲	*2 500*	*7*	*13*
×	*2 860*	*10, 10b, 10d*	*4*

as the influence of chain length, and the two effects may either oppose or fortify one another. It is perhaps worth noticing that the sequence of indices in Fig. 15 is the same as that observed in the solubility experiments on page 249. C_{1430} and C_{2500} dissolved in the concentration 5 % in xylene at 75° C, whereas C_{360} and C_{2860} required 85° C.

As will be shown in a subsequent paper theoretical considerations indicate that in all probability the hexane absorption by amorphous polymethylene at constant temperature and vapour pressure is independent of the index of polymethylene, provided this is larger than 54. If so, the deviations of the curves in Fig. 15 have to be ascribed exclusively to deviations in crystallinity of the individual films. As previously mentioned the crystallinity probably decreases with increasing index for mechanical reasons, but this variation is overshadowed by chance deviations at least in the extremely high-index polymethylenes.

It follows that intercomparison of hexane absorption by solid polymethylenes is of little value, unless carried out at some well-defined degree of

crystallinity. Even above the "melting point", where the crystallinity should be zero, such comparison may be difficult, because there is evidence that a certain degree of orientation prevails even in the liquid state [9].

Richards [19] by measurements of the swelling of polymethylenes in liquid xylene observed an increasing absorption with decreasing index. 10 % was absorbed by polymethylene of index 2 860 and about 20 % by polymethylene of index *ca.* 700. Similar results were obtained with liquid *n*-hexane at 20° C [19, p. 22]. Solid low index alkanes, however, do not absorb at all. Consequently, accepting Richards results, the absorption should pass a maximum at a certain index and then decrease. If this index lies somewhere between 360 and 1 400 then qualitative accordance is attained between our results and those of Richards, but in view of the uncertainties outlined above little or no importance can be attached to such comparisons.

K. The absorption of different volatile alkanes by polymethylene C_{2860} at constant temperature.

Fig. 16 illustrates the variation of the absorption with the index ν of the volatile alkane, the same polymethylene film (no. 10 d) having been used throughout. The temperature was 22° C. In order to permit direct comparison millimoles absorbed alkane per gram polymethylene is plotted against the saturation fraction. Hexane shows the larger absorption. For increasing index of normal alkanes the absorption decreases as expected. The effect of chain branching, judging from the data of "iso-octane" (8i), seems to be to lower the absorption relative to that of the normal compound, but obviously no general conclusion can be derived from this single experiment. The dotted parts of curve 8 and 8i are given with reserve, because the relevant data are close to the saturation point.

Richards [19 p. 26, Table III] observed an *in*creasing absorption (wt %) with increasing index up to a certain limit. The measurements were carried out at 20° C with liquid alkanes. Richards data are (symbols as elsewhere in this paper):

	ν_1	$\frac{g_{\nu_1}}{g_{\nu_2}}$	$\frac{g_{\nu_1}}{g_{\nu_2} \cdot M_{\nu_1}}$
n-pentane	5	0.06	0.0008
n-hexane	6	0.082	0.00095
n-heptane	7	0.105	0.00105

An extrapolation of the graphs in Fig. 16 to the saturation pressure is very uncertain, but it seems very unlikely that the absorption should vary with the index in opposite ways at saturation and at lower vapour pressures respectively.

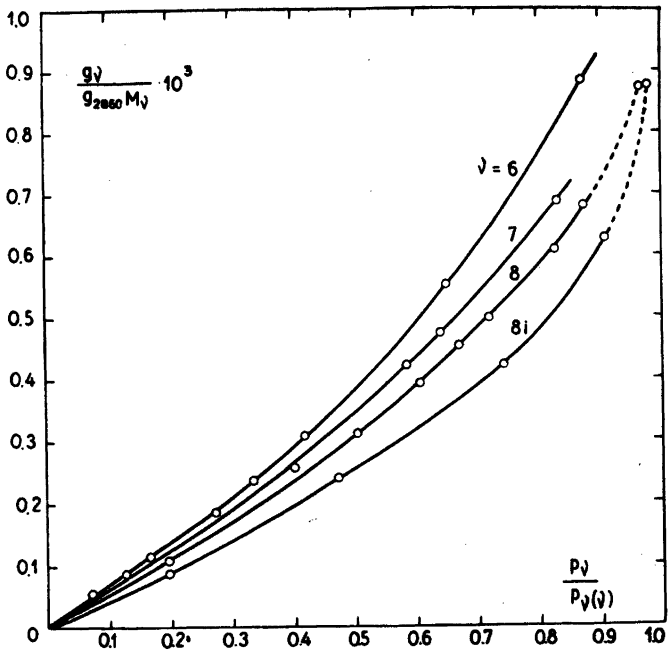

Fig. 16. Absorption of different volatile alkanes by polymethylene C_{2860} at 22°. Film no. 10d. Millimoles volatile alkane per gram polymethylene against saturation fraction.

ν	*Volatile alkane*	*Series*
6	n-*hexane*	*4*
7	n-*heptane*	*14*
8	n-*octane*	*15*
8	"*iso-octane*" *(8i)*	*16*

Even if the molecular weight is taken into account (last column in the above table) the sequence is the opposite to the one found by us for unsaturated vapours. In section L an indirect extrapolation is carried out. It confirmed the discrepancy.

The four series of data collected in Fig. 16 were measured in the sequence 6, 7, 8 and 8i. It is of course important to ensure, that no changes in structure have occurred during the measurements. As mentioned on page 259 there has not been found evidence of such changes at the relevant temperature. This point has been further confirmed by S. Brodersen (private communication), who re-measured the hexane absorption of the film no. 10 d at 22° and obtained data in complete agreement with those in Fig. 16 curve "6".

L. Mathematical representation of the results.

The isotherms in Fig. 12, 15 and 16 roughly give the concentration of the volatile alkane (ν_1) in the swelled phase as a function of the vapour activity $p_{\nu_1}/p_{\nu_1(\nu_1)}$. For the present the deviation of the vapours from ideality will be neglected. Let a_{ν_1} denote the activity and c_{ν_1} the concentration in units of millimoles per gram polymethylene. It has been found that as a first approximation the equation

$$\frac{a_{\nu_1}}{c_{\nu_1}} = \alpha e^{\beta c_{\nu_1}} \tag{I}$$

or in the logarithmic form

$$\log_{10} \frac{a_{\nu_1}}{c_{\nu_1}} = \log_{10} \alpha + 0.4343\ \beta c_{\nu_1}, \tag{II}$$

α and β being constants, is satisfied by the data, since all the curves in Fig. 12, 15 and 16 are transformed into straight lines when $\log \frac{a_{\nu_1}}{c_{\nu_1}}$ is plotted against c_{ν_1}, as has been done in Fig. 17 and 18. The scatter is considerable at low values of c_{ν_1} as should also be expected, because the relative accuracy of the underlying vapour pressure measurements is smaller in this region. For the significant data, however, the graphs leave no doubt about the linear relationship, except perhaps for octane (Fig. 17, 8), which displays a slight curvature. The numerial values of the slopes β and the intersections $\log \alpha$ have been graphically estimated. They are given in Table 5, column 7 and 6.

Table 5.

1	2	3	4	5	6	7	8	9	10	11
ν_1	ν_2	t	series no.	film no.	$\log \alpha$ (± 0.02)	β (± 0.05)	α (± 0.1)	c_{ν_1} sat. vap.	$\left[\frac{c_{\nu_1 \text{ vap.}}}{c_{\nu_1}}\right]_{c \to 0}$	$[c_{\nu_1}]_{a=1}$
6	360	22°	11	8	0.52	−1.31	3.3	0.00721	0.024	
6	1430	22°	5, 6	1	0.08	−0.46	1.2	721	0.009	
6	2500	22°	12	6	0.08	−0.51	1.2	721	0.009	
6	2500	22°	13	7	0.08	−0.44	1.2	721	0.009	
6	2860	22°	4	10, 10b, 10 d	0.18	−0.51	1.5	721	0.011	1.20
7	2860	22°	14	10 d	0.25	−0.58	1.8	214	0.004	1.00
8	2860	22°	15	10 d	0.30	−0.67	2.0	064	0.001	0.92
8 "i"	2860	22°	16	10 d	0.38	−0.81	2.4	233	0.006	0.77
6	1430	17°	2	1	0.08	−0.39	1.2	585	0.007	(1.8)
6	1430	27°	9	1	0.08	−0.48	1.2	881	0.011	

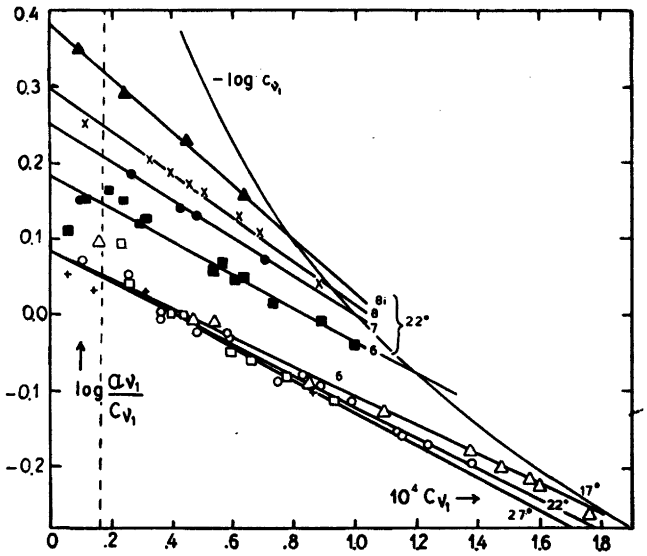

Fig. 17. Dependency of "activity coefficient" upon concentration. Transformation of the curves in Fig. 16 and 12.

	Film no.	*Series*		*Film no.*	*Series*
■	*10, 10b, 10d*	*4*	△	*1*	*2*
●	*10d*	*14*	+	*1*	*5*
×	*10d*	*15*	○	*1*	*6*
▲	*10d*	*16*	□	*1*	*9*

The physical significance of α is realized in the following way. Let $c_{v_1\,\mathrm{vap}}$ be the concentration of alkane vapours in units of millimoles per ml at a vapour pressure p_{v_1} and constant temperature. By $c_{v_1\,\mathrm{sat.\,vap}}$ we denote the concentration of the saturated vapours, then, neglecting the small deviation from ideality, we have

$$a_{v_1} = \frac{c_{v_1\,\mathrm{vap}}}{c_{v_1\,\mathrm{sat.\,vap}}}$$

On substitution of this expression into (I) we obtain

$$\frac{c_{v_1\,\mathrm{vap}}}{c_{v_1}} = \alpha \cdot c_{v_1\,\mathrm{sat.\,vap}} \cdot e^{\beta c_{v_1}}.$$

Hence $\alpha c_{v_1\,\mathrm{sat.\,vap}}$ is the limiting distribution ratio of the volatile component between the vapour phase and the swelled phase for $c_{v_1} \rightarrow 0$. $c_{v_1\,\mathrm{sat.\,vap}}$ is a constant, depending upon temperature, pressure and index of the alkane, its

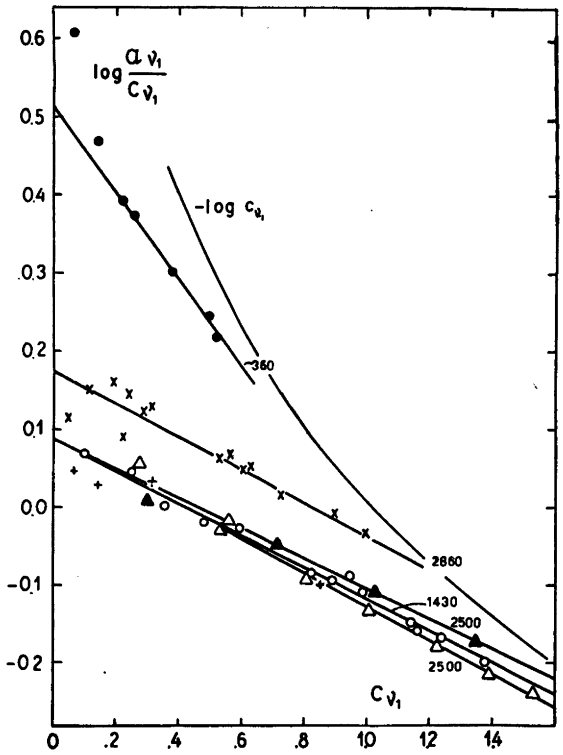

Fig. 18. Dependency of "activity coefficient" upon concentration. Transformation of the curves in Fig. 15 (same notation).

numerical values can be calculated from the saturation vapour pressures in Table 1 by means of the relation $p = RTc$. When p is given in units cm Hg and c in millimoles per ml, the equation becomes $c = p/62.37\ T$, which has been used for calculation of the values of $c_{v_1\ \text{sat. vap}}$ given in column 9. Column 10 contains approximate values of the limiting distribution ratio, obtained as the product of α and $c_{v_1\ \text{sat. vap}}$. This calculation implies the approximation of regarding the unit *millimoles per gram* used for c_{v_1} equal to *millimoles per ml.* As, however, the density of polymethylenes is very close to 1 this is justified for the present purpose. With this approximation the reciprocal values $\frac{c_{v_1}}{c_{v_1\ \text{vap. sat}}}$ are Ostwald's solubility coefficients.

In order to correlate in terms of solubility our vapour swelling data with those obtained by Richards (*vide* p. 267) for swelling in liquid alkanes the curves in Fig. 12, 15 and 16 should be extrapolated to unit activity $p_{v_1}/p_{v_1(v_1)}$

= 1, which is by definition the activity of the pure liquid. For this procedure the transformed isotherms in Fig. 17 and 18 are much more suitable. Inserting $a_{p_1} = 1$ into equation (II) we get

$$-\log c_{p_1} = \log \alpha + 0.4343\ \beta c_{p_1} \qquad \text{(III)}$$

Consequently the extrapolated values of c_{p_1} for $a_{p_1} = 1$ can be read directly on Fig. 17 as the abscissae of the intersection points between the function $-\log c_{p_1}$ and the respective straight lines. The solubilities obtained for the four different alkanes in polymethylene C_{2860} are listed in column 11. Their order of magnitude, 1 millimole per gram, agree quite well with Richard's solubilities of the liquid alkanes in a non-specified polymethylene sample at 20°, but the sequence is the opposite to that observed by Richards.

The intersections are only well-defined for C_{2860}, whereas the lines of C_{2500} and C_{1430} are approached almost asymptotically by the $-\log c$ curve (Fig. 18). For C_{360} there appears to be no intersection at all, which might be indicative of unlimited solubility at room temperature. Although this is true of the low-molecular linear "polymethylenes" up to about C_{16} it is against experimental evidence in the region of solid high polymers. It is probable, therefore, that the non-existency of intersection for C_{360} rather indicates that the equation (II) is a less good approximation at lower indices, and that the curvature suggested by the two points off the line is in fact real. By extrapolation of the C_{360} graph in Fig. 15 to $p_6/p_{6(6)} = 1$ we get $g_6/g_{6(6)} \approx 0.06$ corresponding to an intersection in Fig. 18 at $c = 0.07$.

The bearing of the swelling experiments described in this paper on the theory of congruency will be discussed in a subsequent paper.

The author is greatly indebted to the late professor J. N. Brønsted for suggesting the investigation and for inspiring criticism. My thanks are due to prof. dr. J. A. Christiansen, civiling. A. E. Lansner and mag. scient. Jørgen Koefoed for discussion.

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On the Isomerism of Hydroxyurea

I. Kinetics of the Reaction between Hydroxylammonium Ion and Cyanate Ion

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Two isomeric substances are known to be formed by the reaction indicated in the title, but contradictory views are held on their structures. As a first approach toward preparation of the pure isomers, for the purpose of further investigations, the kinetics of the reaction has been provisionally examined in aqueous solution at 0° C. It could be described as a bimolecular, second order, irreversible reaction. The velocity constant is approximately 8, using the minute as time unit and molarity as unit of concentration. The reaction is much faster than the analogous process leading to urea.

When aqueous solutions of a hydroxylammonium salt and a cyanate are mixed, it is believed that chiefly the following ionic reaction occurs

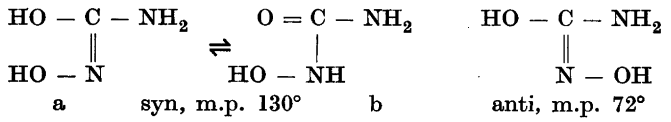
$$HONH_3^+ + {}^-OCN \rightarrow CH_4O_2N_2 \text{ "hydroxyurea"}$$

It is analogous to Wöhler's classical synthesis of urea from ammonium ion and cyanate ion [1], but differs *inter alia* in that crystalline hydroxylammonium cyanate never has been isolated and in that two isomers are known of the "hydroxyurea". Although a number of formulations have been proposed, the structure of the isomers cannot be regarded as finally settled. The problem is particularly interesting because *a priori* both structural and geometrical isomerism as well as tautomerism, or several of these phenomena combined, must be considered possible in an attempt to explain the existence of two discriminate substances. Hydroxylamine and its derivatives, such as oximes, hydroxamic acids, aminooxides etc. have occupied the structural chemists for quite extensive periods and given rise to some of the most violent and fruitful

discussions in the history of chemistry. The same applies to cyanic acid and its derivatives. Some of the interesting problems presented by the reactants may possibly reappear in hydroxyurea.

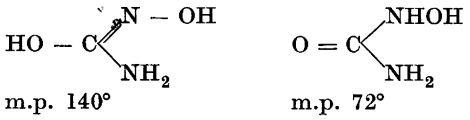
By letting hydroxylammonium nitrate and potassium cyanate react in aqueous solution Dresler and Stein [2] in 1869 first prepared a compound of the empirical formula $CH_4O_2N_2$, which they, on grounds of analogy to the urea synthesis, named "Hydroxylharnstoff". It showed a melting point of approximately 130° C.

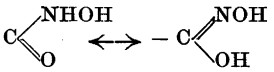
In 1901 the other isomer, m.p. *ca.* 72° C was discovered by Francesconi and Parrozzani [3] by careful fractionation of the reaction mixture from the above process, performed at 0° C between the solid components. The authors suggested that the two compounds were geometrical isomers, similarly to what is known for oximes



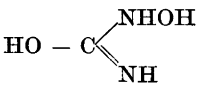
the higher melting isomer being the syn-form, probably in tautomeric equilibrium with the structure b.

In 1907 Conduché [4] observed that the lower melting isomer alone had retained the ability of hydroxylamine derivatives to undergo condensation with oxo-compounds. On these grounds he advocated the following structural isomerism



Hurd and Spence in 1927 [5] found that hydroxyurea did not undergo a Lossen rearrangement to isocyanate, and concluded that it cannot contain a hydroxamic acid structure 

According to Hurd and Spence hydroxyurea should be



There is no allusion to the isomerism in their paper.

Other papers dealing with hydroxyurea have little or no relevance to the structural problem. Cordier [6] has investigated the evolution of nitrogen on treatment with hypobromite, but did not obtain conclusive results. Meyer [7]

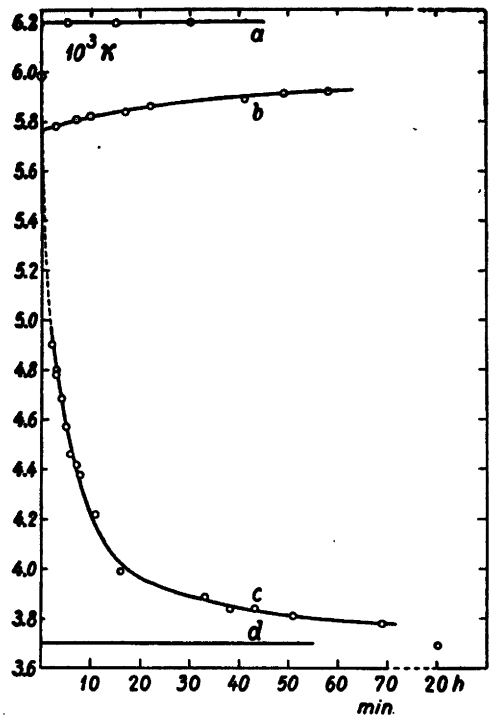


Fig. 1. Specific conductivity at 0° C of aqueous solutions. a) 0.1 M *potassium cyanate; b) 0.1* M *hydroxylammonium chloride; c) Equal volumes of 0.1* M *potassium cyanate and 0.1* M *hydroxylammonium chloride; d) 0.05* M *potassium chloride.*

attempted a condensation of hydroxyurea (mixture of isomers) with β-ketoesters, but failed to isolate the anticipated isoxazolone derivatives. Instead a small yield was obtained of a substance, which analyzed as an equimolar addition product.

A detailed discussion of the proposed structures will be postponed until the experimental material presented in this and the subsequent papers in this series can be included.

For the purpose of further investigation into the structural problem it was desirable to prepare the isomers of hydroxyurea in a pure state. The methods previously described in the literature give extremely small yields. As a first approach towards an improved method of preparation the kinetics of the reaction (1), leading to the formation of hydroxyurea, has been provisionally examined.

$$K^+ + {}^-OCN + HONH_3^+ + Cl^- = CH_4O_2N_2 + K^+ + Cl^- \qquad (1)$$

Since the reactants are ionic, whereas hydroxyurea may be assumed to be undissociated or practically so, the method that presents itself is conductometry. Preliminary experiments showed that at 0° C the isomers were suf-

ficiently stable and the reaction in aqueous solution slow enough to be followed conductometrically.

In Fig. 1. the specific conductivity $\varkappa$ at 0° C of aqueous solutions is plotted against a time basis. In order to secure that no secondary reactions occurred at the platinized electrodes the stock solutions of the two components were first measured. Whereas potassium cyanate (*a*) showed a constant conductivity, a small increase with time was observed for hydroxylammonium chloride (*b*). This is not due to catalytic oxydation, since the process takes place in hydrogen atmosphere as well. The phenomenon has been observed previously by Ross [8], who regarded it as being due to catalytic decomposition on the electrode surface. Curve *c* represents the course of the conductivity of a mixture of the components in stoichiometric proportions prepared by mixing equal volumes of 0.1 *M* solutions. Samples were withdrawn from the reaction vessel at intervals and measured. In this way the influence of the above secondary reaction was reduced to a minimum, which for the present purpose may be neglected. During the first ten minutes the reaction is too rapid for this technique; the corresponding data were therefore measured separately using the conductivity cell as reaction vessel; apart from that, the conditions were identical. As shown by curve *c* the conductivity falls off rapidly to the value of 0.05 *M* potassium chloride (*d*). This result is compatible with the assumptions that the reaction at 0° C follows the scheme (1), that the process is irreversible and that both isomers are only weakly dissociated. It may also be inferred from curve *c* that no detectable degradation of hydroxyurea involving formation of ions occurs at 0° C within 20 hours. The experiment obviously cannot give any information about the proportions of the two isomers formed.

On the basis of the conductometric data the formation of hydroxyurea can be described as a bimolecular, second order reaction. Since the reactants are present in the same molar concentration, initially c_0, we should have

$$kt = \frac{1}{c} - \frac{1}{c_0} \tag{2}$$

k being the rate constant and c denoting the concentration of any reactant at any time t during the reaction. The specific conductivity $\varkappa$ of the reaction mixture at any time is

$$\varkappa = \varkappa_{KCl} + \varkappa_{HONH_3^+} + \varkappa_{-OCN} \tag{3}$$

According to (1) $\varkappa_{KCl}$ is constant during the reaction. Let Λ denote the molar conductivity, then for dilute solutions we have $\varkappa = \Lambda\ c/1000$. Introducing this in (3) we obtain

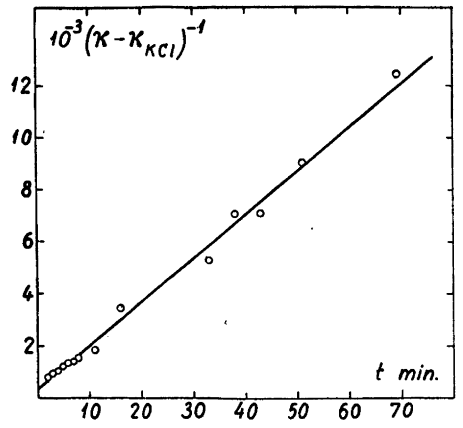


Fig. 2.

$$\varkappa - \varkappa_{KCl} = \Lambda_{HONH_3^+} \cdot \frac{c}{1000} + \Lambda_{-OCN} \cdot \frac{c}{1000}$$

or, writing $\frac{1000}{\Lambda_{HONH_3^+} + \Lambda_{-OCN}}$ as L we obtain $c = L\,(\varkappa - \varkappa_{KCl})$. Inserting this expression for c into (2) this becomes

$$(\varkappa - \varkappa_{KCl})^{-1} = k \cdot t \cdot L + \frac{L}{c_0} \qquad (4)$$

As shown in Fig. 2 the experimental data satisfy equation (4) within the not very great experimental accuracy; the plot of $(\varkappa - \varkappa_{KCl})^{-1}$ against t is linear. The slope of the best line, estimated from the graph, is approximately $k \cdot L = 1.7 \cdot 10^2$; the intersection is $L/c_0 \approx 4 \cdot 10^2$. Introducing $c_0 = 0.05$ in the latter we get $L \approx 20$. As however the intersection in Fig. 2 cannot be determined with any great accuracy a somewhat better value of L is probably provided in the following way. The initial conductivity $\varkappa_0$ of the reaction mixture (curve *c*, Fig. 1) is assumed to be the arithmetic mean of those of the two components, *i. e.* $\varkappa_0 = 0.006$. Since $\varkappa_0 = \varkappa_{KCl} + \frac{\Lambda_{HONH_3^+} + \Lambda_{-OCN}}{1000} \cdot 0.05$

we get $L = \frac{1000}{\Lambda_{HONH_3^+} + \Lambda_{-OCN}} = 22$

Substituting this value for L in $kL = 1.7 \cdot 10^2$ we obtain a rate constant of approximately 8 at 0°, using the minute as time unit and moles per l as unit of concentration. The reaction is thus considerably faster than the analogous process between ammonium ion and cyanate ion leading to the formation of urea. Rate constants for this reaction in aqueous solution were determined by

Walker and Hambly [10] for temperatures between 25° and 80° C. The value at 25° C expressed in the same units as above was 0.00227. By a rough extrapolation to 0° C an order of magnitude of 10^{-5} is obtained.

EXPERIMENTAL

Potassium cyanate was prepared according to Erdmann [11] by oxidation of potassium ferrocyanide with potassium dichromate. It was recrystallized repeatedly from 80 % ethanol, quickly washed with absolute ethanol and ether, dried and stored over sulphuric acid. The product contained only traces of carbonate (weak opalescence with barium chloride solution). On titration with silver nitrate it gave an equivalent weight of 81.20, calculated for KOCN 81.11. Hydroxylammonium chloride of analytical grade was used.

The specific conductivities were measured by means of the conventional bridge method at a frequency of 1 000 cycles. All measurements were made against the standard $\varkappa = 0.002501$ for 0.02 *N* potassium chloride in water at 20° C [9]. The all-glas cell with sealed-in platinized platinum electrodes was thermostated in a Dewar vessel containing a mixture of ice and water. The carbon dioxide was removed from the solution and the temperature equilibration accelerated by passing a stream of carbon dioxide-free air through the cell for about 2 minutes before the first measurement.

The reaction vessel, the stock solutions and the pipettae were precooled to 0° C and the appropriate volumes measured at the same temperature. When the reaction was performed in the measuring cell, the potassium cyanate solution was first introduced and then the hydroxylammonium solution was added quickly, and immediate mixing effectuated by an air current as above. In this way the catalytic decomposition of hydroxylammonium was reduced to a minimum. When a separate reaction vessel was used, 3 ml samples were withdrawn at intervals with a precooled pipette and quickly transferred to the cell.

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The Chlorine Oxidation of Glycosides

III.* Oxidation of Methyl β-Cellobioside

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Parts I and II of this series have dealt with the oxidation of simple methyl glycosides with chlorine water. It was found that the β-glycosides were oxidized to glyconic acids under conditions which precluded initial hydrolysis of the glycoside. The intention of this work is to study the degradation of cellulose by bleaching with chlorine, the methyl β-glycosides being regarded as simple model substances. In this communication similar experiments with more cellulose-like model substances, methyl β-cellobioside and cellobionic acid, are reported.

The reaction of methyl β-cellobioside with chlorine water was carried out and followed by analytical determinations as for the corresponding reaction of methyl β-glucoside [1]. Calculations from optical rotation measurements indicated that two reactions of different velocities took place. The faster reaction occurred at about the same rate as the reaction of methyl β-glucoside with chlorine water, while the second appeared to be two or three times slower than this and predominated during the second week of chlorination.

At intervals portions of the reaction mixture were withdrawn, freed from chlorine and hydrochloric acid, and analyzed in paper partition chromatograms. A solvent mixture of butanol, ethanol, and water was used. The spots were in most cases detected with a silver nitrate reagent according to the method of Trevelyan, Procter and Harrison [2]. This reagent proved very useful and gave dark colourations with all the substances encountered in this work.

The acids and lactones which were present gave chromatograms which were difficult to interpret on account of poor separation of the spots. It was

* Part II. *Acta Chem. Scand.* **6** (1952) 791.

** Billeruds AB Research Fellow, 1951—1952.

therefore decided to convert the acids into derivatives which could be more readily separated, and the phenylhydrazides were found to be convenient for this purpose. Norris and Campbell [3] have used phenylhydrazine hydrochloride to convert keto acids into their phenylhydrazones before chromatographic identification of gluconic acid. In the present investigation samples of the reaction mixture were heated with phenylhydrazine and non-reducing acids thus partly converted into phenylhydrazides. These derivatives travelled much faster than the free acids on the chromatogram, and they gave strong spots, easy to identify. Reducing acids appeared only faintly or were carried into a dark zone near the solvent front.

Examination of the solutions by this technique showed that cellobionic acid is readily formed in the early stages of the chlorination reaction, while gluconic acid is produced more slowly and accumulates during the first two weeks. Traces of methyl β-glucoside appeared transiently. A separate chlorination experiment showed that cellobionic acid itself is oxidized to gluconic acid.

These results indicate that the methyl-glucoside bond in methyl β-cellobioside and the disaccharide linkage in cellobiose derivatives in general are cleaved in the same manner as in methyl β-glycosides, although the latter type of linkage undergoes cleavage less readily than the former.

Gluconic acid is not a stable end product in the reaction with chlorine water; it is slowly oxidized further to 5-ketogluconic acid [1]. Saccharic acid was also detected in chromatograms of chlorinated gluconic acid. Saccharic acid is obviously responsible for the "fifth spot" met with in the previous work [1], though this fact was not demonstrated at the time.

The solutions of chlorinated cellobionic acid and methyl β-cellobioside contain unidentified components, which are not present in solutions of chlorinated gluconic acid or methyl β-glucoside. The positions of these components on the chromatograms suggest that they are derivatives of cellobionic acid. Two of these spots could be developed with a spraying reagent for keto-compounds and thus probably arise from ketocellobionic acids.

Attempts to prepare cellobionic acid phenylhydrazide in a crystalline state failed, although its preparation has been reported [4]. Gluconic acid was isolated in a small yield as the phenylhydrazide from the solution of chlorinated methyl β-cellobioside. Saccharic acid was isolated from a solution of chlorinated gluconic acid as the potassium hydrogen salt in a yield of 6 % and identified as the dibenzimidazole derivative.

Methyl β-cellobioside and cellobionic acid were treated with 2 *N* hydrochloric acid, this corresponding to more than the maximum hydrochloric acid strength produced in a 13-day chlorination. Chromatographic examina-

tion of the solutions showed that it took 10 days before detectable amounts of hydrolysis products appeared. The reaction with chlorine, therefore, cannot take place with an initial hydrolysis of the glucosidic bonds.

EXPERIMENTAL

Action of chlorine on methyl β-cellobioside and cellobionic acid. — A 0.25 *M* solution of methyl β-cellobioside was treated with chlorine and determinations carried out following the earlier procedure [1]. The results are given in Table 1. Samples withdrawn were at once freed from chlorine by aeration and from hydrochloric acid by successive treatment with silver carbonate and hydrogen sulfide.

Table 1. Chlorination of methyl β-cellobioside.

Reaction time		α_D	Conc. of HCl.
days	hours		equiv./l
0	0	− 3.35	0
0	20	− 2.78	
1	20	− 2.28	
2	20	− 1.82	
4	0	− 1.48	
5	20	− 1.08	0.77
9	0	− 0.53	1.08
12	22	− 0.16	
15	21	+ 0.06	1.97
23	0	+ 0.41	

Cellobionic acid (0.25 *M*) was chlorinated in the same way. The α_D-values changed only slightly.

Chromatographic examinations. — The chromatograms were run on Whatman No. 1 paper and developed for 22 hours by the descending technique with a mixture of butanol (40 %), ethanol (10 %), and water (50 %). The spots were detected with a silver nitrate reagent [2] as already mentioned. When reducing acids were studied it was found more convenient to omit the phenylhydrazine treatment, and to develop the spots directly with resorcinol reagent.

Solutions to be treated with phenylhydrazine were heated (1 ml taken) in small, sealed Pyrex tubes with phenylhydrazine (0.1 ml) for one hour on the steam bath. The tubes were turned a couple of times during heating in order to mix the contents. After cooling the mixture was allowed to settle, and the clear solution was applied to the paper.

R_F-values for the pure compounds and for the phenylhydrazides are given in Table 2. The results of the chromatographic experiments are summarized in Table 3.

Table 2. R_F-values.

Compound	Compound	Phenylhydrazide
Methyl β-cellobioside	0.14	
Methyl β-glucoside	0.30	
Cellobionic acid	0.02	0.38
Gluconic acid	0.04	0.57
5-Ketogluconic acid	0.05	0.45—0.60 a)
Saccharic acid	0.02	0.22; 0.85 b)

a) Probably the phenylhydrazone. The spot trailed and was difficult to observe.
b) Most probably the mono- and the diphenylhydrazides respectively.

Table 3. Chromatographic examinations.

Chlorinated solution	Time of chlorination, days	Compound					
		Methyl β-cellobioside	Methyl-β-glucoside	Cellobionic acid	Gluconic acid	5-Ketogluconic acid	Saccharic acid
Methyl β-cellobioside	2	+ +	−	+ +	(+)		
	5	+	(+)	+ +	+		
	9	(+)	(+)	+ +	+ +	+	+
	13	−	−	+	+ +	+	+
Cellobionic acid	9			+	+ +	+	+
	27			(+)	+ +	+	+
Gluconic acid	27				+ +	+	+

Isolation of gluconic acid as the phenylhydrazide. — Methyl β-cellobioside solution, chlorinated for 9 days, (5 ml) was freed from chlorine and hydrochloric acid and evaporated to 0.5 ml. Phenylhydrazine (0.25 ml) was added and the mixture heated 1.5 hours on the steam bath. The excess of phenylhydrazine was extracted with ether (5 × 10 ml) and a brown precipitate (43 mg) collected. One recrystallization from alcohol gave a slightly coloured product (28 mg) with m.p. 187° (decomp.) *. The yield was 7 % calculated on the methyl β-cellobioside taken. Further recrystallizations yielded pure gluconic acid phenylhydrazide, m.p. 195° (decomp.).

Isolation of saccharic acid as the dibenzimidazole. — Gluconic acid (0.5 *M*) in hydrochloric acid (3 *N*) was chlorinated for 27 days. The solution (40 ml) was freed from chlorine

* All melting points uncorrected.

and hydrochloric acid. Potassium hydroxide solution was added until the solution became alkaline, and the mixture was then heated for 45 min. on the steam bath. After cooling acetic acid was added to pH 3.5 [5]. The solution was concentrated to 10 ml and acetic acid (1 ml) was added. After standing for 3 days at 0° the precipitate was collected and washed with 30 % ethanol. 0.53 g of an almost colourless compound was obtained. One recrystallization from hot water gave potassium hydrogen saccharate (0.31 g) which decomposed at 184°. Yield 6.3 % calculated on the gluconic acid taken.

The potassium hydrogen saccharate (0.31 g) was condensed with *o*-phenylene diamine following the procedure of Lohmar, Dimler, Moore, and Link [6]. Saccharic acid dibenzimidazole (0.27 g), identical with an authentic preparation, was obtained. It had m.p. 230° (decomp.) and $[\alpha]_D^{20}$ + 61° (c = 2 in 5 % citric acid; microtube). Lohmar *et al.*[6] reported m.p. 238 (d.) and $[\alpha]_D^{25}$ + 60.3°.

Action of hydrochloric acid on methyl β-cellobioside and cellobionic acid. — A solution of methyl β-cellobioside (c = 1.72) in hydrochloric acid (2 N) was kept at room temperature. The a_D-value changed from — 0.54 to — 0.51 in 16 days (2 dm tube). Paper chromatograms were prepared, and after 10 days a faint spot of glucose became visible.

Cellobionic acid (c = 1.79) in hydrochloric acid (2.1 N) was treated similarly and with the same result.

SUMMARY

Chlorination experiments with methyl β-cellobioside in aqueous solution have indicated that both glucosidic bonds are cleaved in the same manner as has previously been demonstrated for methyl β-glycosides. The methyl-glucoside bond suffers cleavage more rapidly than the bond between the two glucose units. Cellobionic acid is formed as an intermediate which is further oxidized to gluconic acid. Saccharic acid has been isolated after prolonged chlorination of a gluconic acid solution.

A technique for the chromatographic identification of non-reducing carbohydrate acids as their phenylhydrazides has been developed.

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On the Formation and Properties of Globin Plastein

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The background to the experiments reported in the present paper is as follows: Some years ago it was demonstrated in this laboratory that the so-called plastein formation by the action of pepsin on the peptic hydrolysate of proteins is due to the enzymatic synthesis of polypeptides [1,2]. The experiments with zein showed that the average molecular weight of the polypeptide precipitate varies quite considerably in different experiments, the values obtained being from two-three thousand to ten thousand and even more. The polypeptide size depends greatly on the conditions (especially pH) under which the peptic hydrolysis of proteins is carried out. The amino acid composition of the polypeptide precipitate corresponds, at least to a great extent, to the respective composition of the zein used as starting material. Only the amide content of synthetic polypeptides is lower than in zein, and this differs in different plastein preparations depending on the duration and pH of the hydrolysis, or, in other words, on the amount of ammonia split off during the hydrolysis from the $CONH_2$-groups of the asparagine and glutamine present in the protein.

The similarity of the amino acid composition of the polypeptide mixture, formed by the action of pepsin, to that of the original protein, together with the high molecular weight of these polypeptides, has raised the question of the extent to which these polypeptides, attached to active groups, can function as catalysts like enzymes. In order to gain information on this question the present study on hemoglobin was undertaken. The aim was to hydrolyze with pepsin the globin prepared from hemoglobin and to examine whether the plastein prepared from this hydrolysate by means of pepsin would combine with the heme group to give a product with an absorption spectrum similar to that of a compound of the hemoglobin type.

EXPERIMENTAL

From the washed ox erythrocytes a stroma-free solution of hemoglobin was prepared. Crystallization was effected from 25 % alcohol at *ca.* −3° C (10 days). The crystals were centrifuged rapidly in the cold, washed with 25 % alcohol and dissolved in a small amount of water. This solution was dialyzed overnight against running tap water and centrifuged clear. Soluble globin was prepared from this solution by the method of Anson and Mirsky [3]. The saltfree solution obtained after dialysis was dried by the lyophilization process and the product remained soluble in water.

It has been shown by earlier work in this laboratory that when using zein the optimal conditions for plastein formation are hydrolysis at pH 1−2 followed by precipitation at pH 4 [4]. These conditions were used in the experiments described in the present paper. Three separate hydrolyses of globin were performed.

A 3 g sample of the globin was dissolved in 400 ml diluted hydrochloric acid at pH 1.5 and 15 mg crystalline pepsin were added. The solution was incubated at 37° C until determinations of the peptide amino nitrogen showed no further hydrolysis of the globin (3−4 days). All determinations of amino nitrogen in these experiments were made by the copper method [5], and calculated using the factor 0.14. The average size of the peptides in solution was calculated from determination of the total nitrogen (Kjeldahl) and amino nitrogen (by total hydrolysis) present in the globin preparation, and was found to be 6.4 amino acid residues.

Under reduced pressure the hydrolysate was concentrated almost to dryness. Distilled water was added and the solution again concentrated. This procedure was repeated 5 times before the concentrate was adjusted to pH 4 with 1 *N* NaOH. A slight cloudiness was observed and the solution was allowed to stand at room temperature until the next day, when it was centrifuged clear. After 24 hours the solution was still clear and a determination of the amino nitrogen present showed that there had been a negligible loss during the concentration and ensuing operations.

The concentration of total nitrogen in the hydrolysate concentrate was approximately 18 mg N/ml (17.8−18.8 mg/ml). 2 mg of crystalline pepsin per ml was used for precipitation of the plastein, the formation of which was observed to commence almost immediately. The mixture was, however, set aside for 24 hours at room temperature and the precipitate was then separated by centrifugation and washed with a small amount of water. A second and smaller precipitate had separated 48 hours later and was collected as before.

Nitrogen estimations on the precipitates showed that they contained a little more than 12 % total N (12.17−12.33 %), and more than 1.1 % amino N (1.11−1.20 %) of dry matter, the latter figure being equivalent to more than 9 % of the total N (9.0−9.7 %). The average size of the peptides constituting the plastein was thus approximately 9 amino acids. The weight of plastein obtained was approximately 10 % of the weight of globin (9.28−10.98 %) whereas the loss of amino N through pepsin at pH4 was approximately 13 % (12.00−13.44 %) of the total N present in the hydrolysate concentrate.

It was thus evident that the plastein synthesized under these conditions consisted of smaller peptides than did the zein plasteins which had been synthesized earlier in this laboratory, and which, as raw plasteins, had been found to contain approximately 3−5 % of their total N as amino N corresponding to an average peptide size of about 25−30 peptides. By careful extraction with water a number of small peptides could be removed from these zein plastein precipitates after which the average size of the remaining poly-

peptides corresponded to about 40-peptides. No attempts were made with the globin plasteins to separate the mixture of peptides into fractions containing different amounts of amino N.

As a basis for comparison between the relative average sizes of the plasteins synthesized from different proteins under similar conditions, samples of egg albumin and zein were hydrolyzed for 4 days using the same dilution, pH, and pepsin concentration as for the globin hydrolyses. The figures for the average size of the peptides in the hydrolysates were respectively 6.4 for the egg albumin hydrolysate and 8.3 for the zein hydrolysate (after allowing for 2 % insoluble material present in the zein sample used). The hydrolysates were concentrated and adjusted to pH 4 in the same manner as were the globin hydrolysates and the plastein formation was made from solutions containing respectively 17.2 and 17.4 mg N/ml. The egg albumin plastein contained 13.9 % total N and 0.92 % amino N of dry matter (equivalent to 6.6 % of total nitrogen and corresponding to a peptide size of between 11 and 12 amino acids) and the weight of the precipitate was 14.5 % of the original protein. For the zein plastein the corresponding nitrogen figures were 13.3 % and 0.49 % (equivalent to 3.7 % of total nitrogen and a peptide size of 21—22 amino acids) and the weight was 16.4 % of the original protein.

RESULTS AND DISCUSSION

As was stated earlier in this paper, attempts were made to produce a reaction between the globin plastein and a freshly prepared solution of heme. This latter solution was prepared in 1 *M* K_2HPO_4 and was diluted 50 times in order to give a preparation suitable for readings with the Beckman spectrophotometer. The aqueous solution of plastein containing 5.5 mg dry material per 100 ml at pH 5.7 was mixed with the heme solution in the proportion of 1 : 2, the pH of the mixture being 8.6. The spectrum obtained for this solution was identical to that given by a mixture of 2 parts of the heme solution with 1 part of distilled water. Under similar conditions the spectrum obtained by mixing the heme solution and a solution of the soluble globin possessed the typical maxima of a compound of the hemoglobin type. Thus no compound resembling hemoglobin was formed from globin plastein and heme.

The plastein yield from the peptic hydrolysate of globin was smaller than from the corresponding hydrolysate of zein. The decrease of amino nitrogen in the plastein synthesis was distinct even in the former case. It is of interest that the average peptide size of globin plastein corresponded to only about 9-peptides. Although this would probably have risen through the extraction of the lower peptides, it is, however, at least obvious that no large amounts of high-molecular peptides are formed from globin plastein. Thus a distinct difference exists between the zein and globin hydrolysates in regard to the polypeptide synthesis. This synthesis proceeds gradually in the zein hydrolysate [1,2], small polypeptides being first formed during the rapid decrease of amino nitrogen and remaining in the solution. The synthesis continues as the small

polypeptides join to bigger ones whereby an intensive precipitation takes place. Both reactions can be so rapid that they are difficult to distinguish from each other but when they proceed at a slower rate they can be distinguished. In a previous experiment, in which the average molecular size of the zein hydrolysate corresponded to 5-peptides, only 2.8 % of the total N of the hydrolysate was precipitated by pepsin at pH 4 during an hour, while the decrease of amino nitrogen was 22.9 % of the amino nitrogen of the hydrolysate. The average peptide size of the solution rose from 5-peptides to about 12-peptides. In 22 hours 19.0 % of the total N of the solution was precipitated. The average peptide size of the precipitate corresponded to about 37-peptides. The small plastein precipitate formed during 1 hour also had the same peptide size. The growth of the polypeptides during the reaction becomes evident from this experiment. The small peptide size of the plastein precipitate obtained from globin hydrolysate can perhaps be explained by assuming that the relatively low-molecular polypeptides, formed at the start of the synthesis, are so difficultly soluble that they are removed from the solution by precipitation. The continuation of the synthesis is thus prevented and no high-molecular peptides are formed. The structure of the peptides formed from different proteins in peptic hydrolysis would thus determine the chain length of the polypeptides formed in the so-called plastein synthesis.

SUMMARY

A raw plastein has been prepared from soluble globin. The average peptide size is smaller than those obtainable from zein and egg albumin under similar conditions. No reaction between globin plastein solution and a solution of heme in K_2HPO_4 buffer could be detected with the Beckman spectrophotometer. In the discussion, suggestions are put forward to account for the small peptide size of the globin plastein.

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Nitrogen Metabolism of the Alder (*Alnus*) The Absence of Arginase and Presence of Glutamic Acid Decarboxylase

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The predominating free amino acid of the roots and root nodules of the alder species *Alnus glutinosa* and *Alnus incana* is L(+)-citrulline [1]. In summer, the nodules contain citrulline to about 0.2—0.3 % of the dry weight, but in winter even as much as 2 % of the dry weight. In addition to citrulline, the roots of *A. glutinosa* contain in winter considerable amounts of arginine and ornithine which are not found in the nodules.

The role of citrulline in the nitrogen metabolism of alder is not yet known in detail, but judging from the previous results [1] it can be expected to occur as a very central compound of the nitrogen store.

The question now arises as to which are the enzymatic reactions through which the citrulline nitrogen becomes utilized by the plants. A possible reaction mechanism might be the Krebs-Henseleit urea cycle [2] through which the synthesis of arginine and the elimination of nitrogen take place in the liver of animals. The simultaneous occurrence in abundance of all three amino acids of this cycle in free form in the roots of *A. glutinosa* implies that the synthesis of arginine in alder could proceed along this line as it has been proved to proceed in *Neurospora* [3].

In the above mentioned work [1] we were unable to find urea except in uninoculated roots of alder and even then only in minimal amount, about 0.005 % of the dry weight. Hence, it does not seem likely that mobilization of citrulline nitrogen would take place through urea.

Since, however, the absence of urea might also be due to its further rapid decomposition, we thought it important to estimate the arginase in the nodules of alder. Unless the alder possesses arginase activity, citrulline nitrogen cannot, of course, be mobilized through the urea cycle.

The root nodules of *A. incana*, but not those of *A. glutinosa*, also contain abundantly γ-aminobutyric acid, in autumn up to 0.2 % of the dry weight [1]. The only enzymatic reaction so far known which leads to the formation of this amino acid is decarboxylation of L-glutamic acid.

By the manometric technique, whereby one of the two decomposition products of the reaction, CO_2, is determined, this reaction has been demonstrated and the particular enzyme activity has also been determined in many plant tissues [4,5]. The other decomposition product, γ-aminobutyric acid, has also been isolated and identified [6] when split by the use of a plant born enzyme.

Steward *et al.*[7,8] have found only a minimal activity of L-glutamic acid decarboxylase in potato tubers which contain abundantly γ-aminobutyric acid. In this case they held it possible that γ-aminobutyric acid is a precursor of glutamic acid and not its decarboxylation product. In further support for this hypothesis they mention the fact that γ-aminobutyric acid is not found in the protein hydrolysates and — after having disappeared from the potato during the protein synthesis — it is no longer set free as γ-aminobutyric acid, on the hydrolysis of the potato protein.

It was therefore interesting to examine whether L-glutamic acid decarboxylase activity is detectable in the nodules of grey alder. As, in another connection [9], we had found considerable amounts of free γ-aminobutyric acid in all parts of pea at every period of growth we made the same experiment with leaves of pea, and also, for the sake of comparison, with carrot, from which Schales [5] prepared his soluble L-glutamic acid decarboxylase.

ARGINASE EXPERIMENT

Arginase has been studied in detail with preparations of animal origin, which show an optimal activity at about pH 9.8[10]. The optimal pH for yeast-[11] and jack-bean [12] arginase also lies on the alkaline side. In animal preparations, of which information is available, the enzyme commences to lose its stability already at pH 8, being very unstable at the optimal pH but quite stable between pH 6 and 8.

We have therefore carried out the arginase experiments at pH 7.8 where the activity of arginases of animal origin is known to be about 60 % of the maximal. We have used $MnSO_4$ as an activator, adding it as required to make the final reaction mixture about 0.01 *M* in respect to manganese.

Preparation of plant tissue suspension and arginine solution. The nodules, leaves, and roots of grey alder were taken from nature in August. A pipettable suspension was prepared from the nodules and leaves by crushing about 20 g fresh tissue with 100 ml cold *M*/15 phosphate buffer, pH 7.8. The activator was added on crushing. The roots were cut in thin slices. L(+)-arginine (B.D.H.) was dissolved in *M*/15 phosphate buffer pH 7.8 to make a solution of concentration 20 mg/ml.

Performance of the experiment. 2 ml of the plant tissue suspension and 2 ml of arginine solution were mixed and kept under toluene at about + 20° C, occasionally stirring. The

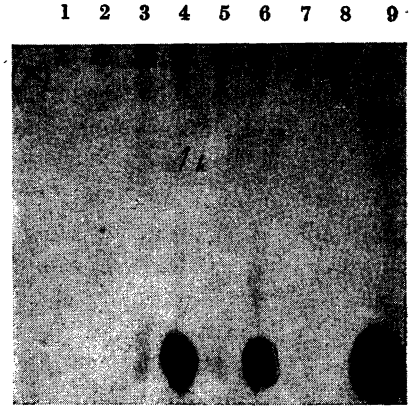

Fig. 1. Absence of arginase activity from the alder. One-dimensional paper chromatogram developed with n-*butanol from samples taken after reaction time of 1 hour. Development of colour with* p-*dimethyl aminobenzaldehyde reagent which gives a yellow spot with urea and citrulline.*

1. *Crushed leaves of A. incana*
2. *The same + arginine*
3. *Crushed leaves of A. incana + crushed cow's liver*
4. *The same + arginine*
5. *Crushed cow's liver*
6. *The same + arginine*
7. *Arginine*
8. *Ornithine*
9. *Urea*

(Citrulline control missing, but the location of citrulline marked with x. Crushed liver contained traces of citrulline.)

reaction was followed by determining paper chromatographically the possible formation of both reaction products, ornithine and urea.

Preparation of paper chromatograms. A 10 μl spot of mother liquor was pipetted on Whatman no. 1 paper straight from the reaction mixture. Four chromatograms were prepared from each sample, two by developing with water-saturated *n*-butanol for 2 days and two by developing with water-containing phenol (500 g phenol + 183 ml distilled water) for 36 hours in the atmosphere made by placing 100 ml of 0.5 % ammonia at the bottom of the chamber. In each run traces of HCN were used in the atmosphere.

One of the butanol and phenol chromatograms was used for identification of ornithine, the other for that of urea.

Ornithine was examined by developing the colour with ninhydrin whereby even 1 μg of ornithine could be detected with certainty corresponding to a 10 μl spot when a concentration of 0.1 mg/ml is used. In this way even the decomposition of *ca.* 1 % of arginine to ornithine could have been demonstrated.

Urea was shown by using *p*-dimethyl aminobenzaldehyde (Dent [13]) for the development of colour in a slightly modified [1] form. A spot of about 0.3—0.5 μg urea is detectable by this reagent.

The samples were taken 1, 4, and 24 hours after the start of the reaction.

RESULTS

Arginase activity could not be noted in any tissues, crushed leaves, nodules, or roots, of *Alnus incana*. Formation of neither ornithine nor urea could be shown paper chromatographically.

For control an experiment was made with cow's liver using the same technique. The chromatograms developed with ninhydrin were then rather blurred due to many impurities in the crushed liver, but in the chromatograms sprayed with *p*-dimethyl aminobenzaldehyde a very intensive formation of urea due to the crushed liver could clearly be shown.

In order to make sure that the crushed alder tissues do not contain any arginase inhibitor, an experiment was also made with a mixture of alder (*A. incana*) leaves and liver crushed together. No inhibitory effect on liver-arginase could be demonstrated with the crushed leaves of alder (see Fig. 1).

Since a formation of even a minute amount of ornithine could have been detected with the very sensitive and specific paper chromatographic method, the arginase activity of the alder tissues examined must be considered as being very low or nil.

Many bacteria contain arginine desimidase [14–16] which splits off an imino group from arginine forming citrulline. The alder tissues did not affect formation of citrulline under the conditions used in our experiments.

L(+)-GLUTAMIC ACID DECARBOXYLASE

Okunuki [4] and Hasse [6] have found that L(+)-glutamic acid decarboxylase has been entirely localized in insoluble cell particles of the cellular tissues examined by them, but Schales [5] again has found that it is easily extractable *e. g.* from carrot with phosphate buffer. In this work, therefore, we used in all experiments unfractionated crushed plant tissue, cell-free phosphate buffer extract and washed suspension of cell residue, obtained by centrifugation.

In this qualitative study we thought it most suitable to demonstrate the reaction by paper chromatographic determination of γ-aminobutyric acid; the manometric technique is less specific because glutamic acid can catalyze the formation of CO_2 in the crushed material even through reactions other than mere decarboxylation (*e. g.* by accelerating cell respiration).

γ-Aminobutyric acid is a completely specific reaction product which can hardly be formed from glutamic acid in any other way than through decarboxylation. To our knowledge the only method for its microdetermination is paper chromatography.

The decarboxylation experiment was made according to the instructions of Schales [5].

Experimental material. All the plant material was grown in the greenhouse and harvested as follows:

1.series: Leaves and nodules of grey alder. Harvested 28 July.
2.series: Harvested 17 Oct.

Nodules of grey alder. The test plant about 2 years old, grown in N-free sand, leaves just falling.

Root of carrot. Variety "Nantes" fully developed, but parts still green in colour.

Leaves of pea. Test plants grown in pots in N-free sand inoculated with an effective strain of root nodule bacteria. Age approximately 10 weeks; been in flower for approximately 1 week.

Preparation of plant tissue suspension. 4 g of plant tissue was rubbed to a homogeneous pipettable suspension in 20 ml of ice-cold *M*/15 phosphate buffer pH 5.60. pH of the suspension was approximately 5.70.

Preparation of L*(+)-glutamic acid solution.* 250.0 mg of L(+)-glutamic acid (Light & Co.) dried *in vacuo* was dissolved in approximately 15 ml of ~ 0.175 *N* NaOH in a beaker and the pH of the solution obtained was adjusted to 5.8 by dilute HCl. The solution was quantitatively washed into a 25 ml measuring flask and made up to volume.

Performance of the experiment. 5 ml centrifuge tubes, calibrated to 2.0 ml, were placed in ice and the following reagents pipetted to them:

A. 2.0 ml suspension + 1.0 ml water.
B. 2.0 ml suspension + 1.0 ml glutamic acid solution.
C. Precipitate, which was obtained by centrifuging the 2.0 ml suspension 3 times with cold phosphate buffer, pH 5.60, and finally making up the volume to 2.0 ml with phosphate buffer pH 5.60 + 1.0 ml water.
D. The precipitate as in experiment C made up to 2.0 ml + 1.0 ml glutamic acid solution.
E. 2.0 ml clear mother liquor obtained by centrifuging the suspension + 1.0 ml water.
F. 2.0 ml mother liquor + 1.0 ml glutamic acid solution.

The volume of the mother liquor used in experiments E and F corresponds to a slightly greater volume of suspension than the one used in experiments A—D.

Solutions A, C, and E are controls to solutions B, D, and F respectively, the latter ones containing 3.33 mg glutamic acid/ml at the start of the experiment.

After all the solutions had been pipetted, they were shaken and transferred from the ice-bath to a thermostat at 37° C where they were occasionally stirred. The tubes were open during the experiment. The samples, 10 μl each, were pipetted on Whatman no. 1 paper, in series 1 after 1 hour, in series 2 after 30 min, 1 hour and 2 hours, counting from the moment the samples were placed in the thermostat. The samples were pipetted by two persons in 4 minutes. The spot was dried on the paper by a current of warm air in about 30 sec. Whatman no. 1 paper was used in 10.2 mm wide strips.

The chromatograms were prepared by running with water-saturated phenol for 36 hours in a neutral atmosphere containing some HCN. Drying was effected by keeping the papers in a current of air of about 60° C for 10 min. in order to evaporate the major part of phenol and then overnight at room temperature.

The colour reaction was performed by drawing the strips through an acetone solution containing 0.25 % ninhydrin and by allowing the colour to develop overnight at room temperature.

A rough semiquantitative determination of the γ-aminobutyric acid split off was made by visually comparing the spots with a reference series with known amounts of γ-aminobutyric acid.

RESULTS

Series 1, which was performed at the end of July, gave very similar results both in regard to leaves and nodules of *A. incana*. The unfractionated suspension of both tissues and the washed insoluble cell residues decarboxylated distinctly L(+)-glutamic acid. On the other hand, the mother liquor which was centrifuged clear showed no noticeable activity of glutamic acid decarboxylase. The quantitativeness of the results is not good enough for the estima-

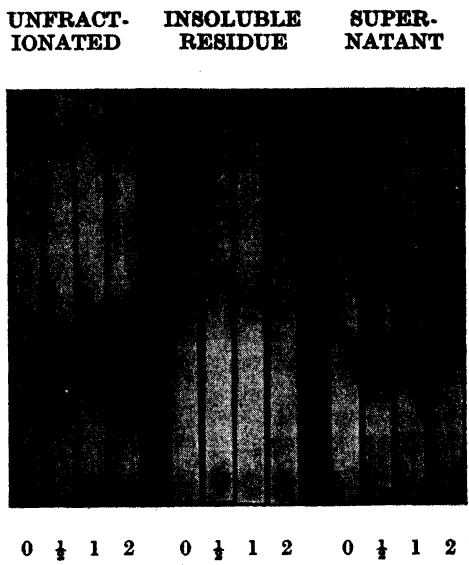

Fig. 2. L(+)*-glutamic acid decarboxylase in the root of carrot. One-dimensional phenol chromatograms for demonstrating γ-aminobutyric acid. Reaction time 0—2 hours at 37° C at pH 5.70.*

tion of the actual enzyme activities, but it can be judged from them that the activities of the unfractionated suspension and the corresponding washed suspension of cell particles are of the same order and relatively weak; about 0.3—0.5 mg γ-aminobutyric acid was split off per ml per hour.

Another experiment, which was carried out in October just when the leaves were falling, gave with the nodules of alder a similar result to the first one but the activities were weaker still; only about 0.1 mg γ-aminobutyric acid was split off per ml per hour, the smallest amount still detectable with certainty.

The activity of the suspension of carrot root was somewhat higher — about 0.3 mg γ-aminobutyric acid/ml/hour was split off — and the activity of the suspension of pea leaves much higher still; noticeably over 1.0 mg γ-aminobutyric acid/ml/hour was split off. A more definite comparison was impossible because the reference series did not contain spots of such high concentrations.

Furthermore, the glutamic acid decarboxylase of carrot and pea — contrary to that of the alder — was s o l u b l e, the washed cell residue containing only traces of activity (Figs. 2 and 3). Thus, this enzyme varies in different plant species from completely insoluble to completely soluble, as also noted in the previous studies.

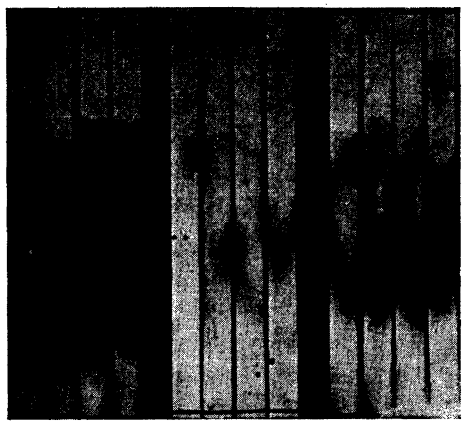

Fig. 3. L(+)*-glutamic acid decarboxylase in pea leaves. Reaction conditions the same as in Fig. 2.*

DISCUSSION

The absence of arginase activity from the alder tissues suggests that the citrulline nitrogen found abundantly in them cannot become utilized *via* urea and so the citrulline must have some other reaction possibilities.

The glutamic acid decarboxylase activity was lower than expected. Although the determinations are semiquantitative only, they seem to indicate that this activity is further lowered from the summer value when the tree prepares itself for the winter rest. An experiment of this kind cannot, of course, conclusively prove whether γ-amino butyric acid is formed *via* decarboxylation or otherwise. Even a low glutamic acid decarboxylase activity suffices to explain the abundant occurrence of γ-aminobutyric acid in the nodules of alder in the autumn [1], providing that the reactions consuming it are retarded very much in the autumn. No direct correlation seems to exist between the concentration of γ-aminobutyric acid and the glutamic acid decarboxylase activity in the plant tissues. The nodules of grey alder contain in October about 0.2 % γ-aminobutyric acid of the dry weight [1], the leaves of pea only about 0.05 % of the dry weight [9] yet the decarboxylase activity in the pea leaves is over 10 times that in the alder. This need not, however, mean that the formation of γ-aminobutyric acid in both cases were not

effected by decarboxylase, because, as indicated above, the enrichment depends both on the velocity of formation and consumption.

SUMMARY

The leaf, root, and nodule tissues of *Alnus incana* were examined for the occurrence of arginase. No arginase activity was found.

The occurrence of L(+)-glutamic acid decarboxylase was examined in the leaf and nodule tissue of *A. incana*. Some activity was noted in summer but very little in autumn at the time the leaves are falling, the activity seeming to concentrate principally in the insoluble cell particles. In comparative semiquantitative experiments carrot and pea possessed a greater activity, the enzyme being easily extractable with phosphate buffer.

The significance of the results is discussed. Utilization of citrulline in the absence of arginase does not seem likely *via* the urea cycle. Formation of γ-aminobutyric acid as a product of glutamic acid decarboxylation is probable although the activity of the corresponding decarboxylase in the leaf and nodule tissue of alder is weak.

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Physico-chemical Investigations on Soap Micelles

II. Sodium lauryl sulfate, potassium oleate, and cetyl trimethylammonium bromide

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The present investigation is a continuation of a previously published study [1] on the degree of association of potassium laurate and myristate micelles in the presence of salts. There it was shown that the micelles grow larger as the ionic strength of the medium increases. The effect is more pronounced with the longer hydrocarbon chain of potassium myristate (14 C atoms) than with potassium laurate (12 C atoms).

In the present study these investigations have been extended to include, besides potassium oleate, also soaps of other types, such as lauryl sulfate and cetyl trimethylammonium bromide, which latter is a cation active soap.

The same methods of investigation as in the study quoted above, namely sedimentation, diffusion, and viscosity measurements, were used for characterizing the size and shape of the soap micelles. All results refer to 30° C. For experimental details, the previous publication should be consulted. The sedimentation constants are expressed in S units ($1\ S = 10^{-13}$ c.g.s.). The diffusion constants have the unit 10^{-7} c.g.s. The limits of error of the molecular weights are estimated, as earlier, to $\pm$ 12 %.

Since the micelles are solvated, the partial specific volume in Svedberg's formula for calculating the molecular weight from sedimentation and diffusion data must refer to the solvated particle ($\overline{V}_{13}$). The following relation holds:

$$\overline{V}_{13} = \frac{\overline{V}_1 + k\ \overline{V}_3}{k+1};\ \text{where}$$

k = amount of H_2O in g/g soap.

$\overline{V}_1$ = pycnometrically determined partial specific volume for the non-solvated particle,

$\overline{V}_3$ = the partial specific volume of the water of solvation. The solvation was determined by ultrafiltration (*cf.* Granath [1]).

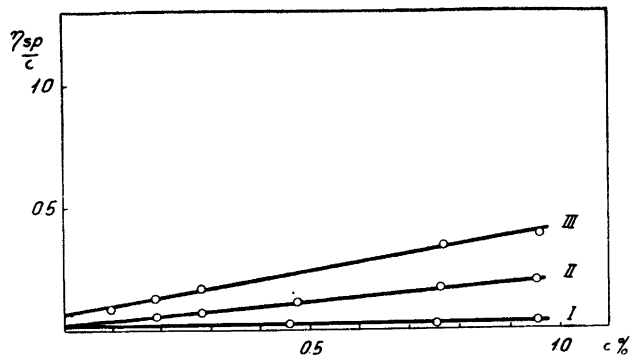

Fig. 1. Specific viscosity as a function of the soap concentration.

I Na-lauryl sulfate in 0.5 M Na_2CO_3
II » » » » 0.8 M NaBr
III Na-myristyl sulfate in 0.8 M NaBr (40° C)

RESULTS

Sodium lauryl sulfate

The Na-lauryl sulfate was prepared from pure dodecyl alcohol (Procter and Gamble) according to a method described by Shedlowsky [2].

The results of the viscosity measurements are seen in Fig. 1, where $\eta_{sp/c}$ is plotted *versus* c (g/100 g solution). The intrinsic viscosity $[\eta]$ is 0.02 in both media (0.5 *M* Na_2CO_3 and 0.8 *M* NaBr). For comparison, a series of measurements were made with Na-tetradecyl sulfate in 0.8 *M* NaBr. The value of $[\eta]$ for this compound was determined as 0.07.

Sedimentation constants of Na-lauryl sulfate were determined in 0.1 *M* and 0.2 *M* Na_2CO_3, and in 0.6 *M* NaBr. The results are given in Table 1.

Table 1. Na-lauryl sulfate. The sedimentation constant as a function of soap concentration.

Medium	c g/100 ml	S_{30}
Na_2CO_3, 0.1 *M*	0.6	1.0
» »	0.4	1.1
» »	0	1.4
Na_2CO_3, 0.2 *M*	0.8	1.0
» »	0.4	1.1
» »	0	1.3
NaBr, 0.6 *M*	1.2	1.1
» »	0.6	1.2
» »	0	1.3

Table 2. Na-lauryl sulfate. The diffusion constant as a function of the salt content at 30° C.

Medium	D_A
NaBr, 0.2 *M*	13.7
» 0.4 »	10.6
» 0.6 »	7.6
» 1.0 »	2.0
Na_2CO_3, 0.05 *M*	9.2
» 0.2 »	7.6
» 0.3 »	7.3
» 0·7 »	3.2

The diffusion constants as functions of the amount of salt (Na_2CO_3 and NaBr, respectively) present are given in Table 2. The diffusion constants changed with concentration within the error limits of these measurements.

The low degree of solvation (< 0.5 g/g) did not enable accurate determinations by ultrafiltration. Therefore, in Table 3 is given the apparent molecular weight M'_A, calculated with $\overline{V}_1$ as the partial spec. volume. In this case M'_A differs only slightly from M_A, the molecular weight of the non-solvated micelle. $M_A = \frac{M_c}{k+1}$, where M_c refers to the solvated particle. In Table 3 the frictional ratio f/f_0 is also given.

Table 3. Molecular constants of Na-lauryl sulfate.

Medium	$\overline{V}_1$	$\overline{V}_{13}$	$1-\overline{V}_1\varrho$	M'_A	f/f_0
Na_2CO_3, 0.1 *M*	0.883	—	0.113	35 000	1.29
» 0.2 *M*	0.883	—	0.103	40 000	1.31
NaBr 0.6 *M*	0.888	—	0.079	54 500	1.29

Potassium oleate.

The potassium oleate was prepared from pure oleic acid and a freshly made up solution of KOH. The product was recrystallized a few times in acetone and dried *in vacuo*.

The viscosity measurements were performed in salt solutions of varying ionic strength. The graphs of $\frac{\eta_{sp}}{c}$ *versus* c are plotted in Fig.2. Extrapolation

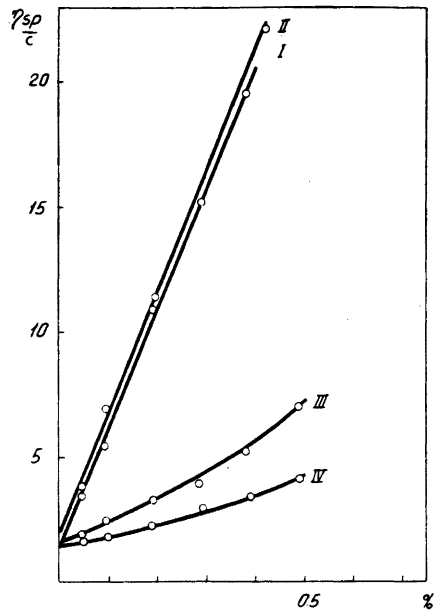

Fig. 2. Specific viscosity as a function of the soap concentration.

I K-oleate + 0.4 *M* KBr + 0.1 *M* K_2CO_3
II » + 0.4 *M* K_2CO_3
III » + 0.2 *M* KBr + 0.1 *M* K_2CO_3
IV » + 0.2 *M* K_2CO_3

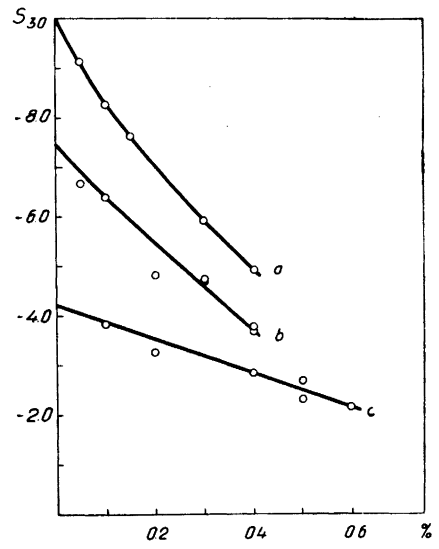

Fig. 3. The sedimentation constant vs. the soap concentration.

a. K-oleate in 0.4 *M* KBr + 0.1 *M* K_2CO_3
b. » » 0.4 *M* K_2CO_3
c. » » 0.2 *M* KBr + 0.1 *M* K_2CO_3

to zero concentration gives notably high values of $[\eta]$ (Table 5) corresponding to very high values of f/f_0. It is apparent that the relative viscosity is a function of the amount of electrolyte present rather than of the ionic strength of the medium.

Fig. 3 illustrates the concentration dependence of the sedimentation constants in a few different media. In all these cases the constants were

Table 4. K-oleate. Diffusion constants in different media.

K-oleate c %	Medium	D_A
0.2/0	0.4 *M* KBr + 0.1 *M* K_2CO_3	0.23
0.2/0	0.2 » » + 0.1 » »	0.52
0.6/0	0.3 » K_2CO_3	1.60
0.6/0	0.1 » »	7.5
0.4/0.1	0.05 » »	9.1

negative, *i.e.* the sedimenting micelles had a lower spec. gravity than the respective media. The diffusion constants in the corresponding media are given in Table 4.

$\overline{V}_1$ of K-oleate was found to be 0.983 in 0.05 *M* K_2CO_3 and 0.996 in 0.4 *M* KBr + 0.1 *M* K_2CO_3. The solvation was determined in the latter medium only and was found to be 1 ± 0.2 g/g soap.

A compilation of the results with K-oleate is found in Table 5.

Table 5. Molecular constants of K-oleate.

Medium	$\overline{V}_1$	$\overline{V}_{13}$	$1-\overline{V}_{13}\varrho$	M	f/f_0	$[\eta]$
0.4 *M* KBr + + 0.1 *M* K_2CO_3	0.996	0.999	− 0.038	29×10^6	5.13	1.6
0.2 *M* KBr + 0.1 *M* K_2CO_3	0.996	0.999	− 0.022	9.4×10^6	3.29	1.6
0.4 *M* K_2CO_3	0.996	0.999	− 0.040	21×10^6	5.22	2.0

Cetyl trimethylammonium bromide

The preparation investigated was an Eastman Kodak product recrystallized in acetone.

Fig. 4 shows the graphs of $\frac{\eta_{sp}}{c}$ *versus* c and Fig. 5 the extrapolation of the sedimentation constants of cetyl trimethylammonium bromide in a number of media.

$\overline{V}_1$ was 0.986 in distilled water and 1.008 in 0.2 *M* KBr. In 0.4 *M* KBr the value of the solvation was $k = 2 \pm 0.2$ g/g soap. $\overline{V}_{13} = 1.004$ was used throughout in calculating the molecular weight of cetyl trimethylammonium bromide in the media used.

Table 6. Molecular constants of cetyl trimethylammonium bromide.

Medium	S°_{30}	D_A	$1-\overline{V}_{13}\varrho$	M_c	f/f_0	$[\eta]$
0.4 *M* KBr	− 8.60	0.94	− 0.034	6.7×10^6	2.11	0.60
0.3 » »	− 4.30	1.10	− 0.026	3.8×10^6	2.16	0.45
0.2 » »	− 3.50	1.43	− 0.017	3.7×10^6	1.68	0.20

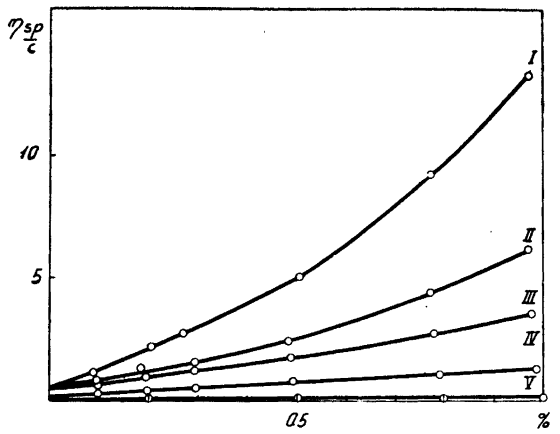

Fig. 4. The specific viscosity vs. the soap concentration.

I Cetyl trimethyl ammonium bromide in 0.5 M KBr
II » » » » » 0.4 M »
III » » » » » 0.3 M »
IV » » » » » 0.2 M »
V » » » » » H_2O

The shape of the micelles

The prevailing opinion concerning the shape of the soap micelles has been that they are cylindrical discs consisting of two parallel layers of molecules oriented with the polar groups out. From light scattering measurements,

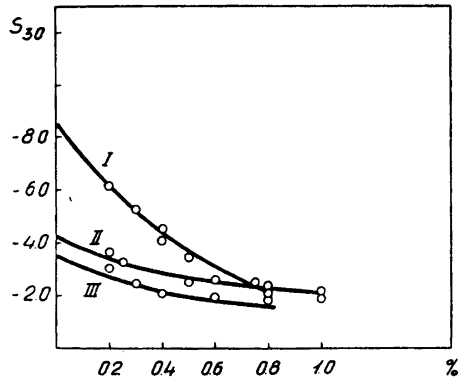

Fig. 5. S_{30} as a function of the soap concentration.

I Cetyl trimethyl ammonium bromide in 0.4 M KBr
II » » » » » 0.3 M »
III » » » » » 0.2 M »

Table 7.

Soap	Buffer	M_c	f/f_0	Rod-shape			Disc-shape		
				a/b	$2b \times 10^8$ cm	$2a \times 10^8$ cm	b/a	$2b \times 10^8$ cm	$2a \times 10^8$ cm
K-laurate	0.8 *M* KBr+ +0.1 *M* K_2CO_3	60 000	1.18	4.0	*36.2*	144	4.3	92	*19.8*
** 35.3 ± 1 Å	1.6 *M* KBr+ +0.1 *M* K_2CO_3	150 000	2.12	23.3	*27.2*	640	35.1	254	*20.0*
K-myristate ** 40.3 ± 1 Å	0.4 *M* KBr+ +0.1 *M* K_2CO_3	130 000	1.49	9.0	*35.8*	320	10.8	164	*15.2*
	0.6 *M* KBr+ +0.1 *M* K_2CO_3	400 000	1.60	11.1	*48.4*	540	13.9	260	*18.8*
	0.7 *M* KBr+ +0.1 *M* K_2CO_3	560 000	1.82	15.8	*48.2*	760	21.9	340	*15.4*
	0.8 *M* KBr+ +0.1 *M* K_2CO_3	840 000	2.22	26.1	*46.8*	1220	40.5	480	*11.8*
	1.0 *M* KBr+ +0.1 *M* K_2CO_3	$1.55 \cdot 10^6$	2.94	49.8	*46.2*	2300	96.6	780	*8.0*
K-oleate	0.2 *M* KBr+ +0.1 *M* K_2CO_3	$9.4 \cdot 10^6$	3.29	63.8	*77.6*	4960	131	1580	*12.0*
	0.4 *M* KBr+ +0.1 *M* K_2CO_3	$29 \cdot 10^6$	5.13	161.4	*83.0*	13400	—	—	—
	0.4 *M* K_2CO_3	$21 \cdot 10^6$	5.22	167.4	*73.0*	12200	—	—	—
Cetyl trimethyl-ammonium bromide	0.2 *M* KBr	$3.7 \cdot 10^6$	1.68	12.7	*97.4*	1240	16.5	580	*35.2*
	0.3 » »	$3.8 \cdot 10^6$	2.16	24.4	*79.0*	1920	37.2	760	*20.6*
	0.4 » »	$6.7 \cdot 10^6$	2.11	23.1	*97.6*	2240	34.5	900	*26.2*
Na-lauryl sulfate ** 37.3 ± Å	0.1 *M* Na_2CO_3	35 000 *	1.29	5.6	*26.0*	146	6.3	85	*13.6*
	0.2 » »	40 000	1.31	5.9	*26.6*	158	6.7	91	*13.6*
	0.6 » NaBr	54 500	1.29	5.6	*30.1*	168	6.3	99	*15.8*

* M'_A values.

** These values are so-called Bragg distances from measurements of electron diffraction [6]. According to these authors, the thickness of the micelle is 10—15 % larger than these values.

Debye [3] has drawn the conclusion that in the presence of salts the micelles of *n*-cetyl trimethylammonium bromide must be rod-shaped. The diameter of the rod should be double the molecular length. The surface of the rod is covered by the polar groups. Debye's values of the molecular weights ($8 \cdot 10^5$ in

0.178 M KBr and $1.86 \cdot 10^6$ in 0.233 M KBr) are in good agreement with the values here obtained ($M_A = 1.23 \cdot 10^6$ in 0.2 M KBr). Thiele [4] in his investigations, among others in the ultramicroscope, arrived at the result that the disc-shaped micelles were converted to positively double refracting rod-micelles in the presence of salts.

The data in this and the previous study have been gathered to throw some light on the question of the shape of the micelles of the soaps investigated under the conditions used. The axial ratio was calculated from the frictional ratio f/f_0 [5], approximating the rod-shape with a prolate ellipsoid of revolution having its major axis $2a$ as the rotational axis, and the disc-shape with an oblate ellipsoid of revolution where the minor axis $2a$ is the rotational axis.

From the relation between f/f_0 and the axial ratio [5], and the equation

$$4/3 \cdot \pi \cdot ab^2 = \frac{M_c \bar{V}_{13}}{N},$$

where N = Avogadro's number, the two half axes, a and b, could be calculated. If the micelles are rod-shaped, the half axis b should be comparable to the length of the corresponding soap molecule. In the other case (disc-shape) the same is true of the half axis a.

From the results in Table 7 it is seen that the assumption of rod-shape of all these soaps in the media investigated gives satisfactory values of the length of the solvated soap molecules. The lengths calculated for the disc-shape are consistently too small.

SUMMARY

This paper reports an investigation of the weights and shapes of the micelles of potassium oleate, sodium lauryl sulfate and cetyl trimethyl ammonium bromide as determined by sedimentation, diffusion and viscosity measurements, in the presence of electrolytes in varying amounts. The degree of association increases strongly with the amount of salt present and with the length of hydrocarbon chain of the respective soaps. The lengths of the shorter half axis of a prolate ellipsoid of revolution, calculated from the frictional ratio, become independent of the medium and are of the same magnitude as the length of the soap molecules. This supports Debye's view that the micelles are rod-shaped. The length of the rod depends on the degree of association, while the diameter is fixed to double the length of the soap molecule.

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The Hydrolysis of Dimethylamido-ethoxy-phosphoryl Cyanide (Tabun)

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The hydrolysis of the cyanide group in the biochemically very active compound dimethylamido-ethoxy-phosphoryl cyanide, generally known as Tabun, has previously been investigated by Holmstedt [1]. The hydrolysis of some other phosphorus compounds, in particular diisopropoxy-phosphoryl fluoride (DFP) [2,3], tetraethylpyrophosphate (TEPP) [4,5], and the dimethylamides of phosphoric acids [6], has been studied closely. In the latter compounds it has been found that the P—N linkage is easily broken by acids, but is extremely stable to alkalis. By means of a qualitative infra-red spectrophotometric method the present author recently established that in the hydrolysis of Tabun both the dimethylamino group and the cyanide group are split off, but that splitting off of the ethoxy group seems to be negligible [7].

The purpose of the present investigation was to determine the reaction mechanism of the hydrolysis of Tabun, and the variation with pH of the rates of removal of the dimethylamino and cyanide groups in acidic solutions. By quantitatively determining the amount of dimethylamine and hydrocyanic acid formed during the hydrolysis and simultaneously following the decrease in toxicity it has been possible to establish that the hydrolytic decomposition of Tabun proceeds in two parallel two-stage reactions.

Most of the organic phosphorus derivatives are powerful inhibitors of the enzyme cholinesterase, and several investigations have been carried out to correlate the inactivation of cholinesterase *in vitro* with the toxicity *in vivo* [1]. Such a relationship is usually present, but some exceptions to this rule are found. In this connection the decrease in toxicity has been compared with the cholinesterase inhibition determined concurrently during the hydrolysis of Tabun in aqueous solution.

EXPERIMENTAL

The hydrolysis of Tabun, synthesized in this institute [1], was measured in McIlvaine's standard buffer solutions at pH 3.0, 4.0, and 5.0 and in one case in aqueous solution. The pH of the solution was measured at the beginning and the end of the hydrolysis, and found to remain constant. 0.200 ml of Tabun, measured out with an AGLA micrometer syringe, was diluted to 100.0 ml with buffer solution or water. The concentration of Tabun was thus $13.29 \cdot 10^{-3}$ *M*. The reaction mixture was kept in a water bath at 25.0 ± 0.2° C.

Determination of dimethylamine. The dimethylamine liberated was determined by the colorimetric method described by Dowden [8]. The principle of this method is to react the dimethylamine with carbon disulfide thereby converting it into dimethyl-dithiocarbamic acid, the cupric salt of which has an amber tint and is rather soluble in benzene. The procedure is described in detail in a paper quoted above [7].

Determination of hydrocyanic acid. The hydrocyanic acid formed during the hydrolysis was determined iodometrically as described by Cupples [9]. At appropriate times 4.00 ml aliquots were diluted with 50 ml of 0.2 *M* sodium hydrogen carbonate solution and titrated with 0.01 *M* iodine solution using starch as indicator. This method, however, is not quite quantitative, so it was standardized against solutions containing known amounts of cyanide. In addition the results were corrected for the small amount of hydrocyanic acid which evaporated from the reaction mixture. This correction was performed at intervals by totally hydrolyzing an aliquot of the sample with sodium hydroxide and then titrating with iodine in the manner above.

Toxicity test. The experimental method adopted to follow the hydrolytic breakdown of the Tabun itself was to determine at appropriate intervals the toxicity of the reaction mixture. This was done by intraperitoneal injection in white mice weighing between 20 and 32 grams. Median lethal doses (LD_{50}) were calculated by the method of Finney [10], using mortality data obtained from groups of ten mice at each of five dose levels. The mortality was estimated 30 minutes after the moment of injection. The LD_{50} value at time zero was obtained by extrapolation. From the LD_{50} values obtained the amount of hydrolyzed Tabun, expressed in per cent, was calculated from the expression:

$$\frac{LD_{50_t} - LD_{50_0}}{LD_{50_t}} \cdot 100$$

Determination of anticholinesterase activity. The anticholinesterase activity of the reaction mixture was determined by the electrometric method described by Tammelin and Strindberg [11]. The principle of this method is to determine the acetic acid produced by the splitting action of cholinesterase on the substrate acetylcholine in terms of the change in pH in the presence of a standard buffer solution. Cobra venom was chosen as the source of cholinesterase, as it gives the most reproducible values and has a high enzymatic activity [12]. The molar concentration of the inhibitor during the pH-determination which caused 50 per cent inhibition of the enzymatic activity (I_{50}) was used as the measure of the anticholinesterase activity. The I_{50} values were calculated according to Augustinsson [13] by plotting the ratio v/v' against the inhibitor concentration, where v and v', expressed by $d(pH)/dt$, represent the observed velocities of the hydrolysis of acetylcholine in the absence and presence of inhibitor respectively.

Samples were withdrawn from the hydrolysis solution and diluted to a suitable concentration for the pH-determination. The I_{50} values were then calculated on the initial

Tabun concentration. The hydrolysis of Tabun in per cent was calculated from the expression:

$$\frac{I_{50_t} - I_{50_0}}{I_{50_t}} \cdot 100$$

The reaction mixture in which the pH-determinations were made, had the following composition: 3.00 ml of Michel's buffer solution [14] + enzyme (according to Augustinsson [12]: 0.1 mg of cobra venom per 2.00 ml of reaction mixture); 2.34 ml of redistilled water; 0.66 ml of redistilled water or of inhibitor, which was added 30 minutes before the acetylcholine solution; and 0.60 ml of acetylcholine solution (0.110 g of acetylcholine iodide per 5.00 ml of water).

RESULTS AND DISCUSSION

Figures 1 and 2 illustrate the liberation of dimethylamine and hydrocyanic acid respectively at pH 3.0, 4.0, and 5.0. From the curves it is obvious that the fission of the P—N linkage is catalyzed by hydroxonium ions, in agreement with the results obtained by Heath and Casapieri [6]. On the other hand the velocity of hydrolysis of the cyanide group increases with increasing pH as demonstrated by Holmstedt [1].

The reaction mechanism of the hydrolysis was studied at pH 5.0. Because both dimethylamine and hydrocyanic acid are liberated, there are three alternative courses for the hydrolysis:

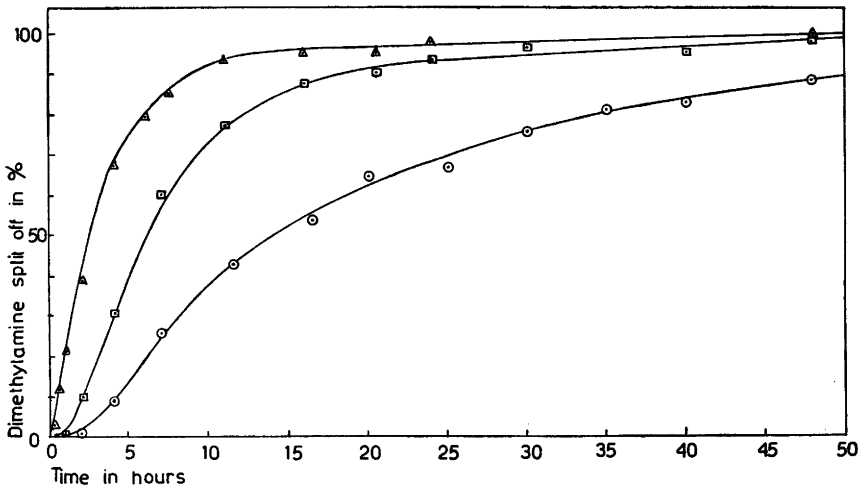
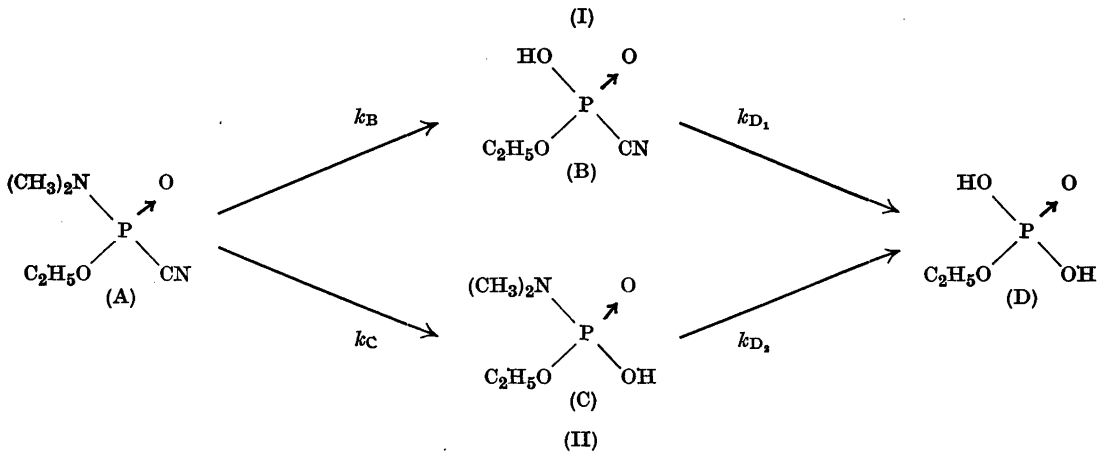


Fig. 1. The rate of formation of dimethylamine during the hydrolysis of Tabun. △ *at pH 3.0,* ⊡ *at pH 4.0,* ⊙ *at pH 5.0.*



Either it can proceed as a single two-step reaction (I) in which the dimethylamino group is first split off forming B which is then broken down to D and hydrocyanic acid, or inversely (reaction II), or finally by a combination of both these routes. To establish in which of these possible ways the hydrolysis proceeds, the decreasing toxicity of the reaction mixture was studied. The toxicity of Tabun is much higher than that of the hydrolysis products and therefore the toxicity of the reaction mixture depends essentially only on the

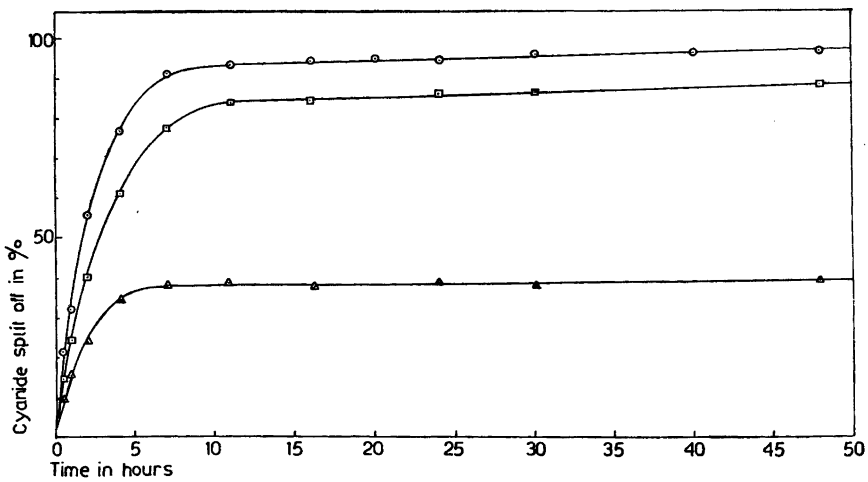


Fig. 2. The rate of formation of hydrogen cyanide during the hydrolysis of Tabun. △ *at pH 3.0,* ⊡ *at pH 4.0,* ⊙ *at pH 5.0.*

Tabun concentration. Near the end of the reaction when the Tabun concentration is very low, the hydrolysis products will however influence the toxicity. If the hydrolysis proceeds by either route (I) or (II), the dimethylamine or hydrocyanic acid respectively should be liberated at the same rate as the toxicity decreases. However, this is not the case, and consequently the hydrolysis must proceed by both of the two parallel routes in the scheme above.

At constant pH all the individual reactions would be expected to proceed as first order reactions. If log c_A (c_A calculated from toxicity tests) is plotted against time (t), a straight line is obtained as expected. From the experimentally determined values of dimethylamine and hydrocyanic acid the concentrations c_B, c_C, and c_D can be calculated:

$$c_{(CH_3)_2NH} = c_B + c_{D_1} + c_{D_2} \tag{1}$$

$$c_{HCN} = c_C + c_{D_1} + c_{D_2} \tag{2}$$

$$c_{A_0} - c_A = \Delta c_A = c_B + c_C + c_{D_1} + c_{D_2} \tag{3}$$

From these equations we obtain:

$$c_B = \Delta c_A - c_{HCN} \tag{4}$$

$$c_C = \Delta c_A - c_{(CH_3)_2NH} \tag{5}$$

$$c_D = c_{D_1} + c_{D_2} = c_{HCN} + c_{(CH_3)_2NH} - \Delta c_A \tag{6}$$

The amount of A measured as LD_{50} and the amounts of B, C, and D as calculated from these equations are recorded in Fig. 3.

For the different reaction steps the following differential equations can be set up:

$$dc_A/dt = -k_B c_A - k_C c_A = -(k_B + k_C)c_A = -k_A c_A \tag{7}$$

$$dc_B/dt = k_B c_A - k_{D_1} c_B \quad (8a) \quad \text{and when } c_A = 0 \quad dc_B/dt = -k_{D_1} c_B \tag{8b}$$

$$dc_C/dt = k_C c_A - k_{D_2} c_C \quad (9a) \quad \text{and when } c_A = 0 \quad dc_C/dt = -k_{D_2} c_C \tag{9b}$$

Integration gives:

$$c_A = c_{A_0}\, e^{-k_A t} = c_{A_0}\, e^{-(k_B + k_C)t} \tag{10}$$

$$c_B = \frac{k_B c_{A_0}}{k_{D_1} - k_A} \left(e^{-k_A t} - e^{-k_{D_1} t}\right) \tag{11}$$

$$c_C = \frac{k_C c_{A_0}}{k_{D_2} - k_A} \left(e^{-k_A t} - e^{-k_{D_2} t}\right) \tag{12}$$

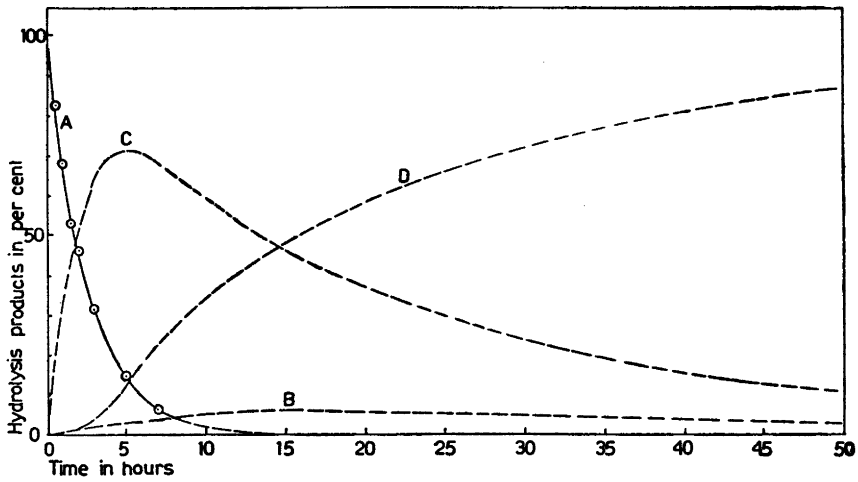
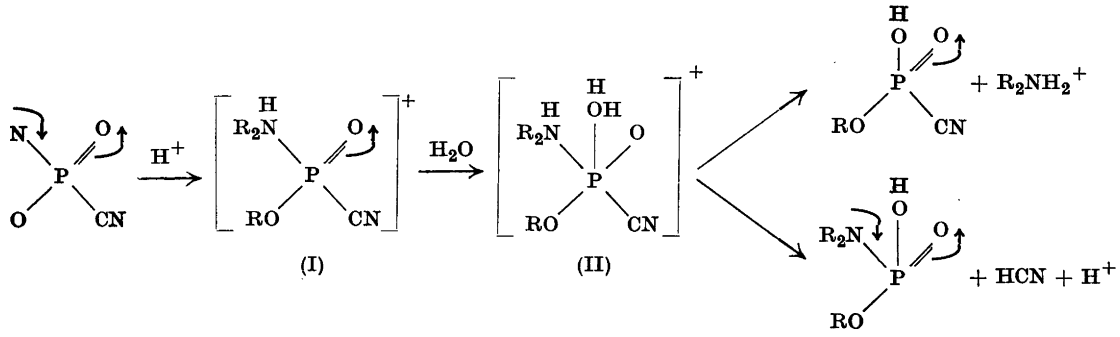


Fig. 3. The hydrolysis of Tabun at pH 5.0. A is measured as LD_{50} *and B, C, and D are calculated from the equations 4—6.*

If log c_A is plotted against t, a straight line is obtained as mentioned above, and from the slope of this line the velocity constant of the decomposition of Tabun was calculated by the method of least squares. The numerical value of k_A obtained was 0.390 hours^{-1}. The velocity constants k_{D_1} and k_{D_2} were calculated in the same way by plotting log c_B and log c_C respectively against t beginning after 16 hours, when all the Tabun is assumed to be hydrolyzed. In agreement with equations (8b) and (9b) straight lines were obtained. The values of k_{D_1} and k_{D_2} so obtained were 0.021 hours^{-1} and 0.043 hours^{-1} respectively. By inserting the experimental values for c_B into equation (11) values for k_B were calculated. The calculation of k_C was performed in the same way from equation (12) and the following numerical average values were obtained: $k_B = 0.01$ hours^{-1} and $k_C = 0.38$ hours^{-1}. The accuracy of the last two values will be rather low because of the nature of the calculations.

The interpretation of the mechanism of hydrolysis of phosphorus halides and organic esters of phosphoric acid is that a molecule of water attacks the phosphorus atom, one of the vacant 3d orbitals of the phosphorus being utilized for this purpose [3,15]. Depending upon the acidity of the solution, the intermediate compound thus formed either accepts or loses a proton forming an unstable ion which decomposes spontaneously to stable products. This mechanism, however, is not applicable to phosphoramides, because the amino group exerts a large electron-donating electromeric effect which will interfere with the coordination of the molecule of water to the phosphorus atom [6,16].

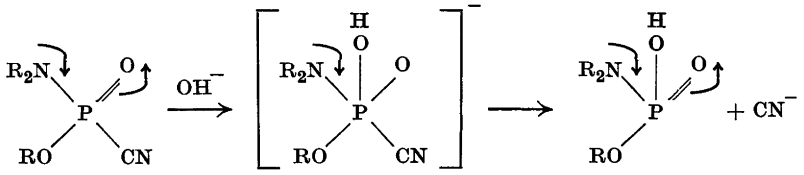
For this reason Day and Ingold's [17] kinetic scheme for the hydrolysis of carbonic esters seems to be more applicable to the hydrolysis of Tabun, and in acid solution the following mechanism is conceivable:



(I) (II)

In the first step which is a fast reaction, a proton is added to the nitrogen atom of the amino group (I). The positive electromeric effect is thus nullified and the hydration of the phosphorus atom is facilitated. The hydrated intermediate (II), which is formed slowly, decomposes spontaneously. In this case either the dimethylamino group or the cyanide group can be split off.

The base-catalyzed hydrolysis probably takes place by the following mechanism:



(III)

The rate-controlling step is a nucleophilic attack on the phosphorus atom by the hydroxyl ion to form the intermediate ion (III). This step is followed by the elimination of the cyanide ion, while the dimethylamino group is not removed in this reaction.

Finally the hydrolysis of Tabun has been studied with regard to decreasing toxicity and anti-cholinesterase activity. From Table 1 it appears that the decrease in toxicity during the hydrolysis agrees well with the decrease in cholinesterase inhibition.

Table 1. The hydrolysis of Tabun determined as LD_{50} and I_{50}.

Time in hours	LD_{50} μM/kg	I_{50} M/l	Hydrolyzed Tabun in % determined as	
			LD_{50}	I_{50}
0	*3.72	*$2.93\ 10^{-9}$	0	0
0.5	4.44	$3.42\ 10^{-9}$	16.2	16.7
1.5	5.95	$4.33\ 10^{-9}$	37.5	32.4
3.0	8.63	$6.75\ 10^{-9}$	56.9	56.6
5.0	16.40	$1.06\ 10^{-8}$	77.3	72.3
6.5	25.60	$1.42\ 10^{-8}$	85.4	79.3
11.5	61.10	$5.02\ 10^{-8}$	93.8	94.5
17.0	101.63	$1.40\ 10^{-7}$	96.3	98.0

* Extrapolated value.

SUMMARY

The velocity of hydrolysis of dimethylamido-ethoxy-phosphoryl cyanide, Tabun, in regard to the dimethylamino and cyanide groups has been measured at pH 3.0, 4.0, and 5.0. It appears that the splitting off of the dimethylamino group is catalyzed by hydroxonium ions and that of the cyanide group by hydroxyl ions.

It has been shown that the hydrolysis of Tabun proceeds in two parallel two-stage reactions. The various velocity constants have been determined at pH 5.0, and the mechanism of the hydrolysis has been discussed.

Finally, it has been demonstrated that the decrease in toxicity during the hydrolysis parallels the decrease in anticholinesterase activity.

The author is indebted to the Chief of the Research Institute of National Defence, Dept. 1, Professor Gustaf Ljunggren, and the Chief of the Institute of Organic Chemistry and Biochemistry, University of Stockholm, Professor Karl Myrbäck, for their kind interest shown in this work.

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Studies on the Hexagonal Tungsten Bronzes of Potassium, Rubidium and Cesium

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Opinions have diverged concerning the chemical character of the product obtained in the form of a dark blue powder or sometimes as hexagonal flaky crystals when mixtures of potassium tungstate and tungsten trioxide rich in the latter are reduced. Thus von Knorre [1] considered it to be potassium octatungstate, $K_2O \cdot 8WO_3$, while Brunner [2] assumed that it consists of a mixture of tungsten bronzes, $K_2W_5O_{15}$, $K_2W_6O_{18}$, and $K_2W_8O_{24}$. Schäfer [3], who synthetized a corresponding rubidium preparation, described this substance as an octatungstate.

In a recent communication from this institute [4] on the preparation and composition of the alkali tungsten bronzes results were given of careful analyses of these blue potassium and rubidium preparations as well as of the corresponding cesium product previously unknown. The compositions of these three substances were found to be in excellent agreement with the general formula Me_xWO_3 common to all hitherto known tungsten bronzes and the idea of von Knorre and Schäfer could thus be definitely excluded. Moreover, X-ray powder analysis showed that each of the three products contained only one phase, these three tungsten bronzes being hexagonal and isomorphous. Unit cell dimensions derived from powder photographs of various preparations, obtained by slight modifications of the experimental conditions mentioned in reference no. 4, are listed in Table 1. (For discussion of the relation between composition and unit cell dimensions *v. infra.*) The observed density values indicate that the elementary cell contains six formula units of Me_xWO_3.

Table 2 gives part of the powder pattern of the rubidium bronze preparation $Rb_{0.29}WO_3$, the other samples only showing minor alterations of the positions and intensities of the reflections. No attempts have been made to find the extension of the homogeneity ranges of the phases.

Table 1. Unit cell dimensions, density and electrical conductivity of hexagonal alkali tungsten bronzes.

Composition	a Å *	c Å	V Å^3	ϱ_{obs}	ϱ_{calc} **	$\varkappa$ ohm^{-1} cm^{-1}
$K_{0.27}WO_3$	7.40	7.56	358			
$K_{0.31}WO_3$	7.37	7.54	354	6.6	6.86	20
$Rb_{0.27}WO_3$	7.39	7.54	357			
$Rb_{0.29}WO_3$	7.38	7.56	357	7.4	7.16	35
$Cs_{0.30}WO_3$	7.38	7.59	358	7.5	7.56	15
$Cs_{0.32}WO_3$	7.42	7.63	363			

* The unit cell dimensions are expressed in true Å units. Probable error $\pm$ 0.01 Å.

** Calculated on the basis of a cell content of six formula units of Me_xWO_3.

Quite recently Brimm and co-workers [5] reported the preparation of a blue sodium potassium tungsten bronze of composition $Na_{\sim 0.08}K_{0.13}WO_3$ giving the same X-ray powder pattern as the blue potassium compound obtained by heating appropriate mixtures of potassium tungstate, tungsten trioxide and tungsten at 1050° C in argon. The symmetry should be hexagonal and the unit cell dimensions $a = 7.44$ Å, $c = 22.80$ Å, the a axis thus being about the same and the c axis three times that of the bronzes described in this communication. No reflections indicating such a multiplication of the hexagonal axis have been observed in our X-ray photographs. It seems probable that the structure of the bronzes prepared by Brimm and co-workers corresponds to a superstructure of the lattice of the hexagonal bronze type described below.

CRYSTAL STRUCTURE

The structure determination was carried out on the basis of single crystal photographs of a minute crystal chosen from the preparation $Rb_{0.27}WO_3$. This crystal was fairly elongated in the [10$\bar{1}$0] direction, which was used as the rotation axis. Rotation and Weissenberg photographs (layer lines 0—4) were taken with Cu-K radiation. The intensities were estimated visually and the correlation between the intensity scales of the layer lines was obtained from a "Weissenberg oscillation photograph" registering appropriate oscillation intervals of the various layer lines on the same film [6].

Table 2. Powder photograph of $Rb_{0,29}WO_3$, obtained in a Guinier focusing camera with Cu-Ka radiation. Comparison between observed (Weissenberg data) and calculated structure factor values.

	Powder data			Single crystal data	
h k i l	Intensity	$\sin^2\Theta_{obs}$	$\sin^2\Theta_{calc}$	$\lvert F_{obs}\rvert_{Wbg}$	F_{calc}
1 0 $\bar{1}$ 0	m	.0144	.0145	80	−110
0 0 0 2	st	.0415	.0415	580	−390
1 1 $\bar{2}$ 0	w	.0436	.0436	80	−110
1 1 $\bar{2}$ 1	v w	.0539	.0540	60	−60
1 0 $\bar{1}$ 2	st	.0558	.0560	240	180
2 0 $\bar{2}$ 0	v st	.0582	.0582	620	430
1 1 $\bar{2}$ 2	st	.0851	.0851	210	170
2 0 $\bar{2}$ 2	st	.0995	.0997	290	−250
2 1 $\bar{3}$ 1	w	.1120	.1122	—	60
3 0 $\bar{3}$ 0	w	.1308	.1309	130	−90
1 1 $\bar{2}$ 3	v w	.1369	.1371	80	60
2 1 $\bar{3}$ 2	m	.1433	.1433	80	100
0 0 0 4	m	.1658	.1662	520	440
3 0 $\bar{3}$ 2	w	.1722	.1724	90	150
2 2 $\bar{4}$ 0	st	.1746	.1746	310	390
1 0 $\bar{1}$ 4	v w	.1808	.1807	150	−80
3 1 $\bar{4}$ 0	w	.1891	.1891	100	−90
2 1 $\bar{3}$ 3	v w	.1950	.1953	—	−50
3 1 $\bar{4}$ 1	w	.1995	.1995	100	−110
1 1 $\bar{2}$ 4	v w	.2095	.2098	120	−80
2 2 $\bar{4}$ 2	st	.2162	.2161	150	−250
2 0 $\bar{2}$ 4	st	.2239	.2244	430	350
3 1 $\bar{4}$ 2	m	.2307	.2306	100	150
4 0 $\bar{4}$ 0	m	.2327	.2328	490	360
4 0 $\bar{4}$ 2	st	.2739	.2743	230	−230
3 2 $\bar{5}$ 0	v w	.2761	.2764	60	−60
3 1 $\bar{4}$ 3	w	.2823	.2826	80	100
3 2 $\bar{5}$ 1	v w	.2869	.2868	—	50
3 0 $\bar{3}$ 4	v w	.2975	.2971	80	−70
4 1 $\bar{5}$ 0	v w	.3056	.3055	80	−60
4 1 $\bar{5}$ 1	v w	.3157	.3159	100	90
3 2 $\bar{5}$ 2	m	.3177	.3179	80	110
2 2 $\bar{4}$ 4	st	.3405	.3408	170	320
4 1 $\bar{5}$ 2	w	.3470	.3470	100	110
3 1 $\bar{4}$ 4	w	.3554	.3553	80	−80
0 0 0 6	v w	.3740	.3739	370	−260
1 0 $\bar{1}$ 6	w	.3879	.3884	120	120

Table 2. cont.

$h\ k\ i\ l$	Powder data			Single crystal data	
	Intensity	$\sin^2\Theta_{obs}$	$\sin^2\Theta_{calc}$	$\lvert F_{obs}\rvert_{Wbg}$	F_{calc}
4 0 $\bar{4}$ 4	st	.3988	.3990	150	300
4 1 $\bar{5}$ 3			.3990	60	−80
3 3 $\bar{6}$ 1	v w	.4032	.4032	100	−130
4 2 $\bar{6}$ 0	m	.4074	.4073	260	250
1 1 $\bar{2}$ 6	v w	.4178	.4175	120	120
2 0 $\bar{2}$ 6	w	.4320	.4321	270	−220
3 3 $\bar{6}$ 2	v w	.4339	.4343	80	110
3 2 $\bar{5}$ 4	v w	.4431	.4426	—	−50
3 1 $\bar{4}$ 5	st	.4487	.4487	80	−90
4 2 $\bar{6}$ 2			.4488	180	−160
5 1 $\bar{6}$ 1	w	.4617	.4614	150	−120
4 1 $\bar{5}$ 4	v w	.4716	.4717	—	−50
2 1 $\bar{3}$ 6	w	.4760	.4757	80	100
3 3 $\bar{6}$ 3	v w	.4866	.4863	—	120
5 1 $\bar{6}$ 2	w	.4928	.4925	150	130

The Laue symmetry was found to be $6/mmm$ and the only reflections regularly missing were $hh\bar{2}hl$ with l odd, which is characteristic of the space-groups D^3_{6h}—$C6/mcm$, C^3_{6v}—$C6cm$, and D^3_{2h}—$C\bar{6}c2$. Furthermore the following regularities of the structure amplitudes could be observed:

1) Reflections $000l$. F^2 uniformly decreasing with l $(= 2n)$ increasing. (Reflections $000l$ absent when $l = 2n + 1$.)

2) Reflections $hkil$. F^2 generally low when l is odd.

These regularities imply that the tungsten atoms must be situated in (or very close to) planes extending normally to the hexagonal axis and $c/2$ apart and that the arrangement of these atoms must be approximately the same in neighbouring planes. In the Patterson function this corresponds to the sections $P(xy0)$ and $P(xy\frac{1}{2})$ containing all the heavy maxima and looking essentially the same.

The section $P(xy0)$ was only found to contain major maxima at the origin and at the centers of the sides and the area of the unit mesh. The height of the first was found to be somewhat less than twice that of the other ones, which among themselves were of equal magnitude. This appearance of the Patterson function is in accordance with the arrangement of six tungsten atoms in the point position $6(g)$ of the space-group $C6/mcm$ [7] (*cf.* Fig. 1a):

$x\ 0\ \frac{1}{4};\ 0\ x\ \frac{1}{4};\ \bar{x}\ \bar{x}\ \frac{1}{4};\ \bar{x}\ 0\ \frac{3}{4};\ 0\ \bar{x}\ \frac{3}{4};\ x\ x\ \frac{3}{4}$, with $x \approx 0.5$.

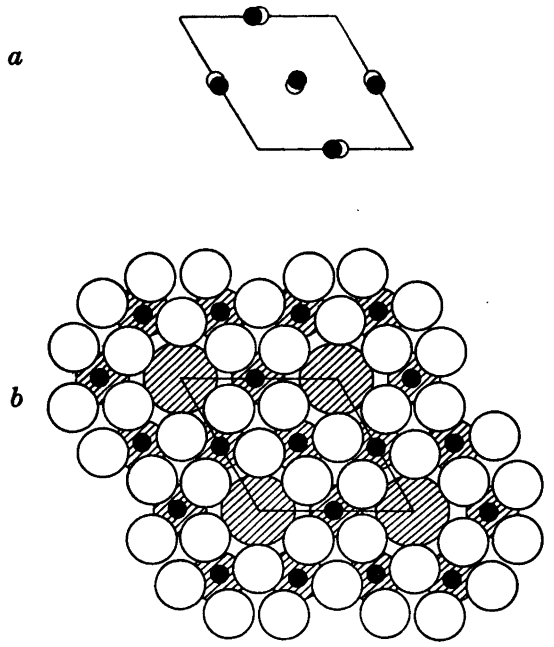

Fig. 1. The crystal structure of the hexagonal tungsten bronzes Me_xWO_3.

a) Positions of the tungsten atoms, the filled small circles representing atoms at $z = \frac{1}{4}$ *and the open ones atoms at* $z = \overline{\frac{1}{4}}$ $(\frac{3}{4})$.

b) Atomic arrangement of one layer of the structure, the circles of medium size corresponding to oxygen atoms at $z = 0$ *(marked with lines) and* $z = \frac{1}{4}$ *and the large ones to alkali atoms at* $z = 0$. *The atoms at* $z = \frac{1}{2}$ *are omitted to show the underlying atoms.*

It was found that a value of x equal to 0.48 gives fair agreement between the observed and calculated values of $F^2(hkil)$. Calculation of the section $\varrho(x0\frac{1}{4})$ of the electron density function gave support to this parameter value.

The interatomic distances corresponding to the positions of the tungsten atoms thus arrived at are 3.78 Å in the direction of the c axis and 3.70 Å parallel to the ab plane. Similar tungsten-tungsten distances have been found in several tungsten oxides and bronzes between the metal atoms of WO_6 octahedra joined by sharing corners [8]. Assuming an octahedral arrangement of the oxygen atoms around the tungsten atoms that should be present in the structure of the hexagonal bronzes a reasonable distribution of the eighteen oxygen atoms of the unit cell could be devised corresponding to the symmetry $C6/mcm$ with six atoms in the point position $6(f)$ and the remaining ones

in 12(*j*) ($x \approx 0.42$, $y \approx 0.22$) [7]. The corresponding tungsten-oxygen distances are 1.89—1.96 Å while the oxygen atoms are mutually 2.56—2.82 Å apart. Due to the relatively low X-ray scattering power of the oxygen atom this arrangement could not be verified by calculations of the electron density function.

This tungsten-oxygen lattice contains holes of considerable width (around the point position 2(*b*)), which were thought likely to accomodate the $6x$ (< 2) alkali atoms of the unit cell in a statistical distribution. This supposition could be verified by investigating the appearance of the electron density function along the line $0y0$, where a proper maximum was found to occur at $y = 0$. For the actual rubidium tungsten bronze these positions of the alkali atoms will lead to distances of 3.28 Å between the rubidium atom and the twelwe neighbouring oxygen atoms. The theoretical upper limit of x in the formula Me_xWO_3 will be 1/3.

The crystal structure thus derived may be summarized as follows (*cf.* Fig. 1 b)[7]:

Cell content: 6 Rb_xWO_3

Space group: D_{6h}^3—$C6/mcm$

$6x$	Rb	in	2(*b*)	: 000; $00\frac{1}{2}$.	
6	W	in	6(*g*)	: $x\ 0\ \frac{1}{4}$; $0\ x\ \frac{1}{4}$; $\bar{x}\ \bar{x}\ \frac{1}{4}$; $\bar{x}\ 0\ \frac{3}{4}$; $0\ \bar{x}\ \frac{3}{4}$; $x\ x\ \frac{3}{4}$.	x=0.48.
6	O	in	6(*f*)	: $\frac{1}{2}00$; $0\frac{1}{2}0$; $\frac{1}{2}\frac{1}{2}0$; $\frac{1}{2}0\frac{1}{2}$; $0\frac{1}{2}\frac{1}{2}$; $\frac{1}{2}\frac{1}{2}\frac{1}{2}$.	
12	O	in	12(*j*)	: $x\ y\ \frac{1}{4}$; $\bar{y}$, $x-y$, $\frac{1}{4}$; $y-x$, $\bar{x}$, $\frac{1}{4}$;	
				$\bar{x}\ \bar{y}\ \frac{3}{4}$; y, $y-x$, $\frac{3}{4}$; $x-y$, x, $\frac{3}{4}$;	$x \approx 0.42$.
				$y\ x\ \frac{1}{4}$; $\bar{x}$, $y-x$, $\frac{1}{4}$; $x-y$, $\bar{y}$, $\frac{1}{4}$;	$y \approx 0.22$.
				$\bar{y}\ \bar{x}\ \frac{3}{4}$; x, $x-y$, $\frac{3}{4}$; $y-x$, y, $\frac{3}{4}$.	

Observed (single crystal data) and calculated structure factor values corresponding to the reflections registered in the powder photographs are given in Table 2. The calculated F values for reflections absent in the powder photograph are generally very low ($|F| < 50$ except for F(5 1 $\bar{6}$ 0), which is equal to —70). The agreement is similar in character also for the remaining interferences registered in the single crystal photographs. There are considerable divergences between the observed intensities of the powder photograph and the calculated ones, which, however, may be qualitatively accounted for as being due to orientation effects.

DISCUSSION OF THE STRUCTURE

The structure is built up of WO_6 octahedra throughout connected by having corners in common and arranged in layers normal to the hexagonal axis. The unit dimension parallel to this axis comprises two such layers, which mutually

only show minor displacements of the atoms. The cohesion between subsequent layers (W—W distances of 3.78 Å) is less pronounced than that within the layers (W—W distances of 3.70 Å), which is reflected in the flaky form of the crystals.

Within the layers the WO_6 octahedra are connected to form a regular network of three- and six-membered rings. The characteristic ability of WO_6 octahedra to join corners forming various polygons has previously been demonstrated in several tungsten-oxygen compounds. Thus, the tetragonal potassium tungsten bronze [9] and the closely related tetragonal sodium bronze [10] are built up of three-, four-, and five-membered rings of WO_6 octahedra, while the tungsten oxide $W_{18}O_{49}$ [11] contains three-, four-, and six-membered rings. From this point of view the arrangement of tungsten and oxygen atoms present in the sodium and lithium tungsten bronzes of the perovskite and perovskite-like types [12–15] as well as in the tungsten oxides WO_3 [16,17] and $W_{20}O_{58}$ [18] may be considered as corresponding to tetragons of WO_6 octahedra. Further examples of the broad extent of the possible combinations of the WO_6 octahedra in tungsten-oxygen compounds are offered by the dioxide [19], crystallizing in a strongly distorted rutile lattice, by the paratungstate ion, $W_{12}O_{46}^{20-}$, the structure of which has recently been determined by Lindqvist [20], and by the heteropolytungstates, investigated by Keggin and others [21]. It is obvious that there exists a remarkable analogy between the coupling together of the WO_6 octahedra in the tungsten-oxygen compounds and that of the SiO_4 tetrahedra of the silicate chemistry. It may also be mentioned that discrete WO_6 octahedra recently have been shown to occur in the alkaline-earth tungstates $Me_3^{II}WO_6$ [22].

The alkali atoms of the tungsten bronzes generally stabilize a highly symmetrical arrangement of the WO_6 octahedra. Thus, while the room temperature form of tungsten trioxide represents a highly distorted, low-symmetrical modification of a lattice of ReO_3-type, the insertion of a proper amount of sodium or lithium atoms gives rise to the cubic tungsten bronzes of the perovskite type. With low contents of sodium and lithium the somewhat less regular, tetragonal bronzes of the degenerated perovskite type occur *.

* A similar modification of the distorted lattice of the room temperature form of tungsten trioxide towards the ideal ReO_3-type lattice is likely to occur in the forms stable at higher temperatures, recently shown to exist by Wyart and Foëx and others [23–25]. The crystalline preparations of tungsten blue, H_xWO_3, investigated by Glemser and Naumann [26], also show a similar transition towards an arrangement of ideal ReO_3-type with increasing content of hydrogen. — The WO_6 octahedra within the big blocks, which constitute the major structural elements of the $W_{20}O_{58}$ lattice, correspond to a fairly regular ReO_3-type structure [18]. In this case the occurrence of recurrent dislocations of certain tungsten atoms is likely to partly eliminate the obstacles preventing the trioxide from acquiring the ideal structure.

In a similar way the bulky potassium, rubidium or cesium atoms seem to stabilize the symmetrical, hexagonal array of WO_6 octahedra present in the actual bronze type. (*Cf.* the highly irregular arrangement of WO_6 octahedra filling up the regions between the six-membered rings of the $W_{18}O_{49}$-lattice [11].)

The unit cell dimensions of the hexagonal tungsten bronzes are approximately the same irrespective of the alkali metal being potassium, rubidium, or cesium (*cf.* Table 1), which indicates that the tungsten and oxygen atoms form a rather rigid three-dimensional network. The interstices in this lattice ought to be somewhat less than the size required by the cesium atom, since the unit cell of the cesium bronze increases slightly with increasing alkali content. On the other hand, increasing the content of the positively charged potassium or rubidium atoms, which are too small to fill up the holes of the tungsten-oxygen lattice, ought to cause a contracting effect on the surrounding oxygen polyhedra. This must result in a reduction of the unit cell volume, since it can hardly be counterbalanced by an expansion of the rigid WO_6 octahedra caused by the simultaneous decrease of the average valency of the tungsten atoms. This idea seems to be verified by the observed data for the potassium bronzes, which show a slight contraction of the unit volume with increasing alkali content. The compositions of the rubidium bronze preparations are too close to each other to allow a corresponding judgment for this group of compounds.

COLOUR

It was pointed out by Hägg [12] that the colour of the cubic sodium tungsten bronzes depends on the proportion of quinque- and sexa-valent tungsten atoms present in the lattice. Subsequently it has been stated that the same relation between the colour and average valency of the metal atoms is valid for a great number of tungsten bronzes, tungsten oxides, and molybdenum oxides [8]. This is also true of the hexagonal tungsten bronzes, the colours of which are comparable to that of the tetragonal tungsten bronzes of composition Na_xWO_3 ($x \sim 0.3$).

ELECTRICAL CONDUCTIVITY

Measurements of the electrical conductivity of the cubic sodium tungsten bronze were carried out by Hägg [12], who stated that this substance is an electronic semiconductor. Straumanis and co-workers [14, 27–29] studied various single and mixed bronze systems and found that the cubic lithium tungsten bronze of highest alkali content shows considerable ionic conductivity. This was interpreted as due to the lithium atoms being small enough to be able to slip through the openings between the WO_6 octahedra of the lattice [14].

Values of the electrical conductivity observed for preparations of the hexagonal tungsten bronzes are listed in Table 1. The measurements, which were made at room temperature only, had to be carried out with the bronze in powder form. The sample was kept in a glass tube between two electrodes under such a high pressure that a maximum reading of the conductivity value was obtained. The figures are thus rather approximate but represent minimum values of the conductivity. They are of the same magnitude as the values observed for samples of tetragonal potassium tungsten bronze [29] and of cubic sodium tungsten bronze of relatively high alkali content [27]. It seems highly probable that the conductivity of the hexagonal tungsten bronzes, containing the largest alkali atoms, is purely electronic in character.

SUMMARY

The dark blue tungsten bronzes obtained by reducing acid mixtures of tungsten trioxide with potassium, rubidium, or cesium tungstate have been shown to have the general formula Me_xWO_3 (x variable with a theoretical upper limit of 1/3). The crystal structure of the three compounds, which are isomorphous, has been elucidated. The lattice may be described as consisting of layers, built up of WO_6 octahedra, which are joined by corners to form a pattern of three- and six-membered rings. The layers are mutually connected by the WO_6 octahedra having corners in common. The alkali atoms are distributed in the major interstices between the layers. — The colour and the electrical conductivity of these bronzes are in fair agreement with the corresponding properties of previously investigated alkali tungsten bronzes.

The author wishes to thank Miss Birgitta Blomberg for her valuable assistance in the experimental work. The investigation forms part of a research program on oxides and oxide systems financially supported by the *Swedish Natural Science Research Council.*

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ACTA CHEMICA SCANDINAVICA 7 (1953) 325–328

Biophysical and Physiological Investigations on Cartilage and other Mesenchymal Tissues

IV. A Semimicro Method for Conductometric Determination of Sulfur *

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The common method of determining sulfur in organic compounds by combustion [1] is inapplicable when the sample is a tissue, because then some of the sulfur remains in the ash as sulfate. When the sample contains a metal it is necessary to choose another way to decompose it. It is possible — more or less easy — to oxidize the sulfur containing compounds with nitric acid, and the sulfur can then be determined as sulfate. When the amount of sulfur to be determined is about 0.1 mg, the gravimetric method demands great care and very good balances, whereas a conductometric titration based upon precipitation of barium sulfate is rapid and less laborious. For references to similar methods see [2,3].

Apparatus. The conductivity bridge did not deviate from the conventional Wheatstone AC type. In the detector circuit a narrowband amplifier tuned to the frequency (1000Hz) supplied by the RC-oscillator was used. A broadband amplifier can also be used, although the balance point of the bridge is then obscured by harmonics. The conductance change could be read directly on an electronic voltmeter. The voltmeter reading was very nearly a linear function of the conductance. A Wagner earth was applied, although it was not indispensable. The oscillator as well as the amplifier and the voltmeter were supplied with a stabilized voltage.

* This is the fourth report of a program of joint investigations on the physiology and nutrition of some mesenchymal tissues under the direction of Karolinska Institutet (Department of Orthopaedic Surgery and the Cancer Research Division of Radiumhemmet) and the Institute of Biochemistry at the University of Upsala.

The conductance cell was a glass cylinder, 6 cm in length and 2 cm in diameter. The two platinum electrodes (area 2 cm^2) were placed just at the bottom of the cell in order to avoid impedance changes originating from slapping of waves on the surface of the solution. The stirring was provided by a motor driven glass rod rotating in the center axis of the cell. The handling of the cell was facilitated by the fact that it was not necessary to platinize the electrodes. The voltage over the cell was about half a volt.

In spite of the rather large temperature dependence of the conductivity, the only thermostating necessary is to place the cell in a vessel with a large volume (2 l) of water. If the solution is titrated after an hour, the temperature usually does not change more than one hundredth of a degree during the titration.

Titration. The titration should be rapid especially because of the simple thermostat used. The precipitation of barium sulfate, however, is a slow process, but if the solution is seeded with about a hundred times as much barium sulfate as to be precipitated and the stirring is sufficient, the precipitation rate is accelerated considerably. The barium sulfate used in seeding was precipitated from hot barium hydroxide solution with sulfuric acid (excess) and then thoroughly washed. The same volume of seed suspension was always used and correction applied for the small amount sulfate in excess. Attention should be paid to the small increase in this correction term particularly during the first time. This increase can probably be explained as a liberation of occluded sulfuric acid.

To prevent precipitation of compounds other than barium sulfate, acetic acid was added to the barium sulfate suspension such that the final solution contained 0.2 % acetic acid.

The standard solution, 0.15 *M* barium chloride, was standardized against potassium sulfate and thus the sulfur content could be directly calibrated to the graduations of the micrometer of the micro buret. From a diagram of the voltmeter reading plotted against the micrometer reading (Fig. 1) it is easy to determine the equivalence point.

EXPERIMENTS

Upon standardization of the barium chloride standard solution duplicate tests agreed within 0.3 %.

It is always possible to burn the sample in a bomb tube with fuming nitric acid according to Carius and then evaporate the nitric acid in excess. In several cases the tedious manipulation with the bomb tubes can be avoided and the process performed at atmospheric pressure in a test tube placed in a small open electric furnace at 140° C. To avoid evaporation of sulfuric acid enough sodium hydroxide or sodium nitrate must be supplied. Because only a fraction of a milliliter conc. nitric acid is required there is little risk of bumping but for safety a little glass powder was added.

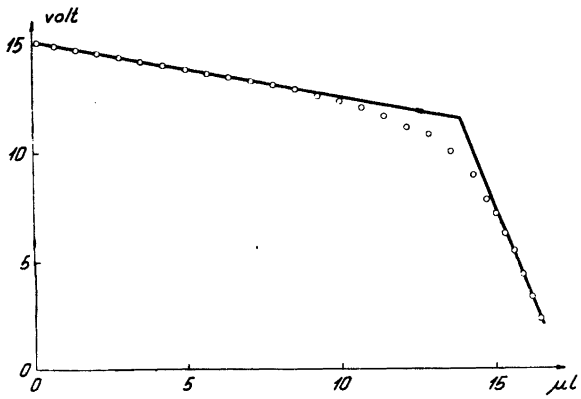

Fig. 1. Voltmeter reading (ordinate) plotted against volume barium chloride standard solution (abscissa). The sample contained 0.0759 mg sulfur.

Procedure: A dried sample containing about 0.1 mg sulfur is weighed. It is then heated together with 0.5 ml conc. nitric acid, 1 mg sodium nitrate and a little glass powder in a test tube for a few hours. The heat supply is increased and the evaporation carried on to dryness. The salt crust is dissolved in 4 ml water, transferred to the conductance cell and titrated together with 1.00 ml of barium sulfate sol.

In a tissue sulfur appears in appreciable amounts as ester sulfate of polysaccharides and in derivates of the amino acids cystine, cysteine and methionine and as sulfate ions. To control the reproducibility and validity of the method some of these substances were tested:

1. *Reproducibility test.* A chondroitin sulfuric acid preparation. Five tests on different amounts burned three to six hours at atmospheric pressure gave the sulfur percentage: 5.62, 5.64, 5.72, 5.62 and 5.61 %.

2. *Validity test.* L(−)Cystine, Merck, analytical reagent. Burned seven hours at atm. pressure. Found 26.3, 26.7 and 26.6 % sulfur. Calculated 26.68 %.

3. DL-Methionine, Merck. Burned seven hours at atmospheric pressure. Only a small fraction of the sulfur had been oxidized to sulfate. To decompose methionine within a reasonable time it is thus necessary to apply higher temperature.

An experiment to accelerate the oxidation rate with a drop of perchloric acid failed, because then the sulfuric acid partially evaporated.

Cartilage [4], protein free hyaluronic acid containing an admixture of ester sulfate bearing polysaccharides [5] and the cystein containing polypeptide bacitracin hydrochloride [6] may be mentioned as examples of samples determined by this method.

Interference of Calcium.

In the usual gravimetric sulfur determination method calcium is coprecipitated. Even in conductometric methods errors due to calcium have been reported [2]. Under the conditions mentioned above, however, calcium in moderate amounts does not interfere. Sulfate samples with up to 0.4 equivalents calcium per equivalent sulfate were tested and gave correct sulfur values within the normal limits of error. The calcium ions adsorbed on the seed crystals do not seem to change during the titration and the solution is so dilute that it is very far from saturation with respect to calcium sulfate.

DISCUSSION

From the experiments performed it can be concluded that it is possible to determine down to 0.1 mg sulfur with 1 % error with this method. Before the titration the sulfur must be converted to sulfate. The oxidation with nitric acid can be performed at ordinary pressure when the sample is a polysaccharide sulfate or cystine derivate. When the sulfur is connected to two carbon atoms as in methionine, high pressure methods must be applied.

Many other methods for volumetric determination of sulfate have been reviewed by Thomson [7]. The conductometric method described here, however, seems to be comparable to the best of them.

A conductometric titration method using benzidine dihydrochloride instead of barium has recently been proposed by Fischer *et al.*[8]. This way, however, is more time-consuming.

SUMMARY

Determination of sulfur in tissues is discussed. Conductometric titration in the presence of a very large excess of barium sulfate permits rapid determination of 0.1 mg sulfur with 1 % error. Moderate amounts of calcium do not interfere.

The author is indebted to Professor A. Tiselius for the privilege of working in his laboratory and to Associate Professor O. Snellman for valuable discussions. This investigation was financially supported by *Konung Gustaf V:s 80-årsfond*, and *Eli Lilly Comp.*, Indianapolis, Ind.

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ACTA CHEMICA SCANDINAVICA 7 (1953) 329–334

On Apparatus for Chromatography

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Hellström and Borgiel [1] have given some practical advice for the manipulation of the automatic analyser for chromatographic work, designed by Claesson [2]. Some disadvantages caused by the construction of the cuvette, are discussed in the present paper and a new design is therefore suggested. In addition to this the author suggests a new type of cuvette with a measuring device, which is specifically intended as an aid for preparative rather than analytical work.

I. Cuvette for Claesson Apparatus. In Claesson's apparatus the adsorption filters are placed on a block having a horizontal boring with two plugs. The beam of light passes through the apparatus by means of a second boring in the plugs. Two round glass windows, one in each plug, and another glass window between the plugs form two cells, one for the reference liquid (solvent) and one for the liquid that issues from the filters. The latter cell is connected by borings in the plug and in the block, on one hand with the filters, and on the other to a tube through which the liquid from the filters is transferred to a collecting vessel. The plugs are cut at an angle of 45° and so the third window, situated between them, becomes oval. The plugs and this window are held together by screws which are tightened parallel to the axis of the boring. The pressure in the apparatus then operates at an angle of 45° to the surface of the oval window. The reference cell is thus quite closed, and hence the plugs and windows must be taken apart each time the reference liquid is changed. One could of course facilitate the changing of the liquid by connecting borings and tubes as in the measuring cell, but it is rather troublesome to align the borings accurately, and it is also difficult to make the two cells sufficiently tight to prevent the diffusion of liquid from one chamber to the other. This is in part due to the difficulty of finding suitable sealing material. The author believes, nevertheless, that the fundamental cause of the drawbacks in the instrument is the pressure, which, holding the cuvette together, acts at an

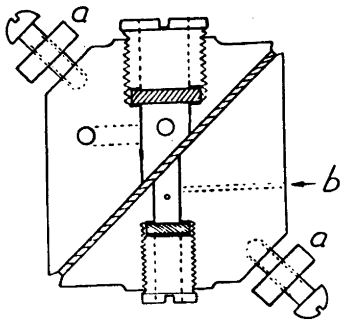

Fig. 1. The cuvette described in text. a indicates the bracket surrounding the cuvette, b the boring to the cell containing the liquid coming from the filter.

angle of 45° to the oval window and thereby develops strains which render more difficult the sealing of the apparatus.

In view of this conclusion a new cuvette was designed. A boring was made through the centre of a cubic block, at right angles to one of the surfaces. The block was then cut diagonally into two parts, as shown in Fig. 1. The corners opposite to the diagonal surfaces on both halves were chamferred, and the holes threaded *etc.* to enable circular windows to be fitted. Borings were made to and from the "inner parts" of the holes. Thus the third window corresponding to the oval window in Claesson's cuvette consist, in this apparatus, of a rectangular glass plate. Films of poly-ethylene 0.2 mm thick with suitable oval holes were used successfully as washers between the glass and metal parts. The two sections of the cube, the window and washers were held together by two screws in a loose bracket surrounding the apparatus in such a way that the screws operate at right angles to the chamferred corners, and hence the pressure keeping the apparatus together works at right angles to the oval window.

The whole cuvette assembly with its connecting tubes was mounted in a holder as shown in Fig. 2.

The tubes were connected with an upper plate threaded to hold the filters. The wide tubes through the plate in Fig. 2 were used for emptying or filling the reference cell. The narrow tubes, approx. inside diam. = 1 mm, conduct the liquid from the filter to the collecting vessel. A circular grove was also made in the plate, in order that a cylinder of any desired height might be fitted. A hole leading to a horizontal drain tube was drilled through the plate, to enable any liquid inside the cylinder to be drawn off.

In operation the apparatus was hung in the thermostat. The filters then stood on top of the thermostat. In Claesson's original apparatus the filters were immersed in the thermostat fluid, which meant that in order to change the filters the whole apparatus had to be removed from the thermostat vessel.

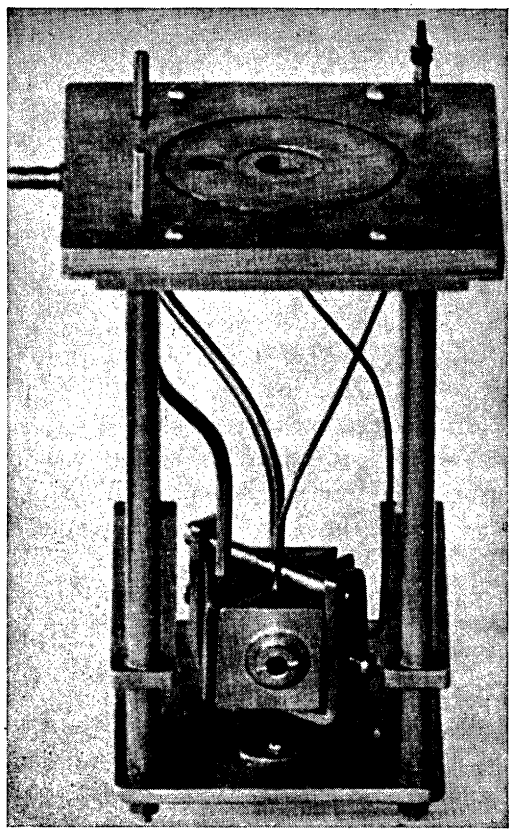

Fig. 2. The cuvette mounted in the holder with the device for the filter on the top plate.

By this operation the thermal equilibrium in the thermostat was disturbed, and there are other disadvantages. With the apparatus designed by us, however, the filters can be changed without lifting the cuvette out of the thermostat vessel. It is true that the filters will then operate at room temperature, but by using a cylinder of suitable height and by means of a second thermostat with spiral tubing, thermostatic control of the filter can be achieved. By such a device it was possible to attain greater variation of temperature for adsorption measurements than was practicable when both filters and measuring cuvette were in the same thermostat, and were therefore, at the same temperature.

II. Cuvette with measuring device. The type of cuvette suggested in the previous section is very easy to manufacture, and a specimen was made in

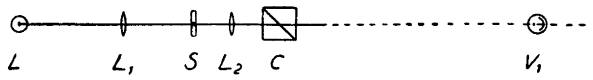

Fig. 3. The lamp L, lenses L_1 and L_2, cuvette C and phototube, V_1. The slit S (text S_1) is focussed on a slit S_2, not given in the fig., just before the phototube.

which both the measuring cell and reference cell were equally small, in order that these two cells might be connected, so that the liquid from the filters should run first through one, then through the other. A beam of light passed through such a cuvette will, disregarding certain diffusion, be deflected when the "front" enters the first measuring cell. When the "front" also passes the second cell — liquid of the same refractive index being present in both cells — the beam of light will revert to its original position. Provided that the volume of fluid passing through the cuvette is measured, one can, using the above instrument, obtain an idea of the derivative of the change in refractive index as a function of traversed solution volume. — The arrangements thus allow the use of the crossing over technique proposed by Holman [3] for the Tiselius-Claesson apparatus.

The author has been satisfied in this connection with trying to develop an automatic device that signals each time a "front" passes the cuvette. In the case under consideration a photo-tube, screened in such a way that it is illuminated by the beam of light only when the fluids in the two cells have the same refractive index, was used to this end. The photo-tube was coupled to an amplifier tube operating an electronic switch for an ordinary doorbell. The apparatus was adjusted in such a way that the bell was silent when the tube was illuminated, and a signal was obtained when the "front" entered one cell.

In Fig. 3 the relative positions of the lamp and necessary lenses is given and Fig. 4 is a circuit diagram of the apparatus with both the photo-tube and bell.

In the example shown the distance from the cuvette to the slit in front of the photo-tube is approximately 2 700 mm. (Fig. 3) A 10 mm deflection of the beam thus corresponds to a difference in refractive index, $\Delta\mu$, between the two measuring cells of nearly 0.004 units. In practice it was found that in the apparatus a deflection was obtained for a 3—4 mm movement of the beam of light, corresponding to $\Delta\mu \approx 0.0013$. When the front passes a measuring cell "schlieren" appear, causing the image of the slit, S_1, on the slit of the photo-tube, S_2, to be blurred, and thereby decreasing the intensity of light. This presents another means of increasing the sensitivity of the instrument.

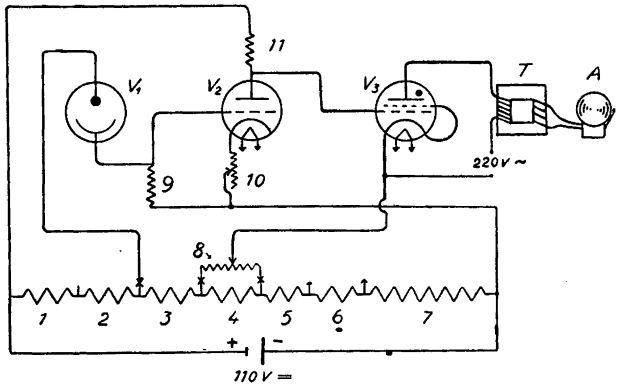

Fig. 4. Circuit diagram with the phototube, RCA 868, V_1. Triode tube, RCA 6SF5, V_2. Thyratrone tube, "Standard" G4S5, V_3. Transformer, T. Bell, A. Resistances: 1 — 4: 2 K Ω, 5: 1 K Ω, 6: 0.5 K Ω, 7: 10 K Ω, 8: 0.1 M Ω, 9: 10 M Ω, 10: 6 K Ω, 11: 10 K Ω.

The above apparatus was, as stated in the introduction, primarily intended as an aid for preparative work. As such work is generally performed at high concentrations and by the technique of "flowing chromatograms", the solvent is frequently changed. This involves a considerable change in refractive index, and the sensitivity indicated will be more than sufficient.

The apparatus has the further advantage that it should be possible to use any volume suitable to the experimental requirements between the two cells. Time may be saved in changing collecting vessels, or if desired, the volume of solvent comprising the "front" itself can be extracted. The model of the cuvette designed for experimental purposes was not equipped with permanent tube connections from cell to cell. For that reason increase in volume between the two cells mentioned above can easily be effected. The cuvette can also be connected in such a way that the liquid passes only one cell, should, for example, one only want to know when a "front" is passing.

It is evident that above apparatus can be used with a variety of measuring devices. Thus this type of cuvette may be used with an alternative registering device or the amplifier may be made to control an automatic movement of the collecting vessels. It might also be possible to combine a current apparatus that continuously changes collecting vessels with an instrument, based on the above design of cuvette, that undertakes the collection of the volume of liquid corresponding to the front itself.

SUMMARY

A new cuvette for the Claesson's self registering apparatus [2] is designed. In addition to this a cuvette with measuring device is also suggested.

The author wish to thank the "*Statens Naturvetenskapliga Forskningsråd*" (State Natural Science Research Board) whose support made this work possible, and Mr. Sigvard Fransson, who has manufactured the apparatus and devices described above. The author gratefully acknowledges the assistance of Agriculturist Erik Eriksson, First Assistant, in designing the circuit digram.

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The Free Amino Groups of the Proteus Flagella Protein

Quantitative Determination of Dinitrophenyl Amino Acids Using Paper Chromatography

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The method of Sanger [1] for the identification and estimation of the terminal amino acids of proteins using 1,2,4-fluorodinitrobenzene as a reagent for the free amino groups has now been applied to a great number of proteins. A review of the method is found in [2]. In most cases chromatography on silica gel has been used for the separation of the different dinitrophenyl (DNP-) derivatives. However, this method requires a comparatively large amount of the protein to be investigated. If only small amounts of material are available a possible separation on paper would be more suitable. Actually, some reports on paper chromatography of DNP-amino acids have been published [3–7]. One of them [7] also deals with the quantitative estimation of the derivatives but no figures are given to show the accuracy of the adopted procedure. It will be shown below that at least some of the DNP-amino acids can be accurately determined using paper chromatography when a simple but effective elution of the spots is used. Thus, only about 10 mg of protein are required for the determination of the free amino groups. The method has been applied to the flagella of the bacterium *Proteus vulgaris*, which earlier has been shown to consist almost entirely of protein material [8].

EXPERIMENTAL

The DNP-proteins and DNP-amino acids were prepared essentially according to [2]. In the case of the flagella, a 1—2 % solution of the flagella was added to two volumes of a 10 % (w/v) solution of fluorodinitrobenzene in alcohol. The mixture was shaken for two hours at room temperature, the precipitate sucked off on glass filter, washed and dried [2].

The flagella protein content in the DNP-protein was determined according to [2].

The DNP-proteins were hydrolyzed with 5.7 *N* HCl in an oven at 110° for 12 hours. With small amounts (5—10 mg) of protein in 0.5 ml acid, the hydrolysis was performed in a small test tube, the top end of which had been drawn out so that the opening was reduced to a capillary in order to minimize evaporation.

When the hydrolysis was completed the ether soluble DNP-derivatives were extracted, the acid solution evaporated *in vacuo* and the solid residue dissolved in water. For the qualitative identification of the DNP-amino acids paper chromatography according to [7] and [6] was applied.

For the quantitative determination, chromatography according to [6] was used, since the spots showed the least spreading by this method. Tertiary amyl-alcohol was used as the moving phase. After the chromatogram was run out, the colored spots were cut out and eluted according to the simple method of Aminoff and Morgan [9]. — Usually 10—15 μg of the DNP-derivatives were used for the determinations. They were applied on the paper in the forms of elongated spots, about 0.5 × 5 cm.

The eluates obtained were acidified with dilute H_2SO_4 and the content of DNP amino acids was determined spectrophotometrically at 360 mμ.

RESULTS AND DISCUSSION

In order to estimate the accuracy of the method, chromatograms were first run with pure DNP-amino acids. The derivatives found in the flagella protein, ε-mono-DNP-lysine and DNP-alanine, were used for these quantitative determinations. The results are given in Table 1.

Table 1.

	μg applied	μg found in eluate	Recovery %
DNP-lysin	51.5	52.5	102
»	51.5	51.4	100
DNP-alanine	17.7	16.8	95
»	60.8	56.0	92

The table shows that lysine is quantitatively eluted, while alanine is eluted to about 94 %.

Of the acid soluble DNP-derivatives, DNP-lysine from haemoglobin (cow), albumin from pea [12] and from the *Proteus* flagella, was quantitatively determined. The flagella were treated in the fluorodinitrobenzene without and after disintegration at pH 3. By this disintegration the flagella structure is entirely destroyed and split products having a molecular weight of approximately 40 000 are produced [8].

Table 2 shows the results obtained. (Only DNP-lysine was found in the chromatograms, besides dinitrophenol and dinitroaniline.)

Table 2.

Protein	Sample	Amount used for determination μg	Eq. DNP-lysine found in sample × 10^7	Eq. lysine per 10^5 g original protein
Haemoglobin (cow)	I	628; 628	33.8; 34.7	78.7; 80.7 M = 80
Pea albumin	I	2355; 1570	80.0; 54.0	74.4; 75.5 M = 75
Proteus flagella	I	806; 806	28.5; 28.3	55.5; 56.0 M = 56
» »	II	560; 560	18.0; 18.8	52.0; 54.3 M = 53
» dis-integrated	III	1322	51.2	57.9 M = 58
» »	IV	506; 506	18.9; 18.5	55.1; 53.9 M = 54

The DNP-flagella contained 65—72 % original flagella protein. In calculating the figures in the last column, the decomposition of DNP-lysine during the acid hydrolysis is taken into consideration [2].

The lysine value for haemoglobin is slightly higher than that found by Porter and Sanger, *i. e.*, 73.4 eq. per 10^5 g protein [10]. The lysine contents of the albumin from pea and of the flagella have earlier been determined to 72.5 and 56 eq. per 10^5 g protein by the electrodialysis method [11, 12]. The accordance between the two methods must be considered good.

Further it is of interest to note that the same result is obtained with the DNP-derivatives of whole or disintegrated flagella, respectively. However, by electron microscopical observations, it was found that no flagellar structure could be detected in any DNP-preparation. The flagella structure therefore seems to be destroyed by this reagent as well as by lowering the pH.

Chromatographic determination of the ether soluble DNP-amino acids obtained from the hydrolyzed DNP-flagella have also been carried out. Qualitative chromatography with the solvent mixtures described by Biserte [7] and by Blackburn and Lowther[6] showed that besides dinitrophenol only DNP-alanine was present after hydrolysis of the DNP-flagella. Quantitative determinations were carried out as described above for the DNP-lysine. Table 3 gives the results.

As mentioned earlier, smaller units of a molecular weight of approximately 40 000 are obtained by disintegrating the flagella at pH 3. Since only about

Table 3.

Sample of flagella	Amount used for the chromatography, μg	Eq. DNP-alanine found in sample $\times 10^7$	Eq. alanine per 10^5 g original protein
I	38.5	2.4	1.1
II	32.6	2.2	1.2
III (disintegrated flagella)	3.39	0.22	1.1
IV » »	6.92	0.34	0.8

1 eq. of free amino group is found per 10^5 g flagella protein, ring formations in the peptide chains building up this protein must be assumed.

Determinations of the free amino groups of other fibrous proteins belonging to the keratin-myosin group have been performed by Bailey [13]. No free amino groups are found in these proteins, and ring formations in the peptide chains must therefore occur also here. As is shown above, a relatively low content of free amino groups in the flagella is found. Since the occurrence of small amounts of impurities of a protein nature in the flagella preparations is not quite improbable [8] and since the values obtained above for the DNP-alanine for different samples of flagella seem to vary more than the experimental errors permit [13], the true content of free amino groups in the flagella other than the α-amino groups of the lysine may be still lower than what the figures given above indicate.

SUMMARY

Quantitative determinations of the free amino groups in some proteins have been performed according to the fluorodinitrobenzene method. Using paper chromatography, the determination could be performed on less than 10 mg substance. The *Proteus* flagella protein is found to contain 55 equivalents of free lysine ε-amino-groups per 10^5 g protein, which is in accordance with the earlier determinations by the electrodialysis method. Furthermore, about 1 eq. of free amino groups deriving from alanine is found per 10^5 g protein.

The author is very much indebted to prof. A. Tiselius for facilities put at his disposal, and to Mr. H. Kiessling for preparing the fluorodinitrobenzene. The present investigation is part of a research program on the structure of bacterial flagella which is financially supported by the *Swedish Natural Science Research Council.*

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ACTA CHEMICA SCANDINAVICA 7 (1953) 340–342

Preparation of Hydroxylysine-ε-^{14}C and Lysine-ε-^{14}C *)

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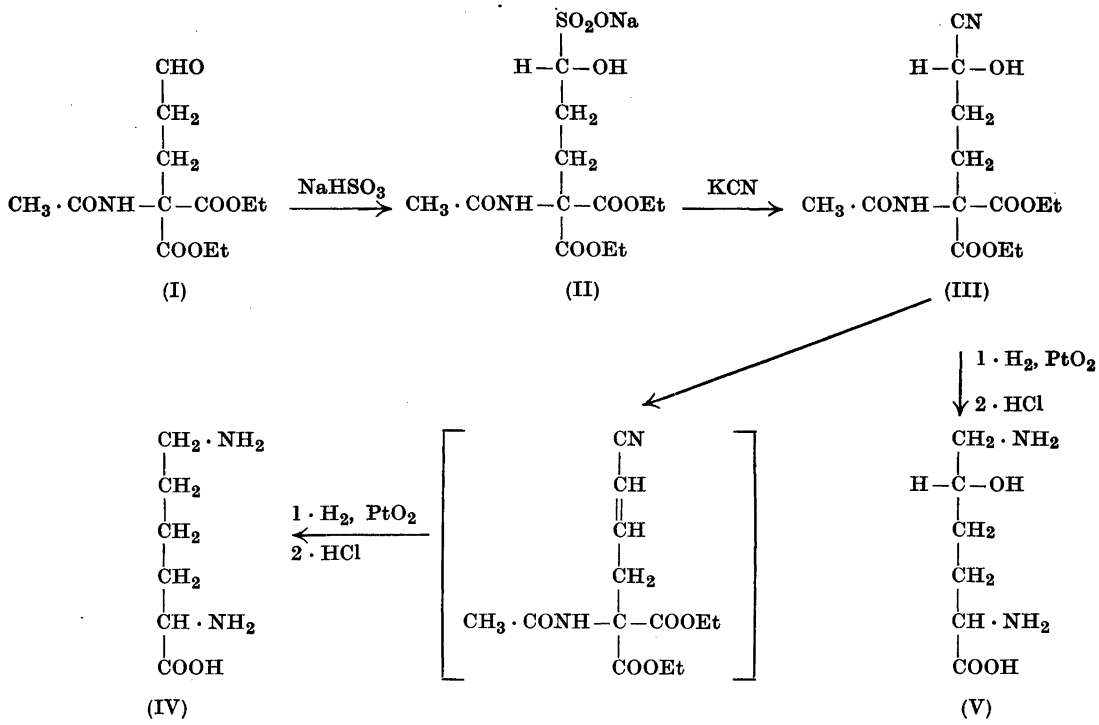
In the course of a study of the metabolism of hydroxylysine and lysine it was desired to have these amino-acids labelled with ^{14}C in the ε-position Lysine labelled in this position has been prepared by Olynyk, Camp, Griffith, Woislowski and Helmkamp [1] and by Borsook, Deasy, Haagen-Smit, Keighly and Lowy [2], but these syntheses give a rather poor yield of radioactive material. No synthesis of labelled hydroxylysine has as yet been published.

The lysine-synthesis according to Warner and Moe [3] and the corresponding hydroxylysine-synthesis of Touster [4] appear to be well suited to this work, as the radioactive carbon can be introduced relatively late (Formulas I—V). In these procedures the cyanohydrine of γ-acetamido γ-γ'-dicarbethoxybutyraldehyde (III) is prepared from the corresponding aldehyde (I) and liquid hydrogen cyanide.

Owing to the difficulty of handling small amounts of radioactive liquid hydrogen cyanide the preparation of the desired cyanohydrine (III) *via* the bisulfite-addition complex and potassium cyanide was investigated and found to give good yields. The hydroxylysine (V) prepared by reduction and hydrolysis of (III) according to Touster [4] gave too low values in periodic analysis. Paper-chromatography showed the presence of both hydroxylysine and lysine. Touster also reports that only 60 per cent of the theoretical value of ammonia was split off by periodic acid in his preparation. It was then investigated whether by altered conditions of reduction the formation of lysine could be avoided. Reduction with Adams' catalyst in glacial acetic acid : ethanol 9 : 1 at ordinary pressure and room temperature proceeded smoothly and gave rise to only a small amount of lysine. To obtain pure hydroxylysine it was, however, always necessary to separate the mixture on

* Part of this work was communicated to the IInd International Congress of Biochemistry, Paris 1952.



a column of Dowex-50 (NH_4-form) according to Weissiger [5]. The material thus obtained was pure as tested by paper-chromatography and periodic acid analysis.

Lysine (IV) was prepared from (III) according to Warner and Moe [3]. The final product contained a small amount of hydroxylysine and was purified by chromatography as described above. The isotopic yield in both syntheses was 30—40 per cent.

EXPERIMENTAL

Bisulfite-addition complex of γ-acetamido-γ'γ-dicarbethoxybutyraldehyde (I). To 21.7 g diethylacetamidomalonate and 2 ml sodium ethoxide (0.5 *N*) in 35 ml benzene was added 7 ml acrolein in 10 ml benzene with stirring. After two hours a solution of 12.6 g $Na_2SO_3 \cdot 7H_2O$ in 35 ml water and 5.8 ml glacial acetic acid was added. The reaction mixture was stirred overnight and filtered from inorganic material and some crystalline bisulfite complex. The resulting clear solution of (II) was diluted to 100 ml with water and used in the following.

Cyanohydrine of (I). To 5 ml of the solution of bisulfite complex was added 0.210 g K ^{14}CN in 2 ml of water. The mixture was shaken for half an hour and then extracted with ethyl acetate. The ethyl acetate solution was dried over sodium sulfate and eva-

porated. Addition of a little ether gave 0.840 g crystallin cyanohydrine. The crystals gave a total of 85×10^7 c.p.m. while the mother liquor gave a total of 10×10^7 c.p.m. After one recrystallization from benzene the cyanohydrine melted at 82°.

Hydroxylysine. 0.650 g cyanohydrine (total 65×10^7 c.p.m.) was hydrogenated in 20 ml glacial acetic acid: ethanol 1 : 9 with 0.1 g Adams' catalyst at ordinary pressure and room temperature. After six hours the reduction was complete. The oil resulting after evaporation of the solvent *in vacuo* was hydrolyzed with 25 ml 6 *N* hydrochloric acid for 15 hours. The hydrochloric acid was removed by repeated evaporation *in vacuo*. The material was dissolved in 100 ml of water and passed through a column of Dowex-50 (35 $mm^2 \times 350$ mm) 100—200 mesh which had been converted to the ammonium salt with *N* ammonia and subsequently washed with water. The column was washed with water until the washings were chloride-free and then eluted with 0.15 *N* ammonia in 4 ml fractions which were tested by paper-chromatography. The fractions containing pure hydroxylysine were combined and evaporated to dryness. The oil was treated with 10 ml *N* hydrochloric acid and the excess removed *in vacuo*. After dissolving in the minimum amount of 50 per cent ethanol, 0.5 ml pyridine was added. The monohydrochloride of hydroxylysine crystallized on scratching and addition of a little acetone. Yield 0.207 g. (total $29 \cdot 10^7$ c. p. m.) Calc. for $C_6H_{15}N_2O_3Cl$ (198.66): N 14.10; Cl 17.85. Found: N 14.01; Cl 17.55; Periodic acid equiv. w. found 198.

Lysine. 0.500 g cyanohydrine was boiled with 5 ml acetic anhydride for 90 minutes and reduced in the same medium with Adams catalyst at 100 atm. pressure and room temperature for 24 hours. The resulting oil was hydrolyzed with 25 ml 6 *N* hydrochloric acid for 15 hours. The material was chromatographed as described for hydroxylysine. The fractions containing only lysine were combined and the dihydrochloride crystallized. Yield 0.130 g. Calc. for $C_6H_{15}O_2N_2Cl_2$: N 13.05; Cl 31.5. Found: N 13.09; Cl 30.5;

SUMMARY

Racemic lysine-ε-^{14}C and hydroxylysine-ε-^{14}C have been synthesized.

I am greatly indebted to Dr Owen A. Moe, General Mills, Inc., Minneapolis, Minn., U.S.A., for a sample of the crystalline cyanohydrine.

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An Improved Method for the Synthesis of Potassium Thiocyanate Labeled with Radioactive Sulphur

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Potassium thiocyanate is a normal constituent of saliva. It is formed from cyanide and thiosulphate through the action of rhodanese, an enzyme found in a variety of organs. Because of its pharmacological action potassium thiocyanate is used in the treatment of essential hypertension. The labeling of the compound with radioactive sulphur, therefore, warrants some interest as a tool for the study of its metabolism. However, labeled potassium thiocyanate may also be used for the introduction of radioactive sulphur into other organic molecules, for instance in the preparation of sulphur labeled mercaptoethylamine and taurine as described by this author [1].

Potassium thiocyanate labeled with S^{35} has previously been prepared by Wood [2]. The method was modified by Tabern *et al* [3]. These procedures are based on the well known reaction between elementary sulphur and potassium cyanide. Because of the relatively low yield obtained by these investigators (49 per cent) and the difficulties involved in the handling of small amounts of elementary sulphur, the synthesis described below was developed. This method is well suited for isotopic work, the yield being high (86 per cent) on micro scale runs (30—40 mg of potassium thiocyanate).

The reaction employed was first used by Gutman [4] and can be written as:

$$K_2S + BrCN = KSCN + KBr$$

The best yield was obtained by dissolving one equivalent of potassium sulphide together with two equivalents of potassium hydroxide in water, and adding in portions two equivalents of dry cyanogen bromide to the solution, kept at 80° C on a water bath. The reaction apparently starts with the reduction of the sulphide to colloidal elementary sulphur, as the reaction mixture turns faintly turbid for a few seconds immediately after the addition of cyano-

gen bromide. At lower temperatures the greater part of the elementary sulphur precipitates out. In a series of check runs, the thiocyanate formed was determined as the iron complex according to Snell [5], the extinction being read in a Uvispec spectrophotometer at 4 700 Å against a freshly prepared standard. Yields from 79 to 86 per cent were found. It was suspected that precipitation of small amounts of the intermediary colloidal sulphur might represent a limitation for the synthetic yield. Other solvents (dioxane, acetone, xylene) were therefore tried, but without success.

Since labeled sulphur is available as sulphuric acid at one tenth the price of labeled sulphide, sulphate was used as starting material. In the conversion of barium sulphate to barium sulphide as described by Kamen [6], hydrogen gas at 900—1 000° C is used as reducing agent. Apart from the explosion hazard, this method also suffers from the disadvantage that some of the sulphur is reduced to hydrogen sulphide. We have therefore worked out a different procedure, in which carbon monoxide at 850—900° C is used for the reduction. According to Houben [7] the reduction of calcium sulphate with carbon monoxide at this temperature gives carbon disulphide in quantitative yield. This is, however, incorrect, and seems to be due to a misinterpretation of the original Polish paper [8]. Calcium sulphate as well as barium sulphate are reduced to the corresponding sulphides under these conditions. In check runs with reduction times from 20—30 minutes yields of barium sulphide of 99.5—100 per cent of the theoretical were found.

The conversion of barium sulphide to potassium sulphide was performed in the diffusion apparatus shown on Fig. 1. The barium sulphide was transferred to the small cup A and acidified with sulphuric acid through the syringe needle. A one hundred per cent excess of potassium hydroxide dissolved in water was placed in the outer compartment. Yields of 97—98 per cent of potassium sulphide were obtained. Using porcelain equipment, approximately 1 mg of barium sulphide was lost in the transfer from the reduction ship to the cup A in the diffusion apparatus. The same amount of barium sulphate was lost in the transfer from the ashing crucible to the reduction ship.

According to the results cited above, one can expect an overall yield of approximately 75 per cent of thiocyanate from sulphate as starting material. In a check run, a yield of 72 per cent was found.

EXPERIMENTAL

The "carrier free" labeled sulphuric acid was obtained from the Isotope Division, Atomic Energy Research Establishment, Harwell, England in a vial containing 10 mc S^{35} in approximately 0.5 ml of solution. By means of a curved Pasteur pipette inserted to the bottom of the opened and inverted vial, the solution and the subsequent washings were

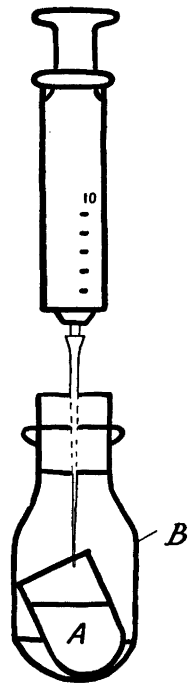

Fig. 1. Diffusion apparatus for the conversion of barium sulfide to sodium sulfide.

blown into a carrier sulphate solution containing 64.2 mg of sodium sulphate. After precipitation and ignition according to Kolthoff [9], a quantitative yield of 105.5 mg barium sulphate was achieved. The barium sulphate was transferred to a porcelain ship and carefully spread to give a greater surface. In this step 1.5 mg of barium sulphate was lost, being burned into the crucible. The carbon monoxide for the reduction was evolved as described by Vogel [10] and passed through a tower filled with potassium hydroxide and a washing bottle containing concentrated sulphuric acid. The silica reduction tube was preheated to 850—900° C and preflushed with carbon monoxide before insertion of the barium sulphate. After passing carbon monoxide at a slow, constant rate through the tube for 30 minutes at 850—900° C, a quantitative yield of 75.5 mg of barium sulphide was found. The reduction ship was cooled in a desiccator over phosphorus pentoxide at atmospheric pressure. Because of the instability of the barium sulphide, the next synthetic step was carried out as soon as possible. The compound was transferred to the small cup A, of the diffusion apparatus, Fig. 1, 0.7 mg being lost in the reduction ship. Into the outer vessel B was introduced 105 mg of potassium hydroxide dissolved in 2 ml of water. Through the rubber stopper was inserted a new, chromium plated injection needle, through which 1.5 ml of 10 per cent sulphuric acid was slowly added to the barium sulphide by means of a glass syringe. The needle was immediately withdrawn to avoid reaction with the metal. Because of the slightly increased pressure inside the diffusion apparatus, the syringe was kept attached with the plunger pressed against the bottom, while withdrawing the needle. The vessel was left for 48 hours with external cooling. The next step was now performed in the diffusion vessel B (Fig. 1). The cup A was removed after

washing the outside walls with 1 ml of water. The alkaline potassium sulphide solution was heated to 80° C on a water bath and cyanogen bromide added in three portions of 43 mg, 22 mg and 22 mg with 10 minute intervals. The cyanogen bromide was freshly prepared according to Hartman and Drager [11].

No attempt was made to isolate undiluted potassium thiocyanate from the above reaction mixture, although this could be done if the highest possible specific activity of the compound were desired. In order to insure a good recovery, 300 mg unlabeled thiocyanate was added as carrier. The solution was neutralized with 1 *N* hydrochloric acid, and evaporated to dryness under reduced pressure. The residue was then extracted with three portions of boiling ethanol (9 ml, 4.5 ml, and 4.5 ml) and filtered. After evaporation of the ethanol, the residue was extracted in the cold with three 50 ml portions of ethyl acetate, and again filtered. The ethyl acetate was then removed under reduced pressure. After four crystallizations of the final residue from absolute ethanol, 266 mg of potassium thiocyanate, with m.p. 172° C (uncorr.), was recovered.

For the preparation of labeled mercaptoethylamine and taurine [1] the isolation of potassium thiocyanate proved to be unnecessary. In this case the reaction mixture containing the radioactive thiocyanate could, after neutralization, be directly utilized for the next step in the synthesis.

SUMMARY

A procedure is described for the preparation of labeled potassium thiocyanate from sulphate S^{35}. The sulphate is reduced by carbon monoxide to sulphide, which is subsequently converted to thiocyanate through the reaction with cyanogen bromide.

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ACTA CHEMICA SCANDINAVICA 7 (1953) 347–351

The Spectrophotometric Determination of Steroid Hormones Solubilized in Aqueous Solutions of Association Colloids *

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In a previous paper [1] we reported on the maximum solubilities of a number of Δ^4-3-ketosteroids in aqueous solutions of certain association colloids. The solubility values were determined polarographically. This method can, however, be utilized only for a limited group of steroid hormones and hence it was found necessary to investigate the applicability of other methods for the accurate determination of steroid hormones solubilized in colloid solutions. The measurement of the ultra-violet absorption was found suitable for this purpose. This procedure made possible the determination of the hormone contents of association colloid solutions directly without previous separation of the components by laborious extractions as in the polarographic method. The present paper describes the spectrophotometric method and gives saturation values for hormones in aqueous sodium lauryl sulphate solutions as determined with this method.

EXPERIMENTAL

Experiments were conducted to determine α-oestradiol, desoxycorticosterone, testosterone, testosterone propionate, and cortisone-21-hemi-succinate spectrophotometrically in aqueous solutions of sodium lauryl sulphate. The ultra-violet absorption spectra of progesterone and desoxycorticosterone acetate in sodium lauryl sulphate solutions were also recorded. Oestradiol possesses a maximum absorption at about 2 800 Å due to its phenolic ring; the other hormones investigated show a maximum at about 2 400 Å which is due to a ketonic group conjugated with a double bond. The aqueous hormone solutions were prepared in the manner earlier described [2,3]. The measurements were carried out with a Beckman Model DU Quartz Spectrophotometer using 1.00 or 0.10 cm cells. The respective pure colloid solutions were used as blanks. Determinations were made both on solutions containing known amounts of the hormones and on saturated solutions.

* A part of a paper read at the 2nd International Congress of Biochemistry at Paris 21—27 July 1952. Abstract in *II^e Congrès International de Biochimie, Résumés des Communications*, p. 127.

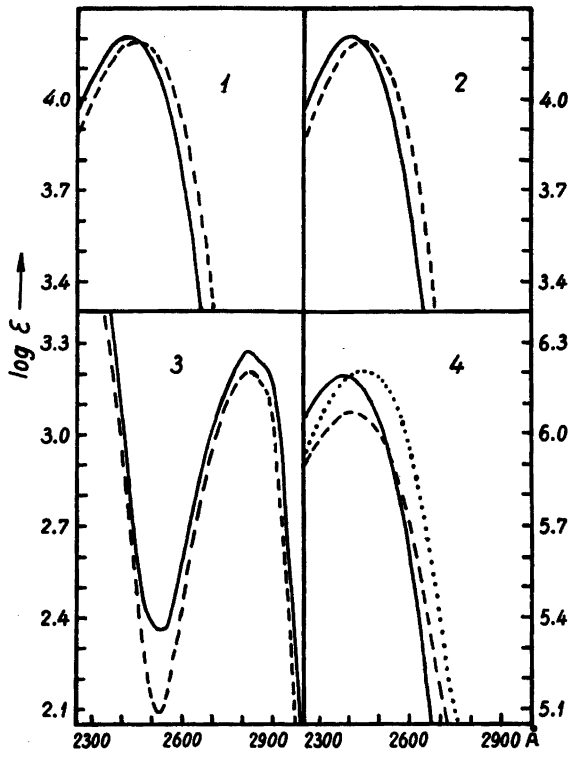

Fig. 1. The ultra-violet absorption of 1. testosterone, 2. progesterone, 3. oestradiol, and 4. cortisone-21-hemi-succinate in ethanol (————), in aqueous sodium lauryl sulphate solution (- - - - -), and in water (· · · · ·).

RESULTS AND DISCUSSION

In aqueous sodium lauryl sulphate solutions the hormones investigated show absorption curves that are similar to those recorded in ethanol (Fig. 1). Except in the case of oestradiol there is, however, observed a shift toward longer wavelengths. A similar shift of the absorption spectrum has previously been observed in the case of solubilized polycyclic aromatic hydrocarbons [4]. In all cases the molar extinction is somewhat lower in sodium lauryl sulphate solutions than in ethanol. The absorption at the maximum increases linearly with the hormone concentration and permits a direct quantitative determination of the amounts of hormones solubilized in sodium lauryl sulphate solutions.

Our spectrophotometric determinations on saturated solutions show that the solubilities of the hormones in sodium lauryl sulphate solutions increase linearly with the colloid concentration, at least in the concentration range

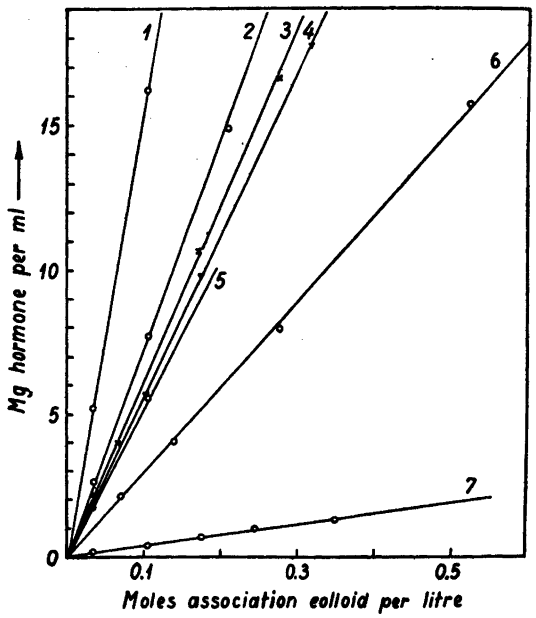

Fig. 2. The solubilities of some steroid hormones in aqueous solutions of sodium lauryl sulphate: 1. desoxycorticosterone, 2. testosterone propionate, 3. testosterone, 4. progesterone 5. cortisone-21-hemi-succinate, 6. desoxycorticosterone acetate, 7. oestradiol.

investigated. The solubility curves are shown in Fig. 2 which also includes two solubility curves determined earlier with the polarographic method (desoxycorticosterone acetate and progesterone).

From the linear solubility curves we have calculated the saturation capacities of the micellar substance for the respective hormones. These values are

Table 1. Maximum solubilizing power of sodium lauryl sulphate for steroid hormones.

Hormone	Moles hormone per mole micellar sodium lauryl sulphate	Moles micellar sodium lauryl sulphate per mole solubilized hormone
Desoxycorticosterone	0.47	2.1
Testosterone	0.21	4.7
Testosterone propionate	0.21	4.7
Progesterone	0.18	5.5
Cortisone-21-hemi-succinate	0.12	8.5
Desoxycorticosterone acetate	0.08	12.5
Oestradiol	0.015	65.8

collected in Table 1. From these figures it is seen that desoxycorticosterone is the most soluble of the hormones in sodium lauryl sulphate solutions. The solubilities of testosterone, testosterone propionate, and progesterone are of the same order, whereas desoxycorticosterone acetate and cortisone-21-hemi-succinate are somewhat less soluble. The solubility of oestradiol is only about a tenth of that of the other hormones investigated *. If the lauryl sulphate micelles are assumed to contain 100 molecules of colloid-forming substance, the number of molecules oestradiol per micelle is about 1 to 2, and that of the other hormones about 10 to 20 molecules. The degree of dispersion of the hormones in aqueous sodium lauryl sulphate is thus fairly high. Of the hormones investigated, cortisone-21-hemi-succinate is the most soluble in pure water, 0.2 mg per ml; its solubility in water is, however, only about a tenth of that in a 1 per cent (0.034 *M*) sodium lauryl sulphate solution.

The new results confirm that the solubilities of the steroid hormones in association colloid solutions are of a magnitude quite different from the solubilities of the polycyclic aromatic hydrocarbons with 3 to 5 rings [4]. In spite of their larger molecules the solubilities of the hormones studied except oestradiol are thus 100 to 500 times greater than that of methylcholanthrene which resembles most closely the hormones in structure. Oestradiol has an intermediate position with a solubility about 10 times greater than methylcholanthrene. As previously pointed out [1], it seems probable that the solubilization of steroid hormones involves an interaction between the hydrophilic groups of the hormones and the colloids.

The spectrophotometric method for direct determination of steroid hormones in association colloid solutions is applicable in all cases where the solvent itself does not possess any appreciable absorption at the wavelengths in question. If the solvent has a strong absorption at these wavelengths, extraction procedures similar to those described earlier [1] must be performed before the spectrophotometric determination can be conducted.

SUMMARY

A direct spectrophotometric determination of steroid hormones solubilized in aqueous association colloid solutions is possible in many cases. The maximum solubilities of a number of steroid hormones in aqueous sodium lauryl sulphate solutions have been determined spectrophotometrically.

* Of the same order as the solubility of oestradiol is that of oestrone which is the subject of a special study by one of us[5].

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Studies on Pyrazolones
II. Condensation Reactions between 1-Phenyl-3,4-dimethyl-5-pyrazolone and 1-Phenyl-3,4-dimethyl-4-halo-5-pyrazolones

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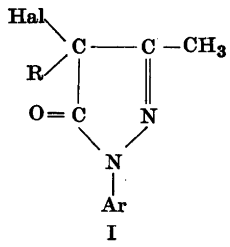
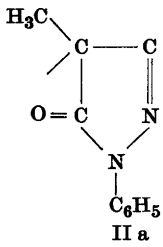
In 1-aryl-3-methyl-4-halo-5-pyrazolones (I a) and its 4-alkyl derivatives the halogen atom is activated by the adjacent C = O and C = N groups and can react with the active hydrogen atom in 1-aryl-3-methyl-4-halo (or alkyl)-5-pyrazolones at room temperature. Interaction between molecules of these kinds has already been described by Knorr [1], who observed the formation of pyrazole blue from 1-phenyl-3-methyl-4-bromo-5-pyrazolone. This reaction has been studied more recently by Smith [2], who discovered that cupric ions strongly catalyze the condensation.



I

a) R = H
b) R = CH_3; Ar = C_6H_5



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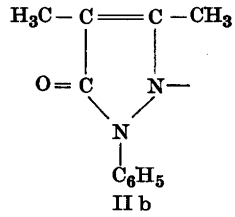
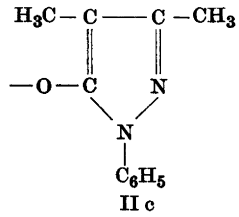


II b



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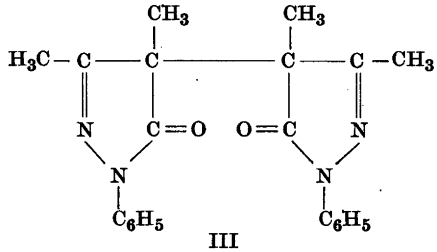

III

The present work was undertaken to study the condensation between 1-phenyl-3,4-dimethyl-4-halo-5-pyrazolones[3] and 1-phenyl-3,4-dimethyl-5-pyrazolone.

In an alcohol solution buffered with sodium acetate, 1-phenyl-3,4-dimethyl-5-pyrazolone and 1-phenyl-3,4-dimethyl-4-halo-5-pyrazolones interact to give a bispyrazolone.

$$Py(CH_3)H\ ^* + Py(CH_3)Hal \rightarrow Py(CH_3) \cdot PyCH_3 + HHal$$

The iodopyrazolone reacts faster than the bromo-compound, the chloropyrazolone slower. When the iodo-compound is used, the yield of bispyrazolone is diminished because of the side-reaction

$$Py(CH_3)I + HI \rightleftharpoons Py(CH_3)H + I_2$$

$Py(CH_3) \cdot PyCH_3$ is only slightly soluble in alcohol at room temperature and precipitates as white crystals. It is a very stable product not readily attacked by either acids or bases.

The constitution of the bispyrazolone is not obvious from its way of preparation. Even if $Py(CH_3)Hal$ has only the one structure I b, there are three possibilities (*cf.* II a, b and c) for $Py(CH_3)H$, and thus $Py(CH_3) \cdot PyCH_3$ may have the structure III or alternative structures obtained by combination of II a with II b or c.

The light absorption curve of $Py(CH_3) \cdot PyCH_3$ (Westöö [3]) lies close to the corresponding curve of 1-phenyl-3,4,4-trimethyl-5-pyrazolone (Biquard and Grammaticakis [4]) and differs from the curves of antipyrine and 1-phenyl-3-methyl-5-methoxypyrazole (Valyashko and Bliznyukov [5]). This shows that the substance is 1,1′-diphenyl-3,3′,4,4′-tetramethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione (III). The light absorption does not change when acid is added which also excludes coupling C to N. Even when $Py(CH_3)Na$ reacts with $Py(CH_3)Br$ in absolute alcohole, the same compound (III) is formed. It is identical with the $Py(CH_3) \cdot PyCH_3$-compound prepared by Knorr[1] by oxidation of $Py(CH_3)H$ with nitrous acid.

EXPERIMENTAL

1,1′-Diphenyl-3,3′,4,4′-tetramethyl-(4,4′-bi-2-pyrazoline]-5,5′-dione (III). A solution of 1-phenyl-3,4-dimethyl-5-pyrazolone (5.8 g) and 1-phenyl-3,4-dimethyl-4-bromo-5-pyrazolone [3] (8.0 g) in alcohol (150 ml) was mixed with 100 ml of acetate buffer (four parts of 1.8 *N* sodium acetate solution per part of 1.8 *N* acetic acid solution) and 5 ml of a 0.01 % solution of $CuSO_4 \cdot 5\ H_2O$. The next day white crystals had formed (by increasing the cupric ion concentration the reaction rate can be increased). They were filtered

* In this paper the radicals II a, b and c will be represented by $PyCH_3$.

by suction and washed successively with 60 % and 96 % alcohol. The combined filtrate and washings were precipitated with water, and the precipitate was recrystallized from alcohol. Yield 95 %. After recrystallization from acetic acid the product melted at 165° C alone or mixed with the $Py(CH_3) \cdot PyCH_3$ product of Knorr. (Found: C 70.4; H 5.9. $(C_{11}H_{11}ON_2)_2$ (374.4) requires C 70.6; H 5.9).

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Studies on Pyrazolones

III. Michael Condensations between Pyrazole Blue and the Addenda 1-Phenyl-3-methyl-5-pyrazolone, 1-*p*-Bromophenyl-3-methyl-5-pyrazolone and 1-Phenyl-3-methyl-4-bromo-5-pyrazolone

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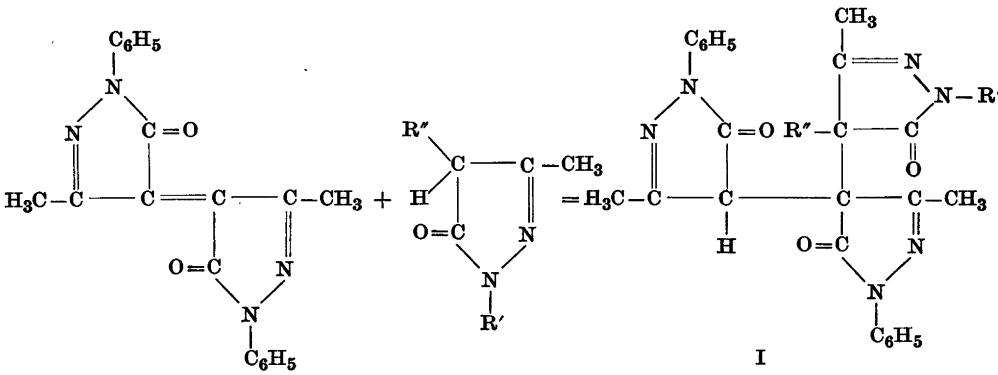
A chloroform solution of pyrazole blue is easily decolorized by 1-aryl-3-methyl-5-pyrazolones, Michael condensations taking place. Reactions of this kind usually require catalyzing by amines or sodium alkoxides (*cf.* Ingold and Powell [1], Kloetzel [2], Kohler and Dewey [3]), but the double bond system of pyrazole blue is so reactive that the additions proceed smoothly without catalyst even at room temperature.



Pyrazole blue

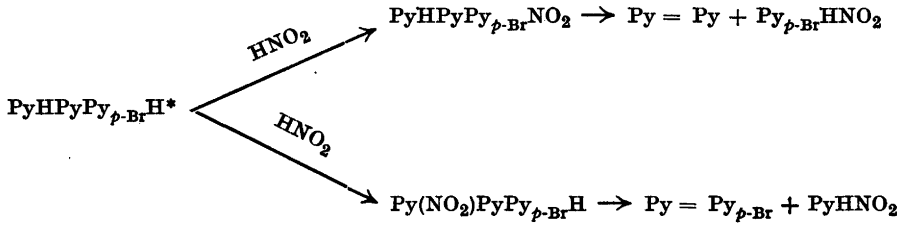
I

a) $R' = C_6H_5$; $R'' = H$
b) $R' = p\text{-}BrC_6H_4$; $R'' = H$
c) $R' = C_6H_5$; $R'' = Br$

From pyrazole blue and 1-phenyl-3-methyl-5-pyrazolone the white 1,1′,1″-triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione (I a) is formed. It is insoluble in ether but markedly more soluble in ethanol than is the corresponding bis-compound, 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione. Like 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione the terpyrazolone gives pyrazole blue with nitrous acid.

Of especial interest is the reaction with nitrous acid of 1,1′-diphenyl-1″-*p*-bromophenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione (I b). Here a mixture of 1,1′-diphenyl-3,3′-dimethyl-[$\Delta^{4,4'}$-bi-2-pyrazoline]-5,5′-dione (pyrazole blue) and 1-phenyl-1′-*p*-bromophenyl-3,3′-dimethyl-[$\Delta^{4,4}$-bi-2-pyrazoline]-5,5′-dione is obtained. This proves that in the adducts C—C-bonds exist between the three carbon atoms at the 4-positions and consequently that the formulas I a and b are correct. The light absorption spectrum (Fig. 1) and the equivalent weight (258) of 1,1′,1″-triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione are in agreement with this view.

The mechanism of the reaction between the terpyrazolone and nitrous acid is supposed to be the following:



Decoloration of a chloroform solution of pyrazole blue by 1-phenyl-3-methyl-4-bromo-5-pyrazolone is possible only with an excess of the bromopyrazolone. Attempts to purify the adduct have not been successful as they cause partial decomposition with recovery of pyrazole blue.

$$\text{PyHPyPyBr} \rightleftharpoons \text{Py} = \text{Py} + \text{PyHBr}$$

The velocities of both addition and decomposition are increased by alkalies, diminished by acids.

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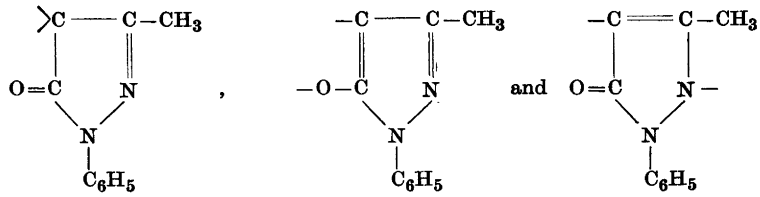
* Py is used to represent the radicals



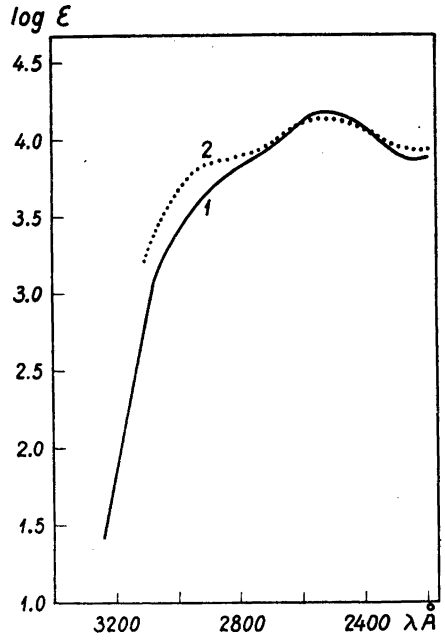


Fig. 1. Log $\varepsilon = \log \log \frac{I_0}{I} - \log c \cdot l$ *is plotted against the wave length. The concentration, c, of the substances is expressed in pyrazolone units per liter. 1) 1,1′1″-Triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione in ethanol (3.2 · 10⁻³, 3.2 · 10⁻⁴ and 3.2 · 10⁻⁵ M solutions). 2) 1,1′-Diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione in ethanol (4.0 · 10⁻⁵ M solution).*

It is likely that 1-phenyl-3-methyl-4-bromo-5-pyrazolone should add to pyrazole blue in the same way as does 1-phenyl-3-methyl-5-pyrazolone, especially as 1-phenyl-3,4-dimethyl-4-halo-5-pyrazolones and 1-phenyl-3-methyl-4,4-dihalo-5-pyrazolones do not decolorize a pyrazole blue solution. Furthermore, when a solution of 1,1′,1″-triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′5″-trione is brominated with the equimolar amount of bromine in buffered ($p_H \sim 9$) solution, pyrazole blue precipitates immediately. It follows that a PyHPyPyBr compound of the structure II c dissociates in the same way as does the adduct from 1-phenyl-3-methyl-4-bromo-5-pyrazolone and pyrazole blue. This supports the view that they are identical. A reduced stability of the bromo compound is to be expected for the substitution diminishes the opportunities for resonance.

EXPERIMENTAL

1,1′,1″-Triphenyl-3,3′3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione (I a) from 1-Phenyl-3-methyl-5-pyrazolone and Pyrazole Blue. Py = Py (2.0 g) and PyH_2 (1.05 g) in chloroform (25 ml) were boiled about ten minutes. A white precipitate and a colourless solution resulted. With excess PyH_2 the reaction took place rapidly also at room temperature. The precipitate was filtered from the cooled mixture and washed with chloro-

form and ether. Yield 2.95 g (97 %). The last traces of solvents were removed by heating the product several hours *in vacuo* (100° C, 0.5 mm Hg) over silica gel. It was then a hygroscopic powder. Found: C 69.3, H 5.0, N 16.20, equiv. wt. 259 on titration against phenolphthalein. $C_{30}H_{26}O_3N_6$ (518.6) requires C 69.5, H 5.0, N 16.21, equiv. wt. 259. Light absorption curve, see Fig. 1. M.p. about 200° C with decomposition.

From the solution in alcohol of 1,1′,1″-triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione a stabler crystal form separates when the walls of the beaker are scratched. Also this stable form is more soluble in ethanol than is 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione.

*1,1′-Diphenyl-1″-*p*-bromophenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione (I b) from 1-*p*-Bromophenyl-3-methyl-5-pyrazolone and Pyrazole Blue.* $HPyPyPy_{p\text{-}Br}H$ was prepared and dried analogously to HPyPyPyH. From 0.246 g of $Py_{p\text{-}Br}H_2$ and 0.331 g of Py = Py in 10 ml of chloroform, 0.543 g of $HPyPyPy_{p\text{-}Br}H$ was obtained (95 % yield). The colourless, hygroscopic product melted at about 200° C with decomposition. It is only slightly soluble in alcohol, insoluble in ether. Found: C 60.2, H 4.1, N 14.05, Br 13.3, equiv. wt. 299. Calc. for $C_{30}H_{25}BrO_3N_6$ (597.5): C 60.3, H 4.2, N 14.07, Br 13.4, equiv. wt. 299.

Reaction between 1,1′,1″-Triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione and Nitrous Acid. HPyPyPyH (1.40 g) and excess sodium nitrite were dissolved in 2.5 *N* sodium hydroxide solution and poured into 5 *N* sulphuric acid with stirring. A few ml of ether were added to prevent foaming. The blue precipitate formed was filtered by suction, washed with water and extracted with boiling ethanol for half a minute. Pyrazole blue (0.92 g, 99 %) remained and was collected by filtration, washed with ethanol and air-dried. It was purified by solution in chloroform and precipitation with ether. (Found: N 16.3. $(C_{10}H_8ON_2)_2$ (344.4) requires N 16.3.) Evaporation of the ethanolic extract gave 0.55 g of yellow needles from which almost colourless 1-phenyl-3-methyl-4-nitro-5-pyrazolone (m.p. 129° C with decomposition; *cf.* Knorr [4]) was isolated (extraction of impurities with ether, recrystallization from ethanol). Found: C 55.1, H 4.3. $C_{10}H_9O_3N_3$ (219.2) requires C 54.8, H 4.1.

*Reaction between 1,1′-Diphenyl-1″-*p*-bromophenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione and Nitrous Acid.* $PyHPyPy_{p\text{-}Br}H$ (5.5 g) was dissolved in 2.5 *N* sodium hydroxide solution containing an excess of sodium nitrite, and the solution was added to 5 *N* sulphuric acid with stirring. The blue reaction product was filtered by suction and washed with water, alcohol and ether. Yield 3.2 g (91 % calculated according to the equation p. 356). The substance was purified by precipitation with ether from its solution in chloroform. Found: C 63.0, H 4.1, Br 8.8. Calc. for Py = Py, $(C_{10}H_8ON_2)_2$, (344.4): C 69.7, H 4.7. Calc. for Py = $Py_{p\text{-}Br}$, $C_{20}H_{15}BrO_2N_4$, (423.3): C 56.7, H 3.6, Br 18.9.

SUMMARY

1,1′,1″-Triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione and 1,1′-diphenyl-1″-*p*-bromophenyl-3,3′3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione have been prepared for the first time. The analogous 1,1′,1″-triphenyl-3,3′,3″-trimethyl-4-bromo-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione is unstable and dissociates into pyrazole blue and 1-phenyl-3-methyl-4-bromo-5-pyrazolone in not too acid solutions.

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Studies on Pyrazolones

IV. Condensation Reactions with Certain 1-Aryl-3-methyl-4-halo-5-pyrazolones

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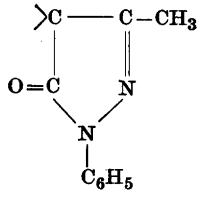
In 1887 Knorr [1] prepared pyrazole blue by the coupling of two molecules of 1-phenyl-3-methyl-4-bromo-5-pyrazolone. In the present investigation the formation of another condensation product (here called furlone yellow) has been achieved by interaction of three molecules of the same bromo-compound.

When an alcoholic solution of 1-phenyl-3-methyl-4-bromo-5-pyrazolone is neutralized with sodium hydroxide solution, crystals of furlone yellow soon precipitate. Furlone yellow can also be obtained from buffered solutions of 1-phenyl-3-methyl-4-halo-5-pyrazolones. Small amounts of cupric ions catalyze the reaction, hydrogen ions retard it (*cf.* Smith [2]). A solubility curve according to Kunitz and Northrup [3] (Fig. 1) indicates that the product is a single compound.

Furlone yellow has the same C-, H- and N-content as pyrazole blue. It is free from bromine and active hydrogen atoms. Ebullioscopic measurements of the molecular weight suggest a $Py_3^{x)}$-formula. Because of these facts it is a ring product formed according to the equation

$$3\ PyHBr \rightarrow Py_3 + 3\ HBr.$$

* Py represents the radicals



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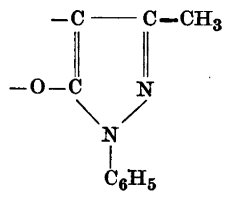

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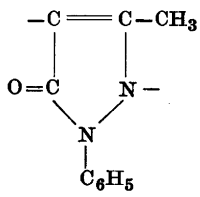


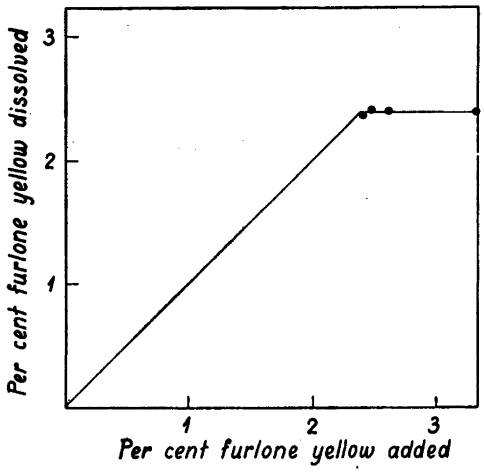


Fig. 1. Solubility diagram of furlone yellow in benzene-petroleum ether (1 : 1).

It is soluble in benzene, acetone, chloroform and ether but only slightly soluble in ethanol at room temperature. When heated it melts with decomposition at 158° C. Consequently it is not identical with the yellow substance prepared by Smith[2,p. 7].

Furlone yellow decomposes rapidly in alkaline solution yielding an orange-red colour. With concentrated sulphuric acid and ferric chloride it gives a red colour. With zinc chloride, hydrogen chloride and water a colourless, very unstable double compound of the composition $Py_3 \cdot ZnCl_2 \cdot 2HCl \cdot 2H_2O$ is formed.

Furlone yellow reacts slowly with hydrogen chloride in ether solution with formation of the slightly soluble salt PyHPyH · 2HCl. The reaction proceeds via several steps. Initially a colourless addition product from furlone yellow and hydrogen chloride is formed, from which unchanged furlone yellow is easily regained. After a while white crystals separate, which are probably the hydrochloride of PyHPyCl. The chloro-compound is then slowly reduced to PyHPyH · 2HCl. The mother liquor from the filtration of PyHPyH · 2HCl gives pyrazole blue when diluted with alcohol.

From the decomposition with hydrogen chloride, it is evident that two of the three pyrazolone groups of furlone yellow are coupled together at their C-4 positions, but *a priori* very little can be deduced about the way the third pyrazolone unit is attached to the rest. A study of the intermediate products in the formation of furlone yellow will give further information.

At first sight it seems probable that furlone yellow is formed according to the equations:

$$2\ \mathrm{PyHHal} \rightarrow \mathrm{PyHPyHal} + \mathrm{HHal};\mathrm{PyHPyHal} + \mathrm{PyHHal} \rightarrow \mathrm{PyHPyPyHal} + \mathrm{HHal};$$
$$\mathrm{PyHPyPyHal} \rightarrow \mathrm{Py_3} + \mathrm{HHal},$$

1,1′-diphenyl-3,3′-dimethyl-4-halo-[4,4′-bi-2-pyrazoline]-5,5′-dione being an intermediate. However, repeated attempts to prepare PyHPyBr only resulted in rapid precipitation of pyrazole blue. PyHPyCl was obtained by addition of hydrogen chloride to pyrazole blue in ether, but on dilution pyrazole blue was recovered. Thus PyHPyCl, which is undoubtedly more stable than PyHPyBr, can exist only in an environment too acid to allow a reaction with PyHHal. Accordingly, the velocity of the splitting off of HHal from PyHPyHal is very great in not too acid medium and must far exceed the velocity of the condensation with PyHHal. This indicates that furlone yellow is not formed by a reaction between PyHPyHal and PyHHal but via pyrazole blue and consequently by reaction between pyrazole blue and 1-phenyl-3-methyl-4-halo-5-pyrazolone.

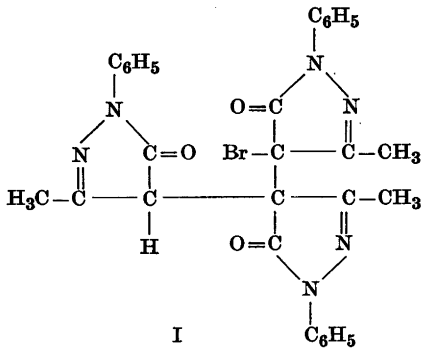
$$2\ \mathrm{PyHHal} \rightarrow \mathrm{Py} = \mathrm{Py} + 2\ \mathrm{HHal};\ \mathrm{Py} = \mathrm{Py} + \mathrm{PyHHal} \rightleftharpoons \mathrm{PyHPyPyHal};$$
$$\mathrm{PyHPyPyHal} \rightarrow \mathrm{Py_3} + \mathrm{HHal}.$$

With some precautions — addition of chloroform to dissolve the pyrazole blue, alkaline solution to get the addition reaction sufficiently catalyzed — it was possible to prepare furlone yellow from pyrazole blue and 1-phenyl-3-methyl-4-bromo-5-pyrazolone in 70 per cent yield (calculated on the total amount of pyrazolone). This yield is too great to have had its origin from the PyHBr alone.

In a previous paper (Westöö [4]) it was shown that the adduct PyHPyPyBr is a 1,1′,1″-triphenyl-3,3′,3″-trimethyl-4-bromo-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione (I). Consequently C—C-bonds must exist between all three pyrazolone units in furlone yellow.

The formulas II, III and IV represent potential structures for furlone yellow. Formula IV can be rejected at once for it violates Bredt's rule [5] and does not explain the decomposition reactions with alkali or acid. In favour of III is the fact that in the case of other substances prepared by similar reactions, such as pyrazole blue and 1,1′-diphenyl-3,3′,4,4′-tetramethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione [6], only C—C-bonds are formed. On the other hand formula II is preferable for steric reasons.

A possible way of distinguishing between the formulas II and III is to prepare a furlone yellow product substituted in one of the pyrazolone groups



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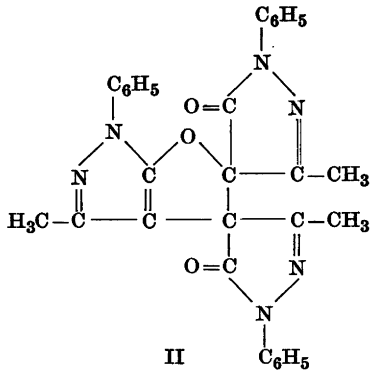
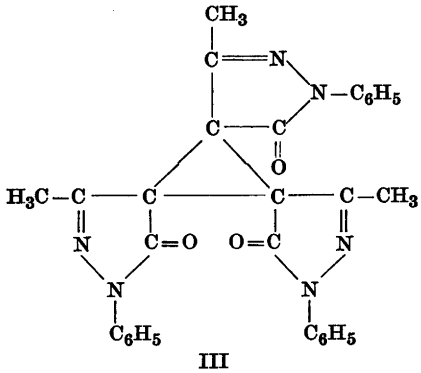
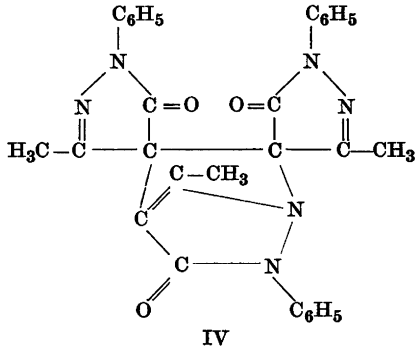


II



III



IV

in two ways so as to place the substituent in different pyrazolone nuclei in the two preparations. If formula III is correct for furlone yellow, identical products or *cis-trans* isomers will be obtained from the two syntheses. Otherwise, two structural isomers will be formed. As substituent bromine in *para* position of the phenyl group was chosen. Thus the two syntheses to be achieved are

$$\mathrm{Py{=}Py} + \mathrm{Py}_{p\text{-Br}}\mathrm{HBr} \rightarrow \underset{\llcorner\!\!\lrcorner}{\mathrm{Py\text{-}Py}}\text{-}\mathrm{Py}_{p\text{-Br}}\ \mathrm{I} + \mathrm{HBr};$$

$$\mathrm{Py{=}Py}_{p\text{-Br}} + \mathrm{PyHBr} \rightarrow \underset{\llcorner\!\!\lrcorner}{\mathrm{Py\text{-}Py}}_{p\text{-Br}}\text{-}\mathrm{Py}\ \mathrm{II} + \mathrm{HBr}.$$

From pyrazole blue and 1-*p*-bromophenyl-3-methyl-4-bromo-5-pyrazolone a yellow compound of the expected properties (decomposition by alkali with orange colour, moderate solubility in benzene, slight solubility in alcohol) could be prepared. The $\underset{\llcorner\!\!\lrcorner}{\mathrm{Py\text{-}Py}}\text{-}\mathrm{Py}_{p\text{-Br}}$ I was contaminated with the corresponding tri-*p*-bromophenyl-compound, from which it was partly separated by fractional precipitation from benzene solution, using alcohol as precipitant. Final purification was accomplished by chromatography on silica gel.

PyHBr and a mixture of Py = Py and Py = $Py_{p\text{-}Br}$ obtained by reaction between $HPyPyPy_{p\text{-}Br}H$ and nitrous acid [4] were used as starting material for Py-Py-$Py_{p\text{-}Br}$ II. In the mixture of Py-Py-$Py_{p\text{-}Br}$ and Py_3 obtained, the bromo-compound was the less soluble and could be enriched by dissolution in benzene and precipitation with alcohol. Final purification involved chromatography on silica gel.

The two mono-*p*-bromo-furlone yellows prepared are not identical. This is obvious from the fact that although they have the same composition, they have different decomposition temperatures, 147° C and 161° C respectively, and also different light absorption curves (Fig. 2). Their solubilities in ethanol are different as well. Thus of the latter compound 0.5 mg dissolves per ml of ethanol, of the former four times as much.

Cis-trans isomers of III might well show different decomposition temperatures and solubilities, but their light absorption curves should not differ very much. However, the conclusive proof of the relation between the two isomers is given by their decomposition reactions with hydrogen chloride.

When Py-Py-$Py_{p\text{-}Br}$ I prepared from pyrazole blue and 1-*p*-bromophenyl-3-methyl-4-bromo-5-pyrazolone was decomposed with hydrogen chloride, Py = Py and PyHPyH · 2HCl were obtained. After oxidation of PyHPyH · 2HCl to give Py = Py, the total yield of pyrazole blue was 60 per cent calculated according to the equation

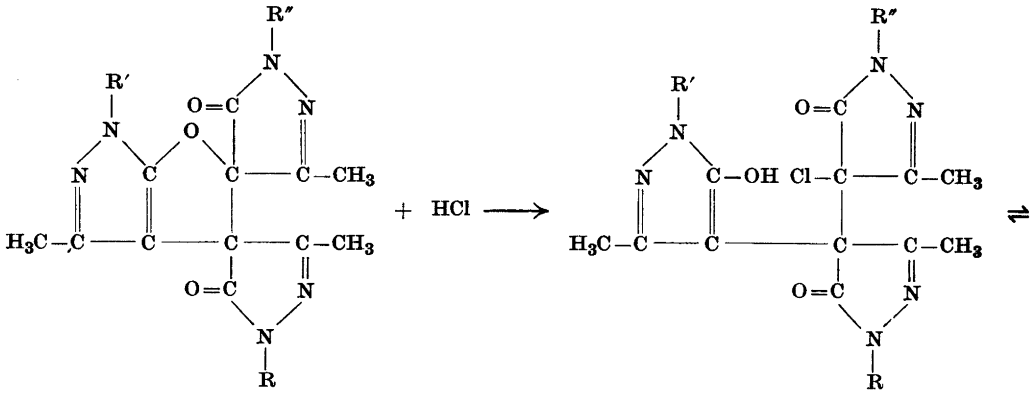
$$\text{Py-Py-Py}_{p\text{-Br}} + \text{HCl} \rightarrow \text{Py} = \text{Py} + \text{Py}_{p\text{-Br}}\text{HCl}.$$

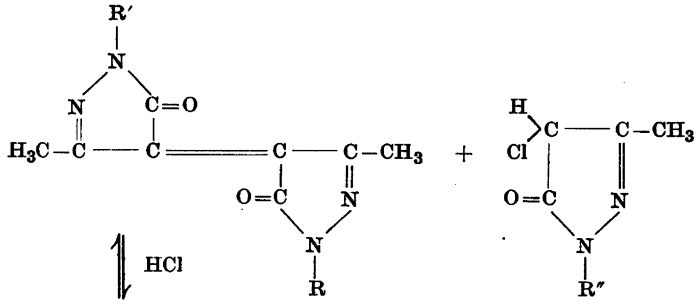
Py-$Py_{p\text{-}Br}$-Py II from Py = $Py_{p\text{-}Br}$ and PyHBr was treated in the same way. Here Py = $Py_{p\text{-}Br}$ and the hydrochloride of $PyHPy_{p\text{-}Br}H$ were obtained in 70 per cent yield.

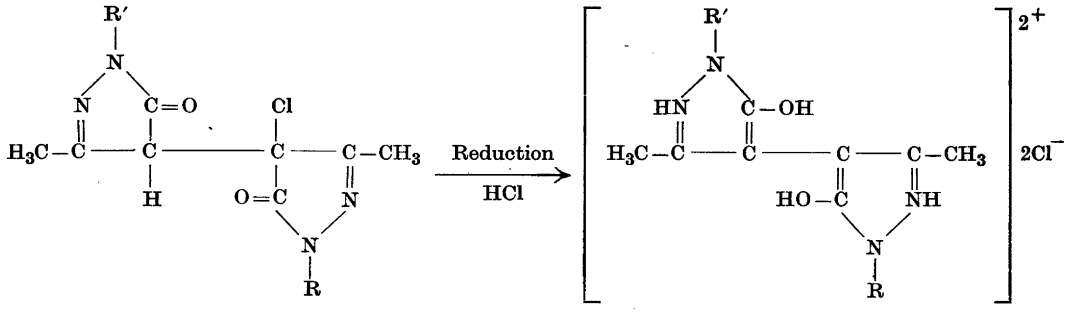
The different products of pyrazole blue type obtained at the decomposition reactions indisputably show that the two mono-*p*-bromo-furlone yellows are neither identical nor *cis-trans* isomers of formula III, but are structural isomers. *Consequently formula II is valid for furlone yellow.* However, formula II allows the existence of a *cis* and a *trans* form of furlone yellow. Whether only one of them or a mixture of both has been obtained is still in the process of research.

The decomposition of furlone yellow by alkali (p. 361) does not prejudice formula II, for an analogy is found in the behaviour of 1-phenyl-3,4-dimethyl-4-halo-5-pyrazolones. These compounds have the same structure as part of formula II for furlone yellow with a polarizable atom in the 4-position and are destroyed by alkali with the appearance of an orange-red colour.

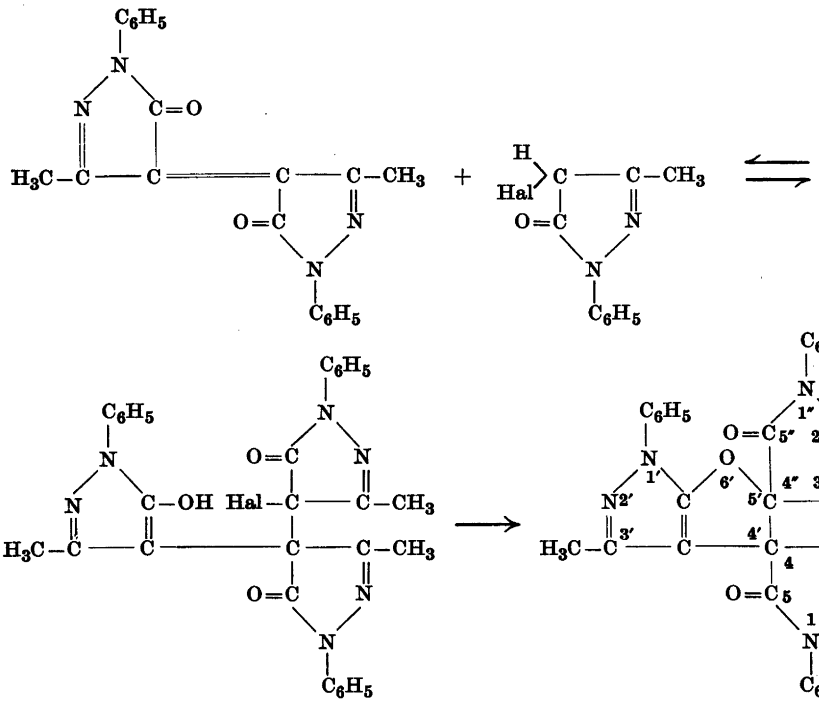
The chief reactions of furlone yellows with hydrogen chloride can now be written:

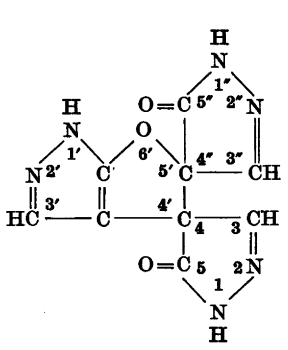




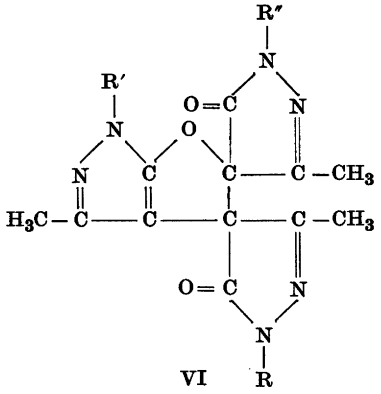


Equations for the formation of furlone yellow can be written as follows:





V



VI

a) R=R′=C_6H_5; R″=*p*-BrC_6H_4
b) R=R″=C_6H_5; R′=*p*-BrC_6H_4
c) R=*p*-BrC_6H_4; R′=R″=C_6H_5
d) R=R′=C_6H_5; R″=*o*-$CH_3 \cdot C_6H_4$
e) R=R′=C_6H_5; R″=*p*-$CH_3 \cdot C_6H_4$
f) R=R′=*p*-BrC_6H_4; R″=*o*-$CH_3 \cdot C_6H_4$

The systematic name of furlone yellow is 1,1′,1″-triphenyl-3,3′,3″-trimethyl-dispiro [pyrazole-4(5*H*),4′(5′*H*)-1*H*-furo [2,3-*c*] pyrazole-5′,4″(5″*H*)-pyrazole]-5,5″-dione. In order to simplify the nomenclature, the diketone V will be called furlone. Thus furlone yellow is a 1,1′,1″-triphenyl-3,3′,3″-trimethylfurlone and the bromo-derivative prepared from pyrazole blue and 1-*p*-bromophenyl-3-methyl-4-bromo-5-pyrazolone is 1,1′-diphenyl-1″-*p*-bromophenyl-3,3′,3″-trimethylfurlone (VI a).

STRUCTURE OF MONO-*p*-BROMO-FURLONE YELLOW II

Although the structures of furlone yellow (II) and of 1,1′-diphenyl-1″-*p*-bromophenyl-3,3′,3″-trimethylfurlone (VI a) are settled, the constitution of the bromo-compound prepared from mono-*p*-bromo-pyrazole blue and 1-phenyl-3-methyl-4-bromo-5-pyrazolone is still unknown. Here the addition to the reactive double bond can take place in two ways:

$$Py_{p\text{-}Br} = Py + PyHBr \rightleftharpoons \begin{cases} Py_{p\text{-}Br}HPyPyBr \\ PyBrPy_{p\text{-}Br}PyH \end{cases}$$

This makes possible the formation of two structural isomers, VI b and c.

By comparing the light absorption curves of some substituted furlone yellows, 1,1′-diphenyl-1″-*o*-tolyl-3,3′,3″-trimethylfurlone (VI d), 1,1′-diphenyl-1″-*p*-tolyl-3,3′,3″-trimethylfurlone (VI e), 1,1′-diphenyl-1″-*p*-bromophenyl-3,3′,3″-trimethylfurlone (VI a) and 1,1′-di-*p*-bromophenyl-1″-*o*-tolyl-3,3′,3″-trimethylfurlone (VI f), with the curve of the mono-*p*-bromo-furlone yellow II obtained, it is possible to arrive at the probable structure of this compound. It is evident from the curves (Fig. 2) that the shoulder at 2 600—3 200 Å changes very little on variation of the 4-bromopyrazolone that is added to pyrazole blue to give the furlone yellow compound. Thus the shoulder is the same when Py = Py has reacted with PyHBr, $Py_{o\text{-}Me}HBr$, $Py_{p\text{-}Me}HBr$ or $Py_{p\text{-}Br}HBr$. Hence a moderate change of the aryl group in the structure VII has no influence on the shoulder, the intensity of which is nevertheless sure to be influenced by the structure VII as a whole.

The light absorption curves of 1,1′-di-*p*-bromophenyl-1″-*o*-tolyl-3,3′,3″-trimethylfurlone (VI f) and mono-*p*-bromofurlone yellow II have shoulders similar to each other, but distinctly different from the corresponding shoulders of all the curves of furlone yellow products prepared from unsubstituted pyrazole blue (VI a, d, e and II). Knowing that the light absorption curves of

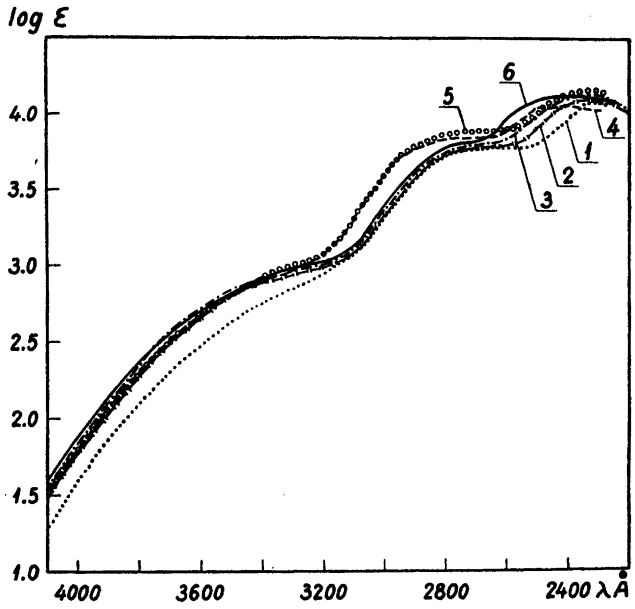
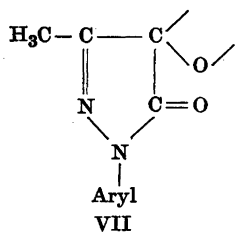
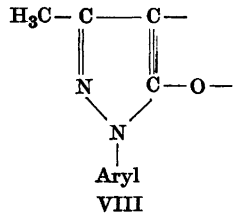
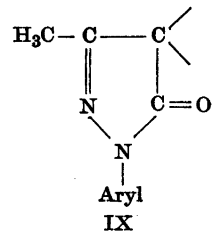


Fig. 2. All the products have been measured in ethanol solution. 1) 1,1'Diphenyl-1"-o-tolyl-3,3',3"-trimethylfurlone (VI d) (2.4 · 10⁻³, 2.4 · 10⁻⁴ and 2.4 · 10⁻⁵ M solutions). 2) 1,1',1"-Triphenyl-3,3',3"-trimethylfurlone (II) (1.4 · 10⁻³, 1.4 · 10⁻⁴ and 1.4 · 10⁻⁵ M solutions). 3) 1,1'-Diphenyl-1"-p-tolyl-3,3',3"-trimethylfurlone (VI e) (2.7 · 10⁻³, 2.7 · 10⁻⁴ and 2.7 · 10⁻⁵ M solutions). 4) 1,1'-Di-p-bromophenyl-1"-o-tolyl-3,3',3"-trimethylfurlone (VI f) (1.3 · 10⁻³, 2.6 · 10⁻⁴ and 2.6 · 10⁻⁵ M solutions). 5) 1-p-Bromophenyl-1'1"-diphenyl-3,3',3"-trimethylfurlone (VI c) (1.9 · 10⁻³, 1.9 · 10⁻⁴ and 1.9 · 10⁻⁵ M solutions). 6) 1,1'-Diphenyl-1"-p-bromophenyl-3,3',3"-trimethylfurlone (VI a) (1.9 · 10⁻³, 1.9 · 10⁻⁴ and 1.9 · 10⁻⁵ M solutions).

substances with stable pyrazole structure (VIII) like 1-phenyl-3-methyl-5-methoxypyrazole (Valyashko and Bliznyukov [7]) show only one absorption band of appreciable intensity (at about 2 400 Å) and no trace of shoulder or band at 2 600—3 200 Å, it is reasonable to suppose that the one third of the furlone yellow with that structure does not influence the shoulder either. The part of the molecule with the structure IX remains as a possibility. Substances of this type present shoulders at the wave length considered, e. g. $Py(CH_3)_2$. (Biquard and Grammaticakis [8]) and $Py(CH_3)PyCH_3$ (Westöö [9]). These facts favour the belief that the latter compound has the constitution VI c and is thus 1-*p*-bromophenyl-1',1"-diphenyl-3,3',3"-trimethylfurlone.


VII


VIII


IX

EXPERIMENTAL

1-p-Bromophenyl-3-methyl-4-bromo-5-pyrazolone, 1-p-Tolyl-3-methyl-4-bromo-5-pyrazolone and 1-o-Tolyl-3-methyl-4-bromo-5-pyrazolone. They were prepared analogously to 1-phenyl-3-methyl-4-bromo-5-pyrazolone [9]. However, the hydrobromide of 1-*p*-bromophenyl-3-methyl-4-bromo-5-pyrazolone could not be decomposed into free pyrazolone and acid by water in acetic acid on account of its slight solubility in dilute acetic acid. Hence it was dissolved in alcohol with an excess of 2.5 *N* sodium hydroxide, acidified with dilute sulphuric acid (bromophenol blue as indicator) and precipitated with water. The free pyrazolone precipitated was filtered by suction and washed with water and small amounts of alcohol and ether. It was purified by extraction with chloroform (boiling half a minute) where it is only slightly soluble, followed by crystallization from alcohol. Found: equiv. wt.: 331 (on titration with sodium hydroxide against phenolphthalein). $C_{10}H_8Br_2ON_2$ (332.0) requires equiv. wt. 332.

According to Dains, O'Brien and Johnsson [10], 1-*o*-tolyl-3-methyl-4-bromo-5-pyrazolone is a yellow substance which melts at 116° C. The product obtained here was colourless after one crystallization from chloroform and then melted at 147 – 149° C with decomposition. Found: C 49.4, H 4.1, Br 29.95, N 10.56, equiv. wt. 267.4. $C_{11}H_{11}BrON_2$ (267.1) requires C 49.5, H 4.15, Br 29.91, N 10.49, equiv. wt. 267.1.

1-*p*-Tolyl-3-methyl-4-bromo-5-pyrazolone could not be purified by recrystallization from chloroform, unlike PyHBr and $Py_{o\text{-}Me}HBr$, on account of the small temperature coefficient of its solubility in that solvent. Crystallization from alcohol gave a colourless product which melted with decomposition at 144° C. Found: equiv. wt. 267.3. $C_{11}H_{11}BrON_2$ (267.1) requires equiv. wt. 267.1.

*1,1'-Di-*p*-bromophenyl-3,3'-dimethyl-[4,4'-bi-2-pyrazoline]-5,5'-dione.* This compound was obtained by oxidation of 1-*p*-bromophenyl-3-methyl-5-pyrazolone with phenylhydrazine in the same way that Knorr [1] prepared PyHPyH from PyH_2.

*1,1'-Di-*p*-bromophenyl-3,3'-dimethyl-[$\Delta^{4,4'}$-bi-2-pyrazoline]-5,5'-dione.* This compound was prepared by oxidation of 1,1'-di-*p*-bromophenyl-3,3'-dimethyl-[4,4'-bi-2-pyrazoline]-5,5'-dione with nitrous acid in the same way that Knorr [1] prepared pyrazole blue; m.p. 248° C (decomposes). Found: C 47.8, H 2.8, Br 31.7, N 11.12. $C_{20}H_{14}Br_2O_2N_4$ (502.2) requires C 47.8, H 2.8, Br 31.8, N 11.16.

Furlone Yellow (II), 1,1',1''-Triphenyl-3,3',3''-trimethylfurlone, from 1-Phenyl-3-methyl-4-bromo-5-pyrazolone. 1-Phenyl-3-methyl-4-bromo-5-pyrazolone (5 g) was dissolved in ethanol (150 ml), and sodium acetate (50 ml of 1.8 *N* solution), acetic acid (50 ml of 1.8 *N* solution) and a 0.01 % solution of $CuSO_4 \cdot 5H_2O$ (2 ml) were added. The amount of acetic acid may be diminished or even omitted. The solution was allowed to stand overnight at room temperature, during which time yellow crystals precipitated. They

were filtered by suction and washed successively with 60 % and 95 % alcohol. Yield 65 %. The product, already quite pure, melted with decomposition at 158° C after recrystallization from ethanol. Found: C 69.7, H 4.7, N 16.30. $(C_{10}H_8ON_2)_3$ (516.6) requires C 69.7, H 4.7, N 16.27. Ebullioscopic measurements of the molecular weight in benzene: M = 512 (1 % solution), M = 507 (2 % solution).

$Py_3 \cdot ZnCl_2 \cdot 2HCl \cdot 2H_2O$. Furlone yellow was dissolved in concentrated hydrochloric acid and an excess of zinc chloride in the same solvent was added. Scratching the side of the beaker caused white crystals to precipitate. The very unstable product was filtered by suction, washed successively with small amounts of concentrated hydrochloric acid and ether, and air-dried overnight. Found C 46.6, H 4.1, Zn 8.6 (determined as sulphate ash), HCl 9.58 (titration against bromophenol blue), Cl^- 18.62 (potentiometric titration with silver nitrate). Calc. for $(C_{10}H_8ON_2)_3 \cdot ZnCl_2 \cdot 2HCl \cdot 2H_2O$ (762): C 47.3, H 4.0, Zn 8.6, HCl 9.57, Cl^- 18.62.

Decomposition of Furlone Yellow with Hydrogen Chloride. Furlone yellow was dissolved in ether, and the solution was saturated with hydrogen chloride. A colourless solution was obtained. On immediate dilution with ether, the colour changed to yellow. Likewise in concentrated hydrochloric acid and in alcohol containing much hydrochloric acid, colourless solutions were obtained from which unchanged furlone yellow could be regained by dilution with water. When furlone yellow was allowed to remain in the acidic ether for some hours, white crystals precipitated. They were stable in the strongly acid solution, but when the mother liquor was diluted or when the crystals were collected and washed with ether, they rapidly turned into pyrazole blue. The unstable product is probably a hydrochloride of PyHPyCl (cf. p. 362). However, after remaining in the mother liquor four days, the crystals could be filtered, washed with ether and air-dried without change. The stable product was almost insoluble in alcohol. With nitrous acid it gave pyrazole blue. Found: C 56.2, H 4.8, Cl 16.74, 0.46 equiv. of weak acid, 0.92 equiv. of total acid per 100 g of substance on titration in acetone-alcohol solution. PyHPyH.2HCl (419.3) requires C 57.3, H 4.8, Cl 16.91, 0.477 equiv. of weak acid and 0.954 equiv. of total acid per 100 g of substance.

From the mother liquor pyrazole blue was obtained on dilution with alcohol.

Furlone Yellow, 1,1′,1″-Triphenyl-3,3′,3″-trimethylfurlone, (II) from Pyrazole Blue and 1-Phenyl-3-methyl-4-bromo-5-pyrazolone. PyHBr (0.669 g) and Py = Py (0.202 g) were shaken 20 hours with alcohol (20 ml), chloroform (20 ml), sodium acetate (13 ml of 1.8 *N* solution) and catalyst (0.25 ml of 0.01 % $CuSO_4 \cdot 5H_2O$ solution).

The chloroform and alcohol were distilled off under reduced pressure. The precipitate formed (wt. 0.621 g) was filtered by suction, washed with water and air-dried. It was recrystallized from alcohol (5 ml), filtered by suction and washed with 5 ml of alcohol. Yellow crystals (0.464 g), which melted with decomposition at 152° C, were obtained. Another crystallization from alcohol (20 ml) resulted in 0.445 g of pure furlone yellow (70 % yield); m.p. 157° C with decomposition.

To prove that the pyrazole blue was really responsible for a considerable amount of the yield, this synthesis was checked by another one with PyHBr alone (0.654 g) as starting material. The procedure was exactly the same as above. A yellow substance weighing 0.420 g was obtained. From the first recrystallization there was obtained 0.296 g of product which melted with decomposition at 152° C. A second recrystallization from 10 ml of alcohol gave 0.259 g of furlone yellow, m.p. 157° C with decomposition.

The comparison of the yields of the two syntheses, 0.445 g and 0.259 g, is significant. The light absorption curves of the two products were identical.

1,1'-Diphenyl-1"-p-bromophenyl-3,3',3"-trimethylfurlone (VI a) from Pyrazole Blue and 1-p-Bromophenyl-3-methyl-4-bromo-5-pyrazolone. $Py_{p\text{-}Br}HBr$ (2.05 g), Py = Py (2.55 g), chloroform (600 ml), alcohol (600 ml), sodium acetate (360 ml of 1.80 *N* solution) and catalyst (12 ml of 0.01 % $CuSO_4 \cdot 5H_2O$ solution) were shaken over night. Decoloration occurred. The organic solvents were distilled off under reduced pressure. The precipitate formed during the evaporation was filtered by suction, washed with water and air-dried. After crystallization from alcohol the yield of yellow substance, partially $(Py_{p\text{-}Br})_3$, was 2.55 g. It was fractionated by solution in benzene and precipitation with alcohol, the tribromo-compound being less soluble than the monobromo-compound. The last fraction was dissolved in benzene and chromatographed on silica gel several times. $(Py_{p\text{-}Br})_3$ was eluted by benzene + ether (20 : 1), $PyPyPy_{p\text{-}Br}$ with ether. M. p. of the monobromo-compound 147° C with decomposition. Light absorption: Fig. 2. Found: C 60.5, H 3.8, Br 13.1, N 14.1. $C_{30}H_{23}BrO_3N_6$ (595.5) requires C 60.5, H 3.9, Br 13.4, N 14.1.

1-p-Bromophenyl-1'1"-diphenyl-3,3',3"-trimethylfurlone (VI c) from 1-Phenyl-3-methyl-4-bromo-5-pyrazolone and 1-Phenyl-1'-p-bromophenyl-3,3'-dimethyl-[$\Delta^{4,4}$-bi-2-pyrazoline]-5,5'-dione. Three and a half g of a mixture of Py = $Py_{p\text{-}Br}$ and Py = Py prepared by oxidation of $PyHPyPy_{p\text{-}Br}H$ with nitrous acid [4] and 2.9 g of PyHBr were shaken with chloroform (340 ml) and alcohol (45 ml) for 10 minutes. More alcohol (295 ml), sodium acetate (210 ml of 1.8 *N* solution) and catalyst (6 ml of 0.01 % $CuSO_4 . 5H_2O$ solution) were added, and the mixture was shaken overnight. Concentration of the decolorized solution *in vacuo*, filtration and crystallization from ethanol resulted in 3.15 g of a mixture of Py_3 and $PyPy_{p\text{-}Br}Py$. It was dissolved in benzene and precipitated with ethanol.

Fraction	Weight, g	% C	Fraction	Weight, g	% C
1	0.68	63.5	3	0.35	67.1
2	0.62	63.8	4	0.75	67.6

Calc. for $PyPy_{p\text{-}Br}Py$: C 60.5. Calc. for Py_3: C 69.7.

The table shows that the bromo-compound was enriched in the first two fractions. These were dissolved in benzene and chromatographed several times on silica gel. The yellow monobromo-compound was eluted with benzene + ether (20 : 1). M. p. 161° C with decomposition. Light absorption: Fig. 2. Found: C 60.7, H 4.0, Br 13,4, N 14.1. $C_{30}H_{23}BrO_3N_6$ (595.5) requires C 60.5, H 3.9, Br 13.4, N 14.1.

Decomposition of 1,1'-Diphenyl-1"-p-bromophenyl-3,3',3"-trimethylfurlone and 1-p-Bromophenyl-1',1"-diphenyl-3,3',3"-trimethylfurlone (VI a and c) with Hydrogen Chloride. 1,1'-Diphenyl-1"-*p*-bromophenyl-3,3',3"-trimethylfurlone (VI a) (0.367 g) was treated with hydrogen chloride in ether solution for three days. The crystals formed were filtered by suction, washed with ether and air-dried. From the bispyrazolone hydrochloride obtained unsubstituted pyrazole blue was prepared by oxidation with nitrous acid (the hydrochloride and excess sodium nitrite were dissolved in 2.5 *N* sodium hydroxide solution and poured into 5 *N* sulphuric acid). Yield 0.070 g (33 %). From the ether solution another 33 % of pyrazole blue was obtained by dilution with ether and alcohol. The products were mixed and purified by solution in boiling chloroform followed by precipitation with

ether, a procedure which does not separate different pyrazole blue substances from each other but eliminates ether-soluble impurities. Found: C 69.5, H 4.5. $(C_{10}H_8ON_2)_2$ (344.4) requires C 69.7, H 4.7. A Beilstein test indicated that the substance was free from bromine.

1-*p*-Bromophenyl-1′,1″-diphenyl-3,3′,3″-trimethylfurlone (VI c) was treated in the same way. Here Py = $Py_{p\text{-}Br}$ was obtained in about 70 % yield. M.p. 219° C with decomposition. Found: C 56.9, H 3.45, Br 19.0, N 13.2. $C_{20}H_{15}BrO_2N_4$ (423.3) requires C 56.7, H 3.6, Br 18.9, N 13.2.

1,1′-Diphenyl-1″-o-tolyl-3,3′,3″-trimethylfurlone (VI d) from Pyrazole Blue and 1-o-Tolyl-3-methyl-4-bromo-5-pyrazolone. Py = Py (0.80 g) and $Py_{o\text{-}Me}HBr$ (0.632 g) were shaken for 1 hour with chloroform (75 ml), alcohol (75 ml) and sodium acetate (45 ml of 1.8 *N* solution) to establish equilibrium with the addition compound. Then a 5 % $CuSO_4 \cdot 5H_2O$ solution (2 ml) was added, and the flask was shaken overnight. Despite the fact that the solution was bluish-brown next morning, the organic solvents were distilled off under reduced pressure. The precipitate formed during the evaporation was filtered by suction, washed with water and air-dried. It was repeatedly crystallized from alcohol. As a green colour was not removed by that procedure, a small crystal of $Py_{o\text{-}Me}HBr$ was added followed by another crystallization. Now a faintly yellow, almost white substance (20 % yield) was obtained, m.p. 151° C with decomposition. Light absorption: Fig. 2. Found: C 70.1, H 5.0, N 15.79. $C_{31}H_{26}O_3N_6$ (530.6) requires C 70.2, H 4.9, N 15.84. As the product has not been purified by chromatography, some 1,1′,1″-tri-*o*-tolyl-3,3′,3″-trimethylfurlone is probably present.

1,1′-Diphenyl-1″-p-tolyl-3,3′,3″-trimethylfurlone (VI e) from Pyrazole Blue and 1-p-Tolyl-3-methyl-4-bromo-5-pyrazolone. Pyrazole blue and an excess of $Py_{p\text{-}Me}HBr$ were treated as in the preceeding synthesis, but only a very small yield was obtained. Hence an excess of Py = Py was tried.

Py = Py (0.66 g) and $Py_{p\text{-}Me}HBr$ (0.37 g) were shaken with alcohol (75 ml), chloroform (75 ml) and sodium acetate (45 ml of 1.8 *N* solution) during one hour. Then cupric catalyst (1 ml of 0.01 % $CuSO_4 \cdot 5H_2O$ solution) was added, and the system was shaken overnight. In spite of the deficiency in $Py_{p\text{-}Me}HBr$, complete decoloration took place. Evaporation, filtering and crystallization from alcohol as in the preceding synthesis gave 0.30 g of yellow product (41 % yield); m.p. 134° C with decomposition. The light absorption curve is shown in Fig. 2. Found: C 70.0, H 5.1, N 15.94. $C_{31}H_{26}O_3N_6$ (530.6) requires C 70.2, H 4.9, N 15.84. The presence of some tri-*p*-tolyl-compound is probable.

1,1′-Di-p-bromophenyl-1″-o-tolyl-3,3′,3″-trimethylfurlone (VI f) from 1-o-Tolyl-3-methyl-4-bromo-5-pyrazolone and Di-p-bromo-Pyrazole Blue. $Py_{p\text{-}Br}$ = $Py_{p\text{-}Br}$ (1.86 g) $Py_{o\text{-}Me}HBr$ (1.54 g), chloroform (430 ml) and alcohol (55 ml) were shaken for 10 minutes. After addition of more alcohol (375 ml) and 1.80 *N* sodium acetate solution (250 ml), the mixture was shaken for 2 hours. Then catalyst (35 ml of 3 % $CuSO_4 \cdot 5H_2O$ solution) was added, and finally the flask was shaken overnight. Concentration of the decolorized solution in vacuo, filtration and crystallization from alcohol resulted in 1.20 g of the faintly yellow $Py_{p\text{-}Br}Py_{p\text{-}Br}Py_{o\text{-}Me}$ (47 % yield) melting at 164° C with decomposition. Light absorption: Fig. 2. Found: C 54.0, H 3.6, Br 23.0, N 12.3. $C_{31}H_{24}Br_2O_3N_6$ (688.4) requires C 54.1, H 3.5, Br 23.2, N 12.2. Some 1,1′,1″-tri-*o*-tolyl-3,3′,3″-trimethylfurlone is present as impurity.

Light Absorption Measurements. The light absorption was measured with a Beckmann spectrophotometer model DU. In the diagrams $\log \varepsilon = \log \log \frac{I_0}{I} - \log c \cdot l$ is plotted

against the wave length, λ, in Ångström units. The concentration, c, of the substances is not expressed in moles per liter but in pyrazolone units per liter.

SUMMARY

Furlone yellow, a substance formed by the intermolecular dehydrohalogenation of three molecules of 1-phenyl-3-methyl-4-bromo-5-pyrazolone, has been prepared for the first time. The structure II containing a dihydrofuran ring is proposed for it. Several bromo- and methyl-substituted furlone yellows have also been prepared.

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Aminoalkyl Ethers of 2-Hydroxythiazoles

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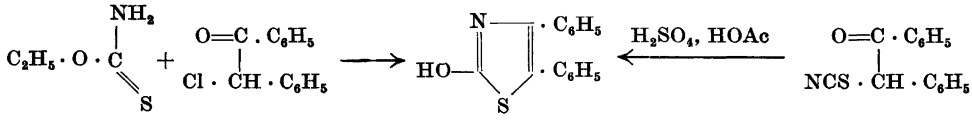
It is known that dialkylaminoalkyl ethers of suitably substituted phenols may exert antihistaminic activity [1,2]. As an extension of work in this laboratory on aminoalkyl compounds containing a thiazole nucleus [3,4] a number of β-dialkylaminoethyl ethers of some 2-hydroxythiazoles were prepared.

The new compounds were smoothly obtained by the reaction of the appropriate dialkylaminoethyl chloride with the sodium salt of a 2-hydroxythiazole. Two of the 2-hydroxythiazoles used as starting materials, 2-hydroxy-4-methylthiazole and 2-hydroxy-4-phenylthiazole, are known in the literature. A new member of this class, 2-hydroxy-4,5-diphenylthiazole, was synthesised in analogous manners, *i.e.* by the reaction of xanthogenamide with desyl chloride or by rearrangement of desyl thiocyanate.



The new aminoalkyl ethers possessed a very weak antihistaminic and antispasmodic action. The diphenyl derivatives showed good local anesthetic activity but were strong irritants.

EXPERIMENTAL

2-Hydroxythiazoles

2-Hydroxy-4-methylthiazole was prepared by rearrangement of thiocyanoacetone according to Hantzsch [5].

2-Hydroxy-4-phenylthiazole was prepared from xanthogenamide and phenacyl bromide [6] or by rearrangement of phenacyl thiocyanate [7]. Both methods yielded a product melting at 210–211° (from chloroform), which is somewhat higher than the m.p. reported in the literature (204°).

2-Hydroxy-4,5-diphenylthiazole. Method A. Xanthogenamide (10.5 g, 0.1 mole) and desyl chloride (23 g, 0.1 mole) were thoroughly mixed and heated on the waterbath for 30 minutes. The mixture melted at first and, after a few minutes began to crystallise. After cooling, the reaction product was triturated with cold ethanol (100 ml) and collected. The crude product (18.2 g, 72 %) melted at 255—256°. Recrystallisation from ethanol did not raise the m.p. (Found: C 71.3; H 4.48; N 5.56; S 12.4. Calc. for $C_{15}H_{11}NOS$ (253.3): C 71.2; H 4.38; N 5.53; S 12.7 %).

Method B. A mixture of desyl thiocyanate[8] (1.5 g), glacial acetic acid (8 ml) and conc. sulphuric acid (0.15 ml) was refluxed for 2 hours. After cooling, the reaction product crystallised from the acid mixture. Water (50 ml) was added to the mixture and the crystals (1.3 g, 87 %) were collected. M.p. 255—256° after recrystallisation from ethanol, undepressed on admixture with the compound prepared by method A above.

2-Chloro-4,5-diphenylthiazole. The hydroxy group in the preceding thiazole was easily replaced by chlorine. The hydroxy compound (2.5 g) was refluxed with phosphorus oxychloride (15 ml) for four hours. After cooling, the mixture was cautiously poured into ice water. The oil which was precipitated crystallised rapidly. The crude product (2.65 g, 97 %) was recrystallised from 50 % acetone; m.p. 73—74°. (Found: C 66.3; H 3.99; Cl 13.2. Calc. for $C_{15}H_{10}ClNS$ (271.8): C 66.3; H 3.71; Cl 13.0 %.)

2-Aminoethoxythiazoles

The compounds described below were all prepared in essentially the same way. As an example, the preparation of 2-(β-diethylaminoethoxy)-4-methylthiazole is given.

2-(β-Diethylaminoethoxy)-4-methylthiazole. 2-Hydroxy-4-methylthiazole (1.5 g, 0.013 mole) was added to a solution of sodium (0.3 g, 0.013 mole) in a mixture of ethanol (10 ml) and toluene (40 ml). The ethanol was removed by distillation until the temperature reached 110°. A solution of β-diethylaminoethyl chloride in toluene, prepared from β-diethylaminoethyl chloride hydrochloride (3.45 g, 0.02 mole) according to the directions given by Cheney, Smith and Binkley[2] for the dimethyl analogue, was added with stirring, and the mixture was refluxed for 4 hours. After cooling, the reaction mixture was filtered and the filtrate extracted with *N* hydrochloric acid. The extract was made alkaline with sodium carbonate solution and the oily base was extracted with ether. By addition of ethanolic picric acid to the extract the *picrate* of the base was isolated (2.55 g, 44 %); m.p. 173—175° (from methanol). (Found: N 16.0. Calc. for $C_{10}H_{18}N_2OS \cdot C_6H_3N_3O_7$ (443.4): N 15.8 %.)

The picrate (2.0 g) was suspended in a mixture of water (65 ml), 5 *N* sodium hydroxide (25 ml) and chloroform (25 ml) and stirred on the water bath for one hour. The chloroform layer was separated and dried and the solvent was evaporated. The residue was distilled *in vacuo* giving a colourless oil (0.8 g) boiling at 150° (bath temperature) at 0.1 mm. (Found: C 56.3; H 8.61; N 13.2. Calc. for $C_{10}H_{18}N_2OS$ (214.3): C 56.0; H 8.46; N 13.1 %.)

2-(β-Piperidinoethoxy)-4-methylthiazole oxalate. This compound was prepared similarly in 58 % yield from 2-hydroxy-4-methylthiazole and β-piperidinoethyl chloride. The oily base was isolated as the oxalate; m.p. 224—225° (decomp., from acetone). (Found: C 49.9; H 6.17; N 8.76. Calc. for $C_{11}H_{18}N_2OS \cdot H_2C_2O_4$ (316.4): C 49.3; H 6.37; N 8.86 %.)

2-(β-Dimethylaminoethoxy)-4-phenylthiazole oxalate. Prepared in 67 % yield from β-dimethylaminoethyl chloride and 2-hydroxy-4-phenylthiazole. M.p. 187—188° (de-

comp.) after recrystallisation from acetone. (Found: C 53.2; H 5.15; N 8.11. Calc. for $C_{13}H_{16}N_2OS \cdot H_2C_2O_4$ (338.4): C 53.3; H 5.36; N 8.28 %.)

2-(β-Diethylaminoethoxy)-4-phenylthiazole oxalate. Prepared in 86 % yield; m.p. 135—136° (decomp., from acetone). (Found: C 55.9; H 6.03; N 7.81. Calc. for $C_{15}H_{20}N_2OS \cdot H_2C_2O_4$ (366.4): C 55.7; H 6.05; N 7.65 %.)

2-(β-Piperidinoethoxy)-4-phenylthiazole oxalate. Prepared in 61 % yield; m.p. 185—187° (decomp., from acetone). (Found: C 57.0; H 5.75; N 7.46. Calc. for $C_{16}H_{20}N_2OS \cdot H_2C_2O_4$ (378.4): C 57.1; H 5.85; N 7.41 %.)

2-(β-Dimethylaminoethoxy)-4,5-diphenylthiazole. Prepared in 60 % yield from β-dimethylaminoethyl chloride and 2-hydroxy-4,5-diphenylthiazole. The solid base was recrystallised from light petroleum; m.p. 104—105°. (Found: C 70.4; H 6.44; N 8.84. Calc. for $C_{19}H_{20}N_2OS$ (324.4): C 70.4; H 6.22; N 8.64 %.)

2-(β-Diethylaminoethoxy)-4,5-diphenylthiazole. Prepared in 50 % yield; m.p. 88—89° (from light petroleum). (Found: C 72.0; H 7.04; N 7.97. Calc. for $C_{21}H_{24}N_2OS$ (352.5): C 71.6; H 6.86; N 7.95 %.)

2-(β-Piperidinoethoxy)-4,5-diphenylthiazole hydrochloride. Prepared in 52 % yield; m.p. 239—241° (from acetone). (Found: C 65.5; H 6.26; N 6.90. Calc. for $C_{22}H_{24}N_2OS \cdot HCl$ (401.0): C 65.9; H 6.28; N 6.99 %.)

SUMMARY

A number of β-dialkylaminoethyl ethers of 2-hydroxythiazoles have been prepared and tested for pharmacological activity.

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The Molecular Weight Distribution in Polymethyl Methacrylate Prepared by Redox Polymerization in Water Phase

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The present study was initiated to investigate possible changes in the molecular weight distribution by redox polymerization of methyl methacrylate. Earlier investigations have indicated that the molecular weight composition of polymethyl methacrylate undergoes considerable changes by polymerization in a pure phase (block polymerization) [1–5]. Thus it has been shown that during the first so-called stationary stage of the polymerization when the net rate of polymerization is constant, molecules with relatively low molecular weights are formed. During the next stage of the reaction — the so-called explosive stage — the net rate of polymerization is multiplied several times while at the same time polymer molecules with higher molecular weights are formed. Herewith the molecular weight distribution is considerably broadened. This increase in the average molecular weight then continues during the whole remainder of the process. The marked change in the particle size during the course of the reaction has been connected with the changes that take place in the reaction medium, above all its increasing viscosity [2,4,6]. Therefore, it was considered of interest to study another type of polymerization in which the conditions of the reaction do not undergo equally great changes. The polymerization of methyl methacrylate in aqueous solution with the redox system $FeSO_4$—H_2O_2 as the initiator was chosen as the new type. The degree of reproducibility that can be achieved by fractionated precipitation and viscometric determination of the molecular weight distribution in a high polymer has in this connection been the object of an investigation.

DERIVATION OF THE DISTRIBUTION FUNCTION FROM THE VELOCITY CONSTANTS

I. *Deactivation by the combination of two radicals.* The mechanism of reaction when methyl methacrylate is polymerized in aqueous solution in the

presence of the redox system $FeSO_4$—H_2O_2 has been thoroughly studied by Evans *et al.* — According to them the course of the reaction can be depicted by the following reaction scheme.

H_2O_2	$+ Fe^{2+}$	$\rightarrow$	$Fe^{3+} + OH + OH^-$	k_1	(I)
OH	$+ Fe^{2+}$	$\rightarrow$	$Fe^{3+} + OH^-$	k_2	(II)
M	$+ OH$	$\rightarrow$	A_1	k_i	(III)
A_1	$+ M$	$\rightarrow$	A_2	k_p	(IV)
A_{r-1}	$+ M$	$\rightarrow$	A_r		
A_r	$+ A_s$	$\rightarrow$	P_{r+s}	k_t	(V)
A_r	$+ OH$	$\rightarrow$	P_r	k_t'	(VI)

Here M represents a molecule of methyl methacrylate, A_1, an activated monomer molecule, A_r a growing polymer consisting of r monomeric units, and P_r a deactivated polymer with the degree of polymerization r. In the presence of a large excess of the monomer as compared to the concentration of $FeSO_4$ and H_2O_2, reaction II can be neglected relative to reaction III. According to Evans it is probable that the constants k_p and k_t are independent of the degree of polymerization. He also assumes that the deactivation of growing molecules occurs solely by combination of two active molecules (reaction V). Concerning the question of the correct termination mechanism, see also references [5] and [10]. In the following derivation, Evans' treatment will be followed after a few errors in his calculations have been corrected [8]. A somewhat more general treatment of the reaction equations can be found in Gee and Melville [11].

Reactions I, III, IV, and V give the following differential equations:

$$\frac{d(OH)}{dt} = k_1 (H_2O_2)(Fe^{2+}) - k_i (M)(OH)$$

$$\frac{d(A_1)}{dt} = k_i (M)(OH) - k_p (M)(A_1) - k_t (A_1) \sum_{n=1}^{\infty} (A_n)$$

$$\frac{d(A_2)}{dt} = k_p (M)(A_1) - k_p (M)(A_2) - k_t (A_2) \sum_{n=1}^{\infty} (A_n)$$

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$$\frac{d(A_r)}{dt} = k_p (M)(A_{r-1}) - k_p (M)(A_r) - k_t(A_r) \sum_{n=1}^{\infty} (A_n)$$

If Bodenstein's condition for the steady state [12, 13, 11, 8] is applied to the concentrations of the radicals (OH), (A_1), (A_2) and (A_r), the following equations are obtained:

$$k_i(\mathrm{M})\ (\mathrm{OH}) = k_1\ (\mathrm{Fe^{2+}})\ (\mathrm{H_2O_2}) \tag{1'}$$

$$k_i\ (\mathrm{M})\ (\mathrm{OH}) = k_p\ (\mathrm{M})\ (\mathrm{A_1}) + k_t\ (\mathrm{A_1}) \sum_{n=1}^{\infty} (\mathrm{A}_n) = \\ = (\mathrm{A_1}) \left[k_p\ (\mathrm{M}) + k_t \sum_{n=1}^{\infty} (\mathrm{A}_n) \right] \tag{2'}$$

$$k_p\ (\mathrm{M})\ (\mathrm{A_1}) = k_p\ (\mathrm{M})\ (\mathrm{A_2}) + k_t\ (\mathrm{A_2}) \sum_{n=1}^{\infty} (\mathrm{A}_n) = \\ = (\mathrm{A_2}) \left[k_p\ (\mathrm{M}) + k_t \sum_{n=1}^{\infty} (\mathrm{A}_n) \right] \tag{3'}$$

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$$k_p\ (\mathrm{M})\ (\mathrm{A}_{r-1}) = k_p\ (\mathrm{M})\ (\mathrm{A}_r) + k_t\ (\mathrm{A}_r) \sum_{n=1}^{\infty} (\mathrm{A}_n) = \\ = (\mathrm{A}_r) \left[k_p\ (\mathrm{M}) + k_t \sum_{n=1}^{\infty} (\mathrm{A}_n) \right] \tag{4'} \ldots\ (r+1')$$

Equation (1′) gives immediately by rearrangement an expression for the concentration of the OH-radical

$$(\mathrm{OH}) = \frac{k_1}{k_i} \cdot \frac{(\mathrm{Fe^{2+}})\ (\mathrm{H_2O_2})}{(\mathrm{M})} \tag{5}$$

By multiplication of equations (2′), (3′) . . $(r + 1')$, one obtains the expression

$$\frac{k_i}{k_p}\ (\mathrm{OH}) \left[k_p\ (\mathrm{M}) \right]^r = (\mathrm{A}_r) \left[k_p\ (\mathrm{M}) + k_t \sum_{n=1}^{\infty} (\mathrm{A}_n) \right]^r \tag{6}$$

$$(\mathrm{A}_r) = \frac{k_i(\mathrm{OH})}{k_p} \left[1 + \frac{k_t}{k_p} \frac{\sum_{n=1}^{\infty} (\mathrm{A}_n)}{(\mathrm{M})} \right]^{-r} \tag{7}$$

The total concentration $\sum_{n=1}^{\infty} (\mathrm{A}_r)$ of the growing radicals thus becomes

$$\sum_{r=1}^{\infty} (\mathrm{A}_r) = \frac{k_i\ (\mathrm{OH})}{k_p} \sum_{r=1}^{\infty} \left[1 + \frac{k_t}{k_p} \frac{\sum_{n=1}^{\infty} (\mathrm{A}_n)}{(\mathrm{M})} \right]^{-r} \tag{8}$$

or after summation and rearrangement

$$\sum_{r=1}^{\infty} (\mathrm{A}_r) = \sqrt{\frac{k_i}{k_t}\ (\mathrm{OH})\ (\mathrm{M})} \tag{9}$$

To be able to express the concentrations of the radicals (OH), (A_r), and $\sum_{r=1}^{\infty} (A_r)$ as functions of the concentration of the monomer (M), one must find a relation between the concentrations of (Fe^{2+}) and (H_2O_2) and the concentration of the monomer (M). The concentrations of (Fe^{2+}) and (H_2O_2) at an arbitrary time t is determined by the differential equation

$$-\frac{d(H_2O_2)}{dt} = k_1 (H_2O_2) (Fe^{2+}) \tag{10}$$

To simplify the mathematical analysis, it is limited to the case when the concentration of $(H_2O_2) = (Fe^{2+}) = a_0$ and $t = 0$. By integration of equation (10) one then obtains

$$(H_2O_2) = (Fe^{2+}) = \frac{a_0}{1 + a_0 k_1 t} \tag{11}$$

The net rate of polymerization is determined by the differential equation

$$-\frac{d(M)}{dt} = k_p (M) \sum_{n=1}^{\infty} (A_n) * \tag{12}$$

which, by combination of equations (5), (9), and (11), gives the expression

$$-\frac{d (M)}{dt} = k_p \sqrt{\frac{k_1}{k_t}} (M) \frac{a_0}{1 + a_0 k_1 t} \tag{13}$$

By integration of equation (13) one obtains

$$\ln \frac{(M)_0}{(M)} = \frac{k_p}{\sqrt{k_1 k_t}} \ln (1 + a_0 k_1 t) \tag{14}$$

where $(M)_0$ signifies the concentration of the monomer at time $t = 0$, or after rearranging

$$(1 + a_0 k_1 t) = \left[\frac{(M)_0}{(M)}\right]^{\beta} \tag{15}$$

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* The correct expression is

$$-\frac{d(M)}{dt} = k_p (M) \sum_{n=1}^{\infty} (A_n) + k_i (M) (OH)$$

or

$$-\frac{d(M)}{dt} = k_p \sqrt{\frac{k_i}{k_t}} (M) \frac{a_0}{1 + a_0 k_1 t} + k_i \frac{a_0^2}{(1 + a_0 k_1 t)^2}$$

where the last term can be neglected compared to the first.

where

$$\beta = \frac{\sqrt{k_1 k_t}}{k_p} \tag{16}$$

Equations (5), (11), and (15) give

$$(\mathrm{OH}) = \frac{k_1 a_0^2}{k_t\,(\mathrm{M})} \left[\frac{(\mathrm{M})}{(\mathrm{M})_0}\right]^{2\beta} \tag{17}$$

If this value is introduced into equation (9), one obtains

$$\sum_{n=1}^{\infty} (\mathrm{A}_n) = a_0 \sqrt{\frac{k_1}{k_t}} \left[\frac{(\mathrm{M})}{(\mathrm{M})_0}\right]^{\beta} \tag{18}$$

The rate of formation of the polymer molecules with the degree of polymerization r is

$$\frac{\mathrm{d}(\mathrm{P}_r)}{\mathrm{d}t} = \tfrac{1}{2}\, k_t \sum_{s=1}^{r-1} (\mathrm{A}_{r-s})\,(\mathrm{A}_s) \tag{19}$$

Equation (7) and (19) give

$$\frac{\mathrm{d}(\mathrm{P}_r)}{\mathrm{d}t} = \tfrac{1}{2}\, k_t \sum_{s=1}^{r-1} \left[\frac{k_i\,(\mathrm{OH})}{k_p}\right]^2 \left[1 + \frac{k_t}{k_p} \frac{\sum_{n=1}^{\infty} (\mathrm{A}_n)}{(\mathrm{M})}\right]^{-r} \tag{20}$$

or after summation

$$\frac{\mathrm{d}(\mathrm{P}_r)}{\mathrm{d}t} = \tfrac{1}{2}\, k_t \left[\frac{k_i\,(\mathrm{OH})}{k_p}\right]^2 (r-1) \left[1 + \frac{k_t}{k_p} \frac{\sum_{n=1}^{\infty} (\mathrm{A}_n)}{(\mathrm{M})}\right]^{-r} \tag{21}$$

in which expression one can replace (OH) and $\sum_{n=1}^{\infty} (\mathrm{A}_n)$ with functions of (M).

$$\frac{\mathrm{d}(\mathrm{P}_r)}{\mathrm{d}t} = \tfrac{1}{2} \frac{\beta^2 a_0^4\, k_1\, (r-1)}{(\mathrm{M})_0^2} \left[\frac{(\mathrm{M})}{(\mathrm{M})_0}\right]^{(4\beta-2)} \left\{1 + \frac{\beta a_0}{(\mathrm{M})_0} \left[\frac{(\mathrm{M})}{(\mathrm{M})_0}\right]^{(\beta-1)}\right\}^{-r} \tag{22}$$

Equation (13) and (15) give

$$-\frac{\mathrm{d}(\mathrm{M})}{\mathrm{d}t} = \frac{k_1 a_0\,(\mathrm{M})_0}{\beta} \left[\frac{(\mathrm{M})}{(\mathrm{M})_0}\right]^{(\beta+1)} \tag{23}$$

After division of equation (22) by equation (23), one obtains

$$-\frac{\mathrm{d}(\mathrm{P}_r)}{\mathrm{d}(\mathrm{M})} = \tfrac{1}{2} \frac{\beta^3 a_0^3}{(\mathrm{M})_0^3} (r-1) \left[\frac{(\mathrm{M})}{(\mathrm{M})_0}\right]^{3(\beta-1)} \left\{1 + \frac{\beta a_0}{(\mathrm{M})_0} \left[\frac{(\mathrm{M})}{(\mathrm{M})_0}\right]^{(\beta-1)}\right\}^{-r} \tag{24}$$

Thus, after integration, the following expression for the concentration of the polymer of the degree of polymerization r is obtained.

$$(P_r) = -\frac{a_0^3\beta^3\ (r-1)}{2(M)_0^2} \int_{\gamma=1}^{\gamma=\gamma'} \gamma^{3(\beta-1)} \left[1 + \frac{\beta a_0}{(M)_0}\gamma^{(\beta-1)}\right]^{-r} d\gamma \qquad (25)$$

where $\gamma = \dfrac{(M)}{(M)_0}$

This expression gives the concentration (P_r) of the polymer of the degree of polymerization r in moles/l as a function of the degree of polymerization r and the amount of remaining monomer γ'. However, it is more convenient to express the concentration in base moles/l, which is obtained by the multiplication of equation (25) by r.

$$r\ (P_r) = -\frac{a_0^3\beta^3\ r\ (r-1)}{2\ (M)_0^2} \int_{\gamma=1}^{\gamma=\gamma'} \gamma^{3(\beta-1)} \left[1 + \frac{\beta a_0}{(M)_0}\gamma^{(\beta-1)}\right]^{-r} d\gamma \qquad (26)$$

This is simply the mass frequency function * which involves only experimentally determined quantities [7-9].

II. *Deactivation by disproportionation between two radicals.* In the previous analysis it was assumed that the deactivation occurred by the combination of two growing radicals. There is, however, no definite reason to assume this to be the termination reaction. An equally probable reaction is deactivation of the radicals by disproportionation, *i. e.*

$$A_r + A_s \rightarrow P_r + P_s \qquad k'' \text{ (VII)}$$

Instead of equation (19) one gets

$$\frac{d(P_r)}{dt} = k_t'' (A_r) \sum_{n=1}^{\infty} (A_n) \qquad (27)$$

If this expression is divided by

$$-\frac{d(M)}{dt} = k_p' (M) \sum_{n=1}^{\infty} (A_n)$$

* In the continued development of this expression, Evans makes the following three errors. Firstly, he integrates from $\gamma = 0$ to $\gamma = \gamma'$ instead of from $\gamma = 1$ to $\gamma = \gamma'$. Secondly, he puts $\beta = 1.67$, whereas the correct expression should be $\beta = \frac{1}{1.67}$ for the conditions of the reaction that Evans gives. Thirdly, he puts $\gamma =$ the degree of conversion, while this ought to be $(1-\gamma)$.

one obtains

$$-\frac{d(P_r)}{d(M)} = \frac{k_t''}{k_p}\frac{(A_r)}{(M)} \tag{28}$$

By combination of this expression with equations (7), (17), and (18), one obtains

$$\frac{d(P_r)}{d(M)} = -\frac{\beta^2 a_0^2}{(M)_0^2}\gamma^{2(\beta-1)}\left[1+\frac{\beta a_0}{(M)_0}\gamma^{(\beta-1)}\right]^{-r} \tag{29}$$

where $\gamma = \frac{(M)}{(M)_0}$

After integration and multiplication with r one obtains

$$r\,(P_r) = -\frac{r\,\beta^2 a_0^2}{(M)_0}\int_{\gamma=1}^{\gamma=\gamma'}\gamma^{2(\beta-1)}\left[1+\frac{\beta a_0}{(M)_0}\gamma^{(\beta-1)}\right]^{-r}d\gamma \tag{30}$$

which expression gives the mass frequency function in the case of deactivation by disproportionation.

Table 1. The theoretical variation of the frequency function

$$\frac{r\,(P_r)}{(1-\gamma')\,(M)_0} = -\frac{\beta^3 a_0^3 r(r-1)}{(1-\gamma')\cdot 2\cdot (M)_0^3}\int_{\gamma=1}^{\gamma=\gamma'}\gamma^{3(\beta-1)}\left[1+\frac{\beta a_0}{(M)_0}\gamma^{(\beta-1)}\right]^{-r}d\gamma$$

for different degrees of polymerization r and different values of γ' (= the fraction of the monomer that is still not polymerized.) In the calculation, the following values have been used $(M)_0 = 0.175$ *mole/l,* $a_0 = 10^{-4}$ *mole/l, and* $\beta^{-1} = 1.67$.

$r \cdot 10^{-3}$	$\frac{r\,(P_r)}{(1-\gamma')\,(M)_0}\cdot 10^3$		
	$\gamma' = 0.7$	$\gamma' = 0.4$	$\gamma' = 0.1$
1	0.0170	0.0220	0.0369
2	0.0474	0.0588	0.0851
3	0.0741	0.0880	0.1122
4	0.0908	0.1035	0.1200
5	0.0976	0.1067	0.1142
6	0.0981	0.1034	0.1027
7	0.0932	0.0941	0.0874
8	0.0838	0.0820	0.0725
9	0.0736	0.0698	0.0589
10	0.0633	0.0579	0.0472
12	0.0440	0.0379	0.0292
15	0.0228	0.0182	0.0133
20	0.0065	0.0047	0.0033

Table 2. The theoretical variation of the frequency function

$$\frac{r\ (P_r)}{(1-\gamma')\ (M)_0} = -\frac{\beta^2 a_0^2 r}{(1-\gamma')\ (M)_0^2} \int_{\gamma=1}^{\gamma=\gamma'} \gamma^{2(\beta-1)} \left[1 + \frac{\beta a_0}{(M)_0} \gamma^{(\beta-1)}\right]^{-r} d\gamma$$

for different degrees of polymerisation r and different values of γ' (= the fraction of the monomer that is still not polymerized). In the calculation, the following values have been used $(M)_0 = 0.175$ *mole/l,* $a_0 = 10^{-4}$ *mole/l, and* $\beta^{-1} = 1.67$.

$r \cdot 10^{-3}$	$\frac{r(P_r)}{(1-\gamma')\,(M)_0} \cdot 10^3$		
	$\gamma' = 0.7$	$\gamma' = 0.4$	$\gamma' = 0.1$
0.5	0.0552	0.0663	0.1034
1	0.0941	0.1095	0.1427
1.5	0.1188	0.1346	0.1651
2	0.1303	0.1452	0.1704
3	0.1370	0.1464	0.1561
4	0.1238	0.1280	0.1270
5	0.1067	0.1064	0.0982
6	0.0892	0.0862	0.0762
7	0.0735	0.0680	0.0575
8	0.0588	0.0521	0.0424
9	0.0450	0.0392	0.0309
10	0.0342	0.0291	0.0224
12	0.0201	0.0162	0.0119
15	0.0083	0.0062	0.0044
20	0.0018	0.0012	0.0009
25	0.0004	0.0002	0.0002

III. *Numerical calculation of the theoretical frequency functions and the mass distributions by graphical integration.* To be able to compare the frequencies given by equations (26) and (30) for different values of the constants $(M)_0$, β, and γ', the frequency functions must first be normalized by referring them to the same total amount of polymer, *i. e.* they must be divided by $[(M)_0 - (M)] = (1-\gamma')\ (M)_0$.

The normalized frequency functions have been calculated by graphical integration for the following cases:

1. $(M)_0 = 0.175$ mole/l $a_0 = 10^{-4}$ mole/l, and $\beta^{-1} = 1.67$
2. $(M)_0 = 0.170$ » $a_0 = 10^{-4}$ » » $\beta^{-1} = 0.64$
3. $(M)_0 = 0.170$ » $a_0 = 10^{-4}$ » » $\beta^{-1} = 0.71$

Table 3. The theoretical variation of the frequency function

$$\frac{r\ (P_r)}{(1-\gamma')\ (M)_0} = -\frac{\beta^3 a_0^3 r(r-1)}{(1-\gamma')\cdot 2\cdot (M)_0^3}\int_{\gamma=1}^{\gamma=\gamma'} \gamma^{3(\beta-1)}\left[1+\frac{\beta a_0}{(M)_0}\gamma^{(\beta-1)}\right]^{-1} d\gamma$$

for different degrees of polymerization r at different values of γ' (= the fraction of the monomer that is still not polymerized) and of β. In the calculation, the following values have been used $(M)_0 = 0.170$ mole/l, and $a_0 = 10^{-4}$ mole/l.

$r \cdot 10^{-3}$	$\frac{r(P_r)}{(1-\gamma')\,(M)_0} \cdot 10^3$			
	$\gamma' = 0.575$ $\beta^{-1} = 0.64$	$\gamma' = 0.31$ $\beta^{-1} = 0.64$	$\gamma' = 0.575$ $\beta^{-1} = 0.71$	$\gamma' = 0.31$ $\beta^{-1} = 0.71$
1	0.1164	0.0952	0.0997	0.0836
2	0.2094	0.1803	0.1876	0.1638
3	0.2064	0.1902	0.1981	0.1832
4	0.1653	0.1638	0.1670	0.1626
5	0.1153	0.1248	0.1234	0.1274
6	0.0766	0.0904	0.0838	0.0924
7	0.0469	0.0618	0.0549	0.0650
8	0.0275	0.0408	0.0342	0.0436
9	0.0161	0.0276	0.0206	0.0285
10	0.0095	0.0182	0.0124	0.0186
12	0.0028	0.0075	0.0040	0.0075
15	0.0004	0.0019	0.0007	0.0017
20	—	0.0002	—	0.0002

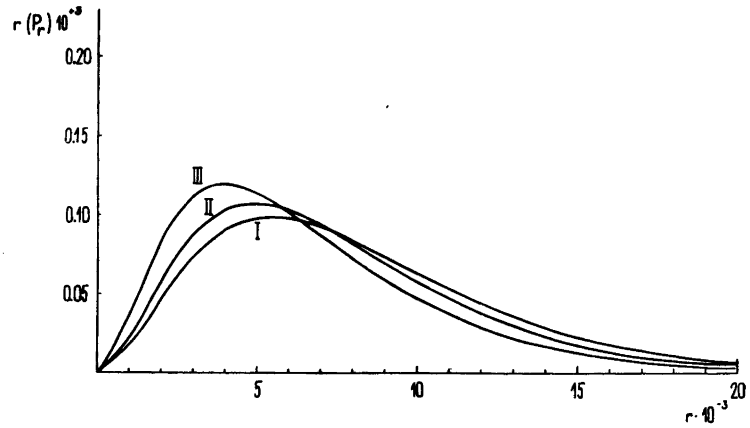

Fig. 1. Mass frequency curve for deactivation of the growing radicals by combination. $(M)_0 = 0.175$ mole/l, $a_0 = 10^{-4}$ mole/l, and $\beta^{-1} = 1.67$.

Curve I $\gamma' = 0.7$. Curve II $\gamma' = 0.4$. Curve III $\gamma' = 0.1$.

Table 4. The theoretical values of the frequency function

$$\frac{r\,(P_r)}{(1-\gamma')\,(M)_0} = -\frac{\beta a_0^2 r}{(1-\gamma')(M)_0^2}\int_{\gamma=1}^{\gamma=\gamma'} \gamma^{2(r-1)}\left[1+\frac{\beta a_0}{(M)_0}\gamma^{(\beta-1)}\right]^{-r} d\gamma$$

for different degrees of polymerization r and different values of γ' (= the fraction of the monomer that is still not polymerized). In the calculation, the following values have been used $(M)_0 = 0.170$ *mole/l,* $a_0 = 10^{-4}$ *mole/l, and* $\beta^{-1} = 0.64$.

$r \cdot 10^{-3}$	$\frac{r(P_r)}{(1-\gamma')\,(M)_0} \cdot 10^3$	
	$\gamma' = 0.575$	$\gamma' = 0.31$
0.5	0.2177	0.1806
1	0.2916	0.2498
1.5	0.2919	0.2580
2	0.2600	0.2382
3	0.1747	0.1730
4	0.1034	0.1125
5	0.0600	0.0721
6	0.0323	0.0438
7	0.0171	0.0263
8	0.0089	0.0156
9	0.0049	0.0097
10	0.0025	0.0058
12	0.0007	0.0020
15	0.0002	0.0011

corresponding to the conditions under which Evans, respectively the author have carried out their experiments. The result of this integration is given in Tables 1—4, where the amount of polymer $\frac{r\,(P_r)}{(1-\gamma')\,(M)_0}$ of a certain degree of polymerization (r) is given as a function of the degree of polymerization and the amount of remaining monomer γ'. Figures 1 and 2 graphically illustrate the functions obtained in case 1) for three different values of γ', and for the deactivation of the radicals by combination, respectively disproportionation. By an additional integration of the frequency curves, the mass distributions in Figs. 6—8 have been obtained. From Figs. 1—2 and Tables 1—4 it is evident that the average molecular weight decreases with the degree of conversion $(1-\gamma')$ for $\beta^{-1} = 1.67$, while it grows for $\beta^{-1} = 0.64$ and $\beta^{-1} = 0.71$ for disproportionation as well as combination. The distributions will be discussed further in relation to the experimentally determined curves.

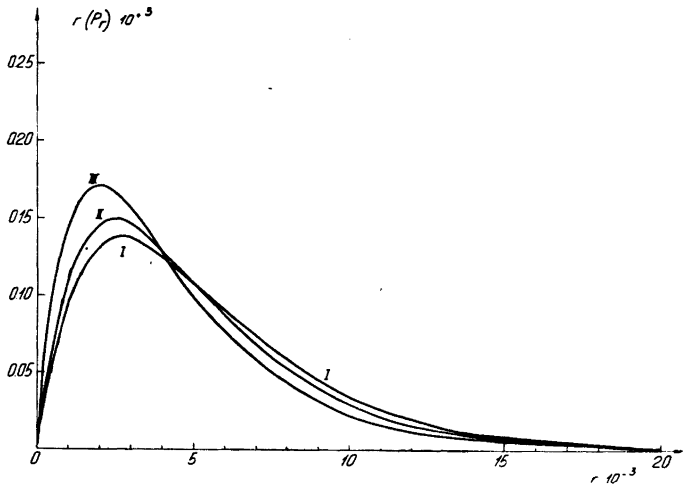

Fig. 2. Mass frequency curve for deactivation of the growing radicals by disproportionation $(M)_0 = 0.175$ *mole/l,* $a_0 = 10^{-4}$ *and* $\beta^{-1} = 1.67$.

Curve I $\gamma' = 0.7$.
» *II* $\gamma' = 0.4$.
» *III* $\gamma' = 0.1$.

EXPERIMENTAL

Preparation of polymethyl methacrylate. To prepare the polymerized products used in determining the molecular weight distribution, essentially the same method as that of Evans *et al.*[7] was employed. The reaction vessel used is shown in Fig. 3. The polymerization was carried out in N_2-atmosphere. First the required amounts of H_2O, $FeSO_4$, H_2SO_4, and cetyl trimethylammonium bromide as the emulsifying medium was introduced into the vessel, after which all O_2 was expelled by leading in O_2-free N_2-gas at E with the stopcocks A, D, and C open and B closed. The O_2-free monomer was added through the funnel D. After dissolving the monomer and expelling possibly remaining O_2 by a continued stream of N_2; the O_2-free H_2O_2 was also introduced through D. After this the stopcocks A, C, and D were closed and B opened. During the entire polymerization N_2-gas was introduced through tube F. This gas could pass out through G via a mercury valve. The solution was kept in constant motion with a stirrer provided with a mercury lock. The polymerization was carried out in a thermostat at a temperature of 25.0° C. In all cases, the concentration of emulsifying agent was 1 % cetyl trimethylammonium bromide based on the amount of monomer. The concentration of H_2SO_4 corresponded to 1 cm^3 N acid in 100 cm^3 H_2O. Concerning the other conditions of the polymerization, see Table 5. The reaction was stopped by bubbling O_2-gas through the flask at the end of the polymerization. The polymer was precipitated by the addition of acetone to the reaction mixture. The precipitate was washed with distilled H_2O in a Büchner funnel and dried *in vacuo* to constant weight.

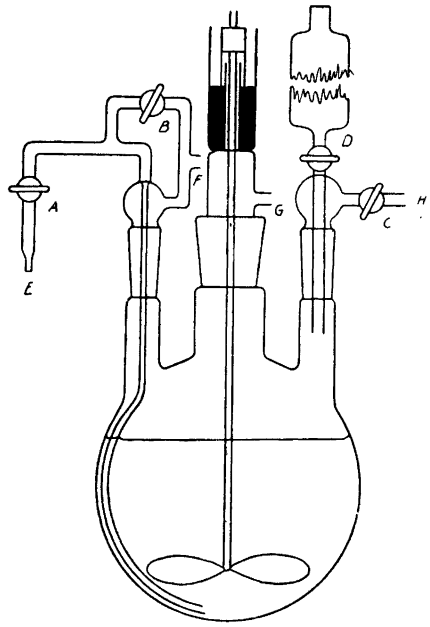

Fig. 3. The reaction vessel for polymerization of methyl methacrylate in N_2-atmosphere.

Fractionation technique. In order to investigate the degree of reproducibility attainable by fractionated precipitation, four different fractionations were carried out on preparations L(5) and L(6) under varying conditions of precipitation, see Table 6.

The precipitation took place in a thermostat. The precipitant was added gradually at 20° C until a turbidity appeared. Then the temperature was raised until the solution was again completely clear after which the temperature was allowed to fall slowly to 20.0° C during the night. The precipitate formed was recovered by decanting the solution and was dissolved in acetone from which it was reprecipitated with an excess of methanol.

Table 5. Experimental data for redox polymerization of methyl methacrylate in water phase.

No. of polymerization	Temp. (°C)	Time of polymerization (min.)	Molarity of methyl methacrylate	Molarity of $FeSO_4$ and H_2O_2	Degree of conversion (%)
L(2)	25	10	0.173	10^{-4}	48.3
L(3)	25	10	0.171	10^{-4}	42.5
L(4)	25	10	0.172	10^{-4}	43.0
L(5)	25	25	0.170	10^{-4}	68.4
L(6)	25	25	0.170	10^{-4}	69.0

Table 6. Conditions of precipitation for fractionation of preparations L(5), L(6), and L(3).

Fractionation	Solvent	Precipitant	Starting concentration g/100 ml
L(5) F(1)	acetone	petroleumether	2
L(5) F(2)	»	»	0.5
L(6) F(1)	benzene	cyclohexane	2
L(6) F(2)	»	»	0.5
L(3) F(1)	»	»	0.5

The fraction obtained was dried *in vacuo* at room temperature until it had reached constant weight. All solutions were recovered and evaporated to dryness. The residue thus obtained has been designated as the last fraction in each series.

Measurement of viscosity. The intrinsic viscosity $[\eta]$ was determined in an Ostwald viscometer. Benzene was used as the solvent. For each fraction, the specific viscosity η_{sp} was determined on solutions of the following concentrations (c) 0.40, 0.30, 0.20, and 0.10 g dry substance per 100 ml solution. The intrinsic viscosity $[\eta]$ was obtained by extrapolation of $\frac{\eta_{sp}}{c}$ to infinite dilution. This was facilitated by the fact that $\frac{\eta_{sp}}{c}$ is a linear function of the concentration c for $c < 0.40$. The molecular weight (M) was calculated from the intrinsic viscosity by the formula [8].

$$(M) = 2.81 \times 10^5 [\eta]^{1.32}$$

RESULTS

The results from the five fractionations have been collected in Tables 7 and 8. In these the weight of each fraction has been recorded and also its intrinsic viscosity and, for most fractions, the average molecular weight calculated from this. From these data the mass distributions of the different preparations have been derived in the usual manner. This calculation is based on the assumption that for each fraction it is true that half of its mass has a lower molecular weight than the average molecular weight determined, while the other half contains molecules with molecular weights greater than the average molecular weight. By summing the masses of all fractions up to a certain fraction and adding to the sum half the weight of this fraction, one obtains the weight of that part of the polymerized product that contains molecules with lower molecular weights than the average molecular weight of the fraction in question.

To estimate the reliability of the experimentally determined mass distribution curves a special study was undertaken. For this preparations L(5) and

L(6) were used. These had been polymerized at different times but under as identical conditions as possible. For the different preparations, different solvent precipitant systems were used. With each preparation two separate fractionations were carried out, in which the initial concentrations were different. The conditions of the precipitations are given in Table 6. Fig. 4 shows the mass distributions obtained by the four fractionations. The intrinsic viscosity $[\eta]$, not the average molecular weight calculated from this, has been used as abscissa in this figure. The solid line is identical in all four cases. The deviations of the points from this line gives an idea of the reliability that can be achieved. The reproducibility must be considered good regarding the errors that can arise in the polymerization itself as well as at the precipitation and the determination of the intrinsic viscosity $[\eta]$ of the different fractions.

Fig. 5 shows the relation between the molecular weight of a fraction and the volume of precipitant required to precipitate the fraction in question. As can

Table 7. Fractionation of polymethyl methacrylate prepared by redox polymerization in water phase under the following conditions: $(M)_0 = 0.170$, $a_0 = 10^{-4}$ *mole/l, temperature of polymerization 25.0° C, time of polymerization 25 min., emulsifying agent 1 % cetyl trimethylammoniumbromide based on the concentration of the monomer. The degree of conversion is 68.4 %, respectively 69.0 % for preparations L(5) and L(6).*

Fraction i	Weight (g)	Weight (%) ω_i	$\frac{1}{2}\omega_i + \sum_{j=1}^{j=i-1} \omega_j$	$[\eta]$benzene	M . 10^{-3}
Preparation L(5)			Fractionation L(5) F(1)		
I	0.1134	1.89	0.95	—	—
II	0.8758	14.63	9.21	1.82	619
III	0.0589	0.98	17.01	1.59	518
IV	0.1541	2.57	18.79	—	—
V	0.3247	5.42	25.54	1.48	472
VI	0.3284	5.49			
VII	0.0575	0.96	31.46	—	—
VIII	0.1932	3.23	33.56	1.37	426
IX	0.1095	1.83	36.09	—	—
X	0.4945	8.26	41.13	1.25	377
XI	0.4099	6.85	48.69	1.09	315
XII	0.1409	2.35	53.29	—	—
XIII	0.8934	14.93	61.93	0.93	255
XIV	0.3551	5.93	72.36	0.79	206
XV	0.5108	8.53	79.59	0.52	119
XVI	0.2522	4.21	85.96	0.29	55
XVII	0.7135	11.92	94.02	—	—

Preparation L(5)			Fractionation L(5) F(2)		
I	0.2400	6.06	3.03	2.15	772
II	0.1467	3.71	7.92	1.96	683
III	0.3080	7.78	13.66	1.79	606
IV	0.3374	8.53	21.82	1.56	505
V	0.2487	6.28	29.22	1.46	463
VI	0.4497	11.36	38.04	1.25	377
VII	0.2126	5.37	46.41	—	—
VIII	0.2728	6.89	52.54	0.96	266
IX	0.3060	7.73	59.85	0.80	209
X	0.3861	9.76	68.59	0.67	166
XI	0.1347	3.40	75.17	0.56	131
XII	0.3184	8.05	80.90	0.46	101
XIII	0.1370	3.46	86.65	0.33	65
XIV	0.4593	11.61	94.19	—	—

Preparation L(6)			Fractionation L(6) F(1)		
I	0.9861	23.68	11.84	1.73	579
II	0.2576	6.17	26.77	1.45	459
III	0.2202	5.29	32.50	1.36	422
IV	0.5867	14.09	42.19	1.22	365
V	0.6316	15.17	56.82	0.99	277
VI	0.8308	19.95	74.38	0.64	156
VII	0.2927	7.03	87.87	0.36	73
VIII	0.3585	8.61	95.69	—	—

Preparation L(6)			Fractionation L(6) F(2)		
I	0.4303	10.75	5.38	2.08	739
II	0.3747	9.36	15.43	1.72	575
III	0.5028	12.56	26.39	1.43	451
IV	0.4604	11.50	38.42	1.30	397
V	0.7504	18.75	53.55	1.00	281
VI	0.7698	19.23	72.54	0.71	179
VII	0.2558	6.39	85.35	—	—
VIII	0.2802	7.00	92.04	0.26	48
IX	0.1783	4.45	97.77	—	—

Table 8. Fractionation of polymethyl methacrylate prepared by redox polymerization in water phase under the following conditions: $(M)_0 = 0.170$ *mole/l,* $a_0 = 10^{-4}$ *mole/l, temperature of polymerization 25.0° C, time of polymerization 10 min., emulsifying agent 1 % cetyl trimethylammonium bromide based on the concentration of the monomer, the degree of conversion is 42.5 %.*

Preparation L(3)			Fractionation L(3) F(1)		
Fraction i	Weight (g)	Weight (%) ω_i	$\frac{1}{2}\omega_i + \sum_{j=1}^{j=i-1} \omega_j$	$[\eta]$ benzene	$M \cdot 10^{-3}$
I	0.4549	15.31	7.65	1.92	665
II	0.2642	8.89	19.75	1.53	493
III	0.4823	16.23	32.31	1.23	369
IV	0.4640	15.62	48.24	0.99	277
V	0.2615	8.80	60.45	0.81	213
VI	0.4147	13.96	71.83	—	—
VII	0.3625	12.20	84.91	0.41	87
VIII	0.2670	8.99		—	—

be expected, the fractions containing the largest molecules are most sensitive to the addition of precipitant, why the resolution is the least for this part. A general experience is also that the first fraction has a tendency to become comparatively large and polydispers.

In Fig. 6, Evans' experimental data are compared with the mass distributions obtained from the frequency functions in equations (26) and (30), when the values of $(M)_0$, a_0, and β, valid for his conditions of polymerization, are introduced. The concentration of cetyl trimethylammonium bromide is here 1 % based on the total weight of the reaction mixture. The upper solid line gives the theoretical mass distribution obtained assuming deactivation of the growing radicals by disproportionation. The lower solid line is the theoretical mass distribution obtained assuming the termination reaction to be a combination of growing radicals. The points in the figure represent Evans' experimental data from three different fractions [8]. The agreement between the experimental and theoretical distributions assuming deactivation by combination is considerably better than Evans claims. From the figure it is also evident that deactivation by disproportionation at least for the lower molecular weights, plays a subordinate role, if any. On the other hand, the deviation from the theoretical distribution is relatively large for higher molecular weights, possibly indicating that long radicals in some way react "abnormally".

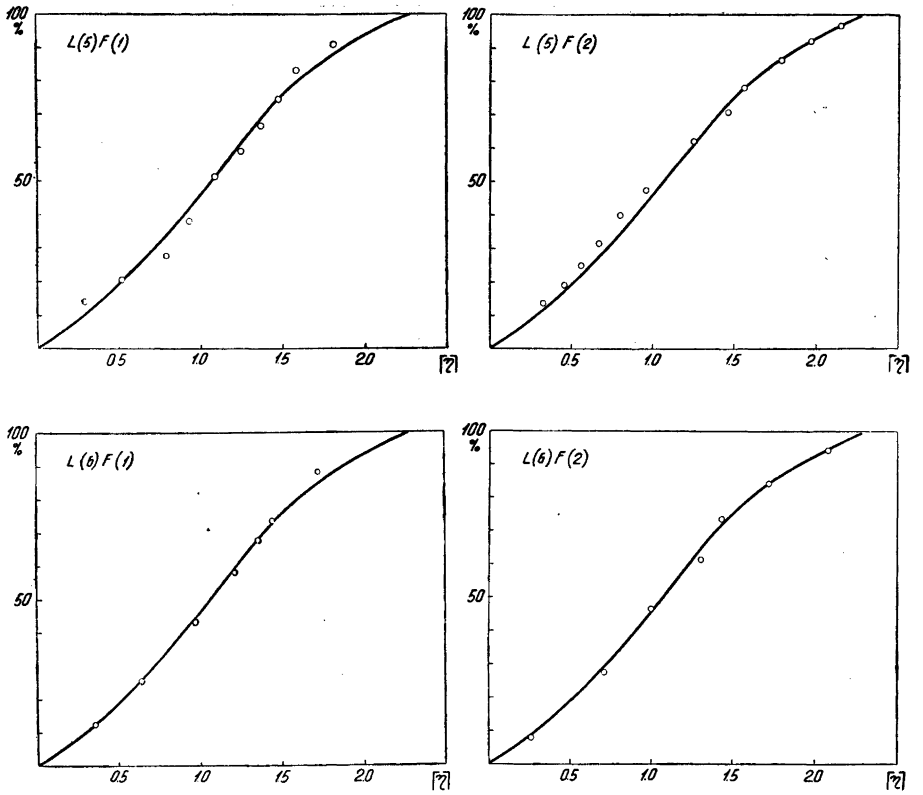

Fig. 4. Mass distribution curves showing the degree of reproducibility achievable by the method employed.

The original purpose of the author's own investigations was only to establish the degree of reproducibility obtainable when determining mass distributions by fractionated precipitation, and to ascertain if the general appearance of the mass distributions undergoes any change during the polymerization. It is, therefore, difficult to compare the experimental and theoretical distributions. As an approximate value of the quantity β, 0.64^{-1}, has been used. The error thus made should not be too large, since the distribution curves are not particularly sensitive to a relatively great change in β; see Figs. 7 and 8, where the theoretical distributions for $\beta = 0.71^{-1}$ are also given. From these figures it is apparent, that the experimental distributions fell approximately in between the theoretical ones obtained for disproportionation and combination, respectively. The experimental distributions have possibly a somewhat erroneous appearance since, owing to the experimental procedure, the lowest

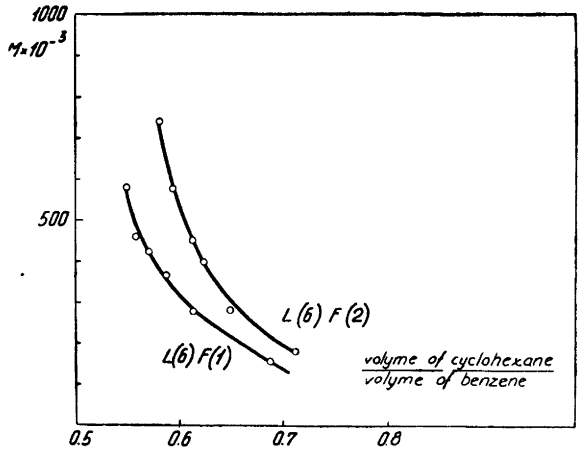

Fig. 5. Precipitation curves for preparation L(6) at two different initial concentrations. See Table 6 concerning the conditions of precipitation.

fraction probably contains considerable amounts of material of high molecular weight, maybe up to about 50 %. It is, however, evident that the deactivation of the active radicals does not take place exclusively by combination at low concentrations of cetyl trimethylammonium bromide. The great effect of a change in the concentration of the emulsifying agent on the rate of polymerization is also notable, see Fig. 9.

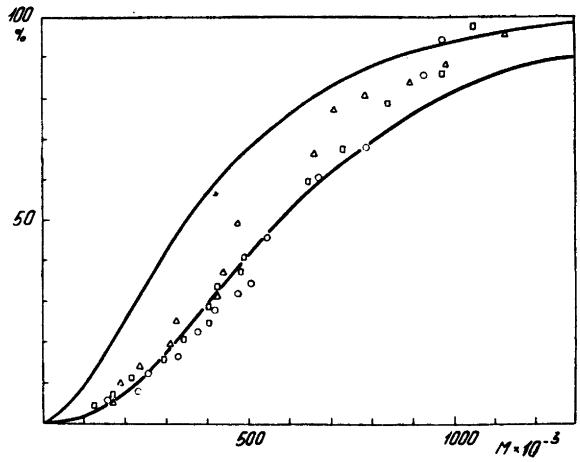

Fig. 6. The mass distribution curve for the case of $(M)_0 = 0.175$ *mole/l,* $a_0 = 10^{-4}$ *mole/l, and* $\beta^{-1} = 1.67$. *The upper solid curve gives the theoretical massdistribution for disproportionation, the lower solid curve the theoretical mass distribution for combination. The points represent Evans' experimental results from three separate fractions.*

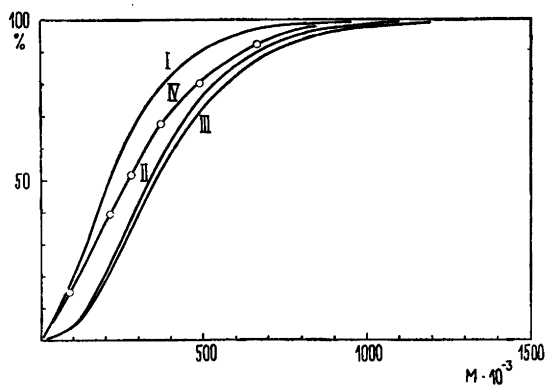

Fig. 7. Mass distribution curves for the case of $(M)_0 = 0.170$ *mole/l,* $a_0 = 10^4$ *mole/l, and* $\gamma_5 = 0.575$.

Curve I. Deactivation by disproportionation $\beta^{-1} = 0.64$.
» *II.* » » *combination* $\beta^{-1} = 0.64$.
» *III.* » » » $\beta^{-1} = 0.71$.
» *IV. Experimentally determined. L(3) F(1).*

It is possible that radicals that can be kept in solution by the amount of emulsifying agent present, are deactivated by combination while the radicals that cannot be kept in solution are deactivated in an abnormal way. If this supposition is correct, deviations from the theoretical distributions should

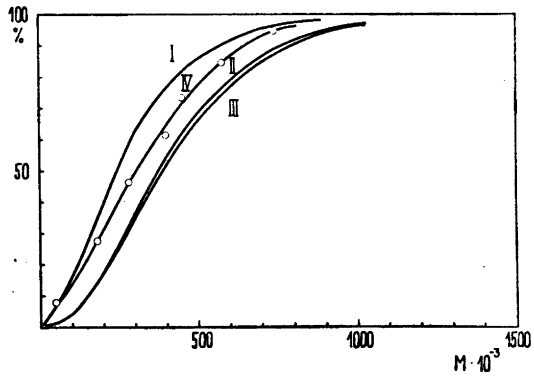

Fig. 8. Mass distribution curves for the case of $(M)_0 = 0.170$ *mole/l,* $a_0 = 10^{-4}$ *mole/l, and* $\gamma' = 0.31$.

Curve I. Deactivation by disproportionation $\beta^{-1} = 0.64$.
» *II.* » » *combination* $\beta^{-1} = 0.64$.
» *III.* » » » $\beta^{-1} = 0.71$.
» *IV. Experimentally determined. L(6) F(2).*

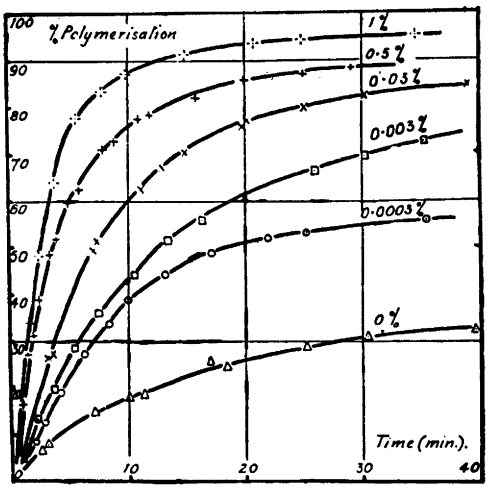

Fig. 9. Effect of varying emulsifying agent concentration on the polymerisation of 0.1 M methyl methacrylate in aqueous solution at 25° C. $a_0 = 10^{-4}$ mole/l (reference 7).

only occur at high molecular weights for high concentrations of emulsifying agent, while a low concentration of emulsifying agent cannot even keep the smaller radicals completely in solution. Finally, when comparing the experimental and theoretical curves, one must remember that the latter are only of an approximate nature, since it is not possible to assume that all radical concentrations are constant. Thus, it can be seen, for example, from equation (19) that according to this assumption the mass distribution cannot change during the polymerization.

SUMMARY

The theoretical mass distributions for the polymerization of methyl metacrylate in water phase in the presence of $FeSO_4$-H_2O_2 as initiators have been derived assuming deactivation by combination and by disproportionation.

The reproducibility in determining mass distributions by precipitation fractionation has been investigated.

The change in the molecular weight composition during the course of the polymerization has been determined and compared to the theoretical distribution at the degrees of conversion in question.

Deactivation occurs, at least mainly, by combination of growing radicals at high concentrations of emulsifying agent, while another termination reaction seems to occur at low concentrations of emulsifying agent or at deactivation of long radicals.

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Cuprous Compounds of Acetylene.

II. Dissolved Compounds in Aqueous Chloride Solutions

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Several solid cuprous compounds can be prepared by passing acetylene into solutions containing cuprous chloride complexes. Dissolved compounds are also formed, as demonstrated by the fact that the solution contains a quantity of acetylene larger than can be attributed to the "physical" solubility. The concentration of such compounds, *e. g.* at 1 atm acetylene pressure, often corresponds to a considerable portion of the copper present.

We have undertaken the investigation of these dissolved compounds for two reasons mainly:

a) probably the dissolved compounds give a somewhat simpler illustration of the chemical relations of acetylene to monovalent copper than do the solid compounds, where complicated lattice forces interfere,

b) to the reactivity of the dissolved acetylene compounds can be traced the catalytic effect of some cuprous salt solutions in the aliphatic polymerization of acetylene (synthesis of monovinylacetylene according to Nieuwland [1]) and in addition reactions as *e. g.* the addition of hydrogen cyanide (preparation of acrylonitrile with Nieuwland's catalyst according to Kurtz [2]).

The solid compounds are of two main types, *viz. addition* compounds where acetylene retains both hydrogen atoms, and *acetylidic* compounds where copper is substituted for the hydrogen atoms. Our own experiments [3] confirm this classification found in earlier literature [1, 4–6], although some substances previously have been placed in the wrong group, due to incomplete analyses. We have tried to find out whether this grouping into addition and acetylidic compounds holds for the dissolved species also. The composition of the dissolved compounds must however be treated as a separate problem, avoiding presumptions of similarities with the solid ones.

Earlier literature gives only scant information on dissolved acetylene-copper compounds.

The first to observe a dissolved compound was Manchot [6] who found reason to suggest the composition C_2H_2CuCl. By absorption measurements on mixtures of cuprous chloride and hydrochloric acid he observed that the maximum amount of acetylene chemically held is 1 mole per mole of cuprous chloride, and concluded that this system represents an equilibrium, displaced towards a complete conversion into C_2H_2CuCl. He seems to believe also in the existence of the dissolved compound $C_2H_2(CuCl)_2$.

Manchot's experiments indicate the existence of a compound with the molar ratio acetylene : copper = n : n. The value of n remains unknown, as does the chlorine content, which latter problem Manchot never discusses. Strictly interpreted the experiments do not even prove that the compound is of addition type.

Also Tzuyrikh and Ginsburg [7] have entered upon the question of dissolved copper-acetylene compounds in a paper dealing mainly with the solid yellow compound formed in cuprous chloride-ammonium chloride solutions. They assume that the dissolved compound is of the same composition as the solid one, and suggest an equilibrium reaction formula giving a qualitative explanation of some effects observed. As earlier mentioned [3] they have however misidentified the solid compound, which is actually an acetylidic and not an addition compound.

In a later paper Chaltykyan [8] rather speculatively deals with the equilibria of the dissolved compounds and their catalytic activity. He regards them all as acetylidic, mainly asymmetrical, and omits simple addition compounds entirely.

TWO TYPES OF DISSOLVED COMPOUNDS

A solution of cuprous chloride in strong hydrochloric acid absorbs acetylene without colour change, whereas solutions containing cuprous chloride and alkali chloride turn intensely yellow. (Earlier literature, especially Manchot [5], gives the impression that a yellow colouration occurs in solutions with a high content of hydrochloric acid also. We assume that what actually has been observed is only the yellow colour characteristic of Cu^{II} in strong hydrochloric acid.) Thus there exist at least one colourless and one yellow compound, possibly several of each kind. The circumstance that the yellow compound forms in neutral but not in strongly acid solutions makes it probable that this compound is acetylidic.

From the introductory experiments given in Fig. 1 is evident that the total concentration of acetylene absorbed is independent of the hydrogen ion concentration when the acetylene pressure, the cuprous chloride concentration, and also the total concentration of potassium and hydrogen chlorides (KCl predominating) are kept constant. The ionic strength is constant, or approximately constant, at a given concentration of cuprous chloride, and the concentration of the varied component (H^+) is a fairly small part (max. 10 %) of the total electrolytic content. Existing dissolved compounds will then have approximately constant activity coefficients. Thus a variation of the hydrogen ion

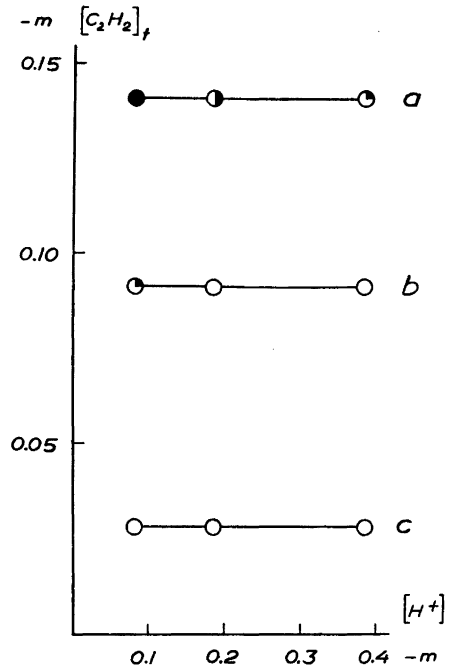


Fig. 1. Acetylene absorption and colour; dependence on hydrogen ion concentration; 1 atm and 20° C. KCl + HCl: 4.00 m.

a) 0.50 m, b) 0.30 m, and c) 0.00 m cuprous chloride

● *denotes strong yellow colour*
◑ » *medium* » »
◔ » *faint* » »
○ *no visible coloration*

concentration should give information on the existence of protolytic equilibria. The assumption that the acetylene absorption corresponds to equilibrium reactions has been confirmed: it is easy to state that the acetylene absorption is reversible, and that a rapid and reproducible shift in the equilibrium occurs when the pressure is altered. The total concentration measured (curves a and b) partly consists of "physically" dissolved acetylene. Presumably this contribution closely equals the acetylene absorption in the blank (curve c) where cuprous chloride is absent. But even if this should not be exactly true it is evident that the concentration of acetylene bound to Cu^I is independent of the hydrogen ion concentration.

But the colour of the solution is markedly changed, the yellow colouration rapidly fading with increasing acidity. Extinction measurements [3] in a similar

system have shown that the extinction depends on the hydrogen ion concentration as could be expected for an *acetylidic* compound.

The experiments of Fig. 1 indicate that the yellow compound, even in intensely coloured solutions, occurs only in a low concentration, and that the main part of the chemically combined acetylene is present as a colourless compound whose concentration is independent of the hydrogen ion concentration, and thus must be of *addition* type. The yellow compound has a very high molar extinction[3] which supports the assumption that its concentration is small.

The concentration of hydrochloric acid has been varied only within 0.1—0.4 m. An appreciably lower concentration than 0.1 m is not feasible as a solid acetylidic compound precipitates. Measurements at higher acidity show a slowly increasing gas absorption. The solutions remain colourless however, and it seems most improbable that a new kind of acetylidic compounds could form in a stronger acid. The conclusion primarily valid for the interval in Fig. 1, that addition compounds predominate, can certainly be generalized to hold also for solutions of higher acidity. In the following we can therefore assume that measurements of the total acetylene absorption can be utilized for determination of the total concentration of addition compounds, at least when the solution is acid and colourless.

In *op. cit.*[3] we have stated that the yellow acetylidic compound of these systems is an anion of the composition $C_2(CuCl)_n^{2-}$, where n is a high number, probably 8. This compound will not be dealt with any further here, the main subject of this paper being the colourless addition compounds and their equilibria.

1. Introduction

a) *Symbols*

P_A	Acetylene pressure in atm
$[C_2H_2]_t$	Total concentration of dissolved acetylene
$[C_2H_2]_{Cu}$	Concentration of Cu^I-bound acetylene
$\pi_A = P_A(1-[C_2H_2]_{Cu}/CuCl_t)$	
α	Absorption coefficient in moles/1 000 g water × atm
$CuCl_t$	Total concentration of cuprous chloride
$\{CuCl\}$	Activity of cuprous chloride. Standard state: solid substance
S	Solubility of cuprous chloride (acetylene absent)
HCl_t	Total concentration of hydrochloric acid
K	Thermodynamic equilibrium constant
γ	Molal activity coefficient
	All concentrations are given in m, *e. g.* moles/1 000 g of water.

b) *Survey.* The primary aim of the experiments was to establish *empirical relations* for the reversible acetylene absorption in acid solutions of cuprous chloride and a dissociating chloride. Secondarily we tried to decide what conclusions could be drawn as to the *composition* of the addition complexes.

Most measurements have been carried out in solutions with hydrochloric acid, this yielding a system with the minimum number of components. For a comparison are included some experiments with alkali chlorides, a small amount of acid being added to suppress the formation of acetylidic compounds. Through absorption measurements in a manometric apparatus according to Fig. 7, the relation between total concentration of acetylene absorbed and the partial pressure was first determined. The concentration of copper-bound acetylene then results by subtraction of the estimated contribution of "physical" solubility. Under d) in this section this assumption is discussed in detail.

We presume that no solid acetylene compounds form and that disturbances from catalytic reactions can be corrected for. These premises are discussed further under e) and f).

The absorption is measured either in a homogenous system or in a system in equilibrium with solid cuprous chloride. In the first case, if the inorganic components are CuCl, HCl and H_2O, the concentration of complex-bound acetylene can be determined as a function of the three variables P_A, HCl_t and $CuCl_t$. When solid cuprous chloride is present the variables P_A and HCl_t are sufficient.

A development of these empiric functions leads to the introduction of S (*cf.* above) as a parameter, so that the degree of saturation $CuCl_t/S$ is used as a variable; S being regarded as an empirical function of HCl_t.

The next step is the introduction of the thermodynamic activity $\{CuCl\}$ as a variable instead of $CuCl_t$ or degree of saturation. Here we utilize potentiometric measurements of the relation between the activity and the concentration of cuprous chloride in hydrochloric acid solutions. In some cases the activity is estimated by other means.

Finally the composition of the addition complexes is discussed. We assume the components to be only acetylene, cuprous ion and chloride ion, and neglect possible hydration. For convenience we use the expression $(C_2H_2)_n(CuCl)_p(Cl^-)_q$ as a general formula for an addition compound. The coefficient q may be negative, but the least sum of p + q is zero, denoting a chloride-free cationic compound.

A given complex stays in equilibrium with its components according to

$$n\ C_2H_2 + p\ CuCl + q\ Cl^- = (C_2H_2)_n(CuCl)_p(Cl^-)_q \qquad (1)$$

and the corresponding thermodynamic equilibrium constant $K_{n,p,q}$ is defined by

$$[(C_2H_2)_n(CuCl)_p(Cl^-)_q] = K_{n,p,q} \cdot P_A^n \cdot \{CuCl\}^p \cdot [Cl^-]^q \cdot \gamma_{Cl}^q/\gamma_{n,p,q} \quad (2)$$

The acetylene partial pressure has here been accepted as a measure of thermodynamic activity; this assumption is discussed under c).

Mixtures of several complexes with different n,p,q-values may occur. For the total concentration of acetylene in addition complexes holds the formula

$$[C_2H_2]_{Cu} = \Sigma n.K_{n,p,q} \cdot P_A^n \cdot \{CuCl\}^p \cdot [Cl^-]^q \cdot \gamma_{Cl}^q/\gamma_{n,p,q} \quad (3)$$

By a comparison between this formula and the empiric function

$$[C_2H_2]_{Cu} = f\ (P_A,\ \{CuCl\},\ HCl_t) \quad (4)$$

we try to determine what terms occur in the sum expression (3) and hence the composition of the complexes present. The experiments were divided into three sections, in each of which one of the three variables P_A, $\{CuCl\}$, and HCl_t was varied while the others were kept constant or allowed to covariate as determined by the experimental conditions.

c) *Acetylene activity and measurement of* P_A. Only a few determinations of acetylene's activity (fugacity), or its deviations from the ideal gas law have been carried out. From the data[10] possible to utilize for a calculation of the activity coefficient (approaching unity as the pressure tends to zero) no more certain conclusion can be drawn than that the activity coefficient at 25° and 1 atm presumably lies within 0.985—0.995. Since the coefficient is uncertain we refrain from corrections. Also, determinations of the physical solubility in water and electrolyte solutions give the impression that correction is unnecessary; Table 1 demonstrates that the absorption coefficient does not vary appreciably with the pressure.

The presence of a minor amount (appr. 0.4 % by volume) of impurities in the acetylene, mainly methane and nitrogen, causes a certain error in the P_A-value. However, it is possible to perform the experiments in such a manner (*cf.* "Apparatus and Procedure") that the relative error in P_A remains constant within each series.

Our conclusion is that the P_A-value, measured as the difference between total and vapour pressures, can be accepted as an activity measure.

d) *Acetylene absorption in water and aqueous electrolyte solutions.* Acetylene is quite soluble in water and electrolyte solutions. Hence there exists in cuprous chloride solutions also a quantity of acetylene not bound to copper. (The term "physical" has been used exclusively to denote this kind of absorption, bearing no assumption as to its nature.) We have assumed that the physical solubility in such systems can be determined by a parallel experiment where the solution has the same content of dissociating chloride as in the main experiment, but

does not contain cuprous chloride. Such an assumption cannot be regarded as generally valid, but for the present experiments no great uncertainty will result.

We take as an example a series (Table 4 b) of measurements on the system 4 m HCl_t and x m $CuCl_t$. For all values of x we have used the same figure for the physical solubility as that measured at x = 0. If x is low, *e.g.* 0.1 m or below, this can be immediately accepted. The concentration of inorganic cuprous complexes and of dissolved acetylene compounds is so small, both absolutely and in comparison with HCl_t, that no important effect on the physical absorption of acetylene can arise, even if these complexes should have high molar effects of salting in or out. When x reaches higher values it is possible, that the physical solubility will be altered to some extent. But the total acetylene absorption also increases with x, almost proportionally, and at the maximum value for x, about 0.75 m, the total absorption is more than 10 times higher than the physical solubility. Thus it is not probable that the relative error in the concentration of complex-bound acetylene, calculated as a difference, will increase appreciably with x.

As is evident from Table 1 the physical solubility in water and electrolyte solutions very closely follows Henry's law. Hydrogen chloride shows less salting out than the other chlorides.

We have found that the absorption coefficient in hydrochloric acid can be expressed by the empiric formula

$$\alpha = \alpha_0 - 2.62 \cdot 10^{-3} \cdot HCl_t \cdot (1 + 0.116\ HCl_t)^{-2} \qquad (5)$$

where $\alpha_0 = 0.0424$ is the absorption coefficient of pure water. Values computed according to this formula agree with the measurements within ± 0.4 %. The formula that Randall and Failey [11] have suggested for salting out effects is also applicable, but the agreement with our measured values will not be quite as good.

The concentration of acetylene bound in copper complexes has been calculated according to

$$[C_2H_2]_{Cu} = [C_2H_2]_t - \alpha P_A \qquad (6)$$

where $[C_2H_2]_t$ is the total concentration of dissolved acetylene, directly determined, and α stands for the absorption coefficient in a corresponding solution without cuprous chloride.

e) *Formation of solid acetylene compounds and measurements in supersaturated systems.* In neutral or weakly acid solutions of cuprous chloride with potassium or ammonium chloride, solid yellow acetylidic compounds of the type $K_2C_2(CuCl)_8$ are formed [3] even at low acetylene pressures, provided that both the cuprous chloride and the alkali chloride activities are sufficiently high, otherwise the violet complex $C_2Cu_2(CuCl)_n$ (which contains less cuprous

Table 1. Solubility of acetylene in water and strong chloride solutions at 25° C. a = absorption coefficient in mole/1000 g water × atm.

Solvent	P_A	a	Solvent	P_A	a
Water	0.281	0.042	2.00 m HCl	0.1–1.1	0.0392 (mean)
	0.356	0.0421			
	0.616	0.0423	3.00 m HCl	0.1–1.4	0.0383 (mean)
	1.003	0.0424			
4.00 m KCl	0.226	0.024	4.00 m HCl	0.173	0.0380
	0.354	0.0250		0.415	0.0379
	0.608	0.0249		0.667	0.0380
	0.928	0.0252		0.799	0.0380
				0.968	0.0378
4.00 m LiCl	0.186	0.023		1.184	0.0379
	0.354	0.0224		1.533	0.0378
	0.680	0.0223			
	1.003	0.0223	6.97 m HCl	0.3–1.6	0.0371 (mean)
1.00 m HCl	0.140	0.0403	10.00 m HCl	0.120	0.0373
	0.310	0.0405		0.372	0.0372
	0.592	0.0406		0.743	0.0373
	0.970	0.0405		1.378	0.0374

chloride and no alkali chloride) is the stable solid phase. The simple red copper acetylide C_2Cu_2 exists only at a fairly low cuprous chloride activity. If the dissociating chloride is NaCl or LiCl, as a rule no solid yellow complexes form, only the violet complex and copper acetylide.

Supersaturation can occur in all these cases (which may explain puzzling information in earlier literature on the conditions for precipitation of different solid compounds).

In a strongly acid solution the formation of solid acetylidic compounds is of course repressed. Preliminary results show that they may, however, exist in a fairly acid medium. Thus at 25° and 1 atm acetylene pressure the violet complex is coexistent with solid cuprous chloride at a hydrochloric acid concentration slightly above 1 m.

The stability of solid addition compounds of the type $C_2H_2(CuCl)_n$ is determined solely by the acetylene pressure and the cuprous chloride acitivity. In manometric experiments such a compound has been observed, coexistent with

solid cuprous chloride at an acetylene pressure of 0.38 atm and 25° C. The relation between acetylene absorbed and original content of cuprous chloride seems to demonstrate that the compound has the composition $C_2H_2(CuCl)_3$. But it should be pointed out that the equilibrium pressure of 0.38 atm is much lower than the value of 0.62 atm which has been reported by Österlöf [4] for a compound of this composition.

In the formation of this addition compound a characteristic supersaturation phenomenon occurs: the acetylene pressure must often be raised to a value more than three times that of the equilibrium pressure before the crystallization takes place (within reasonable time).

The experiments described here only deal with solutions where no solid acetylene compounds form. Thanks to the tendency towards supersaturation it is, however, not necessary to avoid all systems where solid compounds can form, which would impel rather narrow limitations. It is easy to check that no coloured acetylidic compounds occur. But it is difficult to see whether a colourless addition compound precipitates in a system where solid cuprous chloride is present. However, the phase conversion causes a considerable pressure drop, which is easy to observe, and there seems to be no slow formation of solid addition compound before this drop (see Table 5 and commenting text). Measurements in these supersaturated systems are also fully reproducible.

f) *Catalytic acetylene reactions.* In cuprous chloride solutions we have observed catalytic acetylene reactions even at a temperature as low as 20—25° C.

Aliphatic polymerization to mono- and divinylacetylene occurs in concentrated alkali chloride solutions and the reaction is promoted by high concentration of both cuprous and alkali chlorides, low acidity and high acetylene pressure.

Vinyl chloride forms in solutions with high concentration of both cuprous chloride and hydrochloric acid. In a 10 m hydrochloric acid saturated with cuprous chloride we have found a reaction velocity of about 0.015 mole/1000 g water and hour, approximately proportional to the acetylene pressure. The relative acetylene conversion as a rule is rather slow, since the total concentration of acetylene dissolved under the conditions stated amounts to 0.85 mole/1000 g water × atm. But in the manometric experiments the effect is clearly noticeable since there is an enrichment of vinyl chloride in the gas phase.

Even if catalytic reactions occur with perceivable velocity it is possible to determine with fair accuracy the concentration of complex-bound acetylene. But each experiment will yield only one point; a stepwise increase of the pressure is not suitable. If necessary the pressure-time curve of the reaction is read (in the case of vinyl chloride formation it is a linear time function) and the equilibrium pressure is computed by extrapolation to

Table 2. Relation between acetylene absorption and partial pressure in hydrochloric acid saturated with cuprous chloride. 25° C.

$[C_2H_2]_{Cu}$ *computed as* $[C_2H_2]_t - \alpha P_A$. α-values from Table 1.

HCl molality	P_A	$[C_2H_2]_t$	$[C_2H_2]_{Cu}/P_A$
1.00	0.0510	0.00831	0.122
	.0975	.01578	.1214
	.1613	.02604	.1209
	.2360	.03812	.1210
	.3233	.0524	.1216
	.4207	.0682	.1217
	.4793	.0776	.1214
	.5660	.0913	.1208
	.6355	.1030	.1214
	.7338	.1182	.1206
	.7953	.1282	.1207
3.00	0.0649	0.0201	0.271
	.1406	.0437	.2725
	.2507	.0785	.2748
	.3774	.1177	.2737
	.4995	.1556	.2732
	.5940	.1858	.2744
	.6630	.2072	.2742
	.7387	.2309	.2743
	.7960	.2492	.2747
	.8372	.2618	.2744
	.8697	.2726	.2752
	.9123	.2845	.2736
	.9524	.2987	.2754
	.9631	.3024	.2756
10.00 *	0.0836	0.0681	0.778
	.2087	.1694	.774
	.2838	.2320	.779
	.2884	.2327	.769
	.6013	.4925	.782

* Each value from a separate experiment, corrected for vinyl chloride formation.

zero time. Such an extrapolation has been employed where the measurements were carried out with a hydrochloric acid stronger than 6 m, except where the cuprous chloride content is small in comparison with the solubility.

2. Variation of the acetylene pressure

a) *Conditions: Solid cuprous chloride present, constant concentration of hydrochloric acid.* In a solution which is in equilibrium with solid cuprous

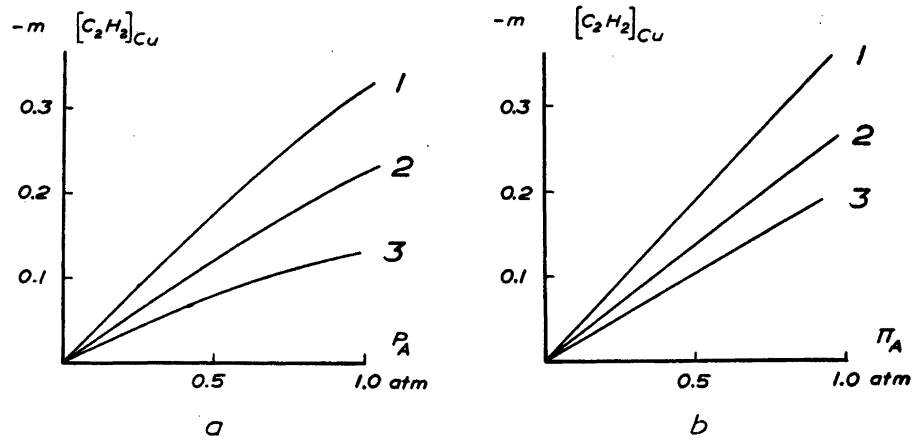


Fig. 2. The acetylene absorption as a function of the pressure: 1: 3.71 m CuCl; 6.00 m KCl 2: 1.86 m CuCl; 9.00 m LiCl 3. 0.40 m CuCl; 4.00 m HCl.

chloride the concentration of Cu^{I}-bound acetylene increases proportionally with the acetylene pressure.

In the experiments summarized in Table 2 the quotient $[C_2H_2]_{Cu}/P_A$ remains constant within $\pm \frac{1}{2}$ % at P_A-variations up to 1 atm. The same holds for the experiment of Table 5.

The variations in the quotient values are no larger than the uncertainty in the experimental data. (According to the estimation of the accuracy, in the experimental section, the ratio should have an uncertainty of $\pm$ 0.25 % at 1 atm, $\pm$0.5 % at 0.1 atm, then rapidly increasing with diminishing pressure.)

Even in systems containing 10 m hydrochloric acid, where vinyl chloride formation interferes and where each value is determined in a separate experiment, we obtained ratios of excellent constancy. We conclude:

$$[C_2H_2]_{Cu}/P_A \text{ constant at } \begin{cases} \{CuCl\} = 1 \\ HCl_t \text{ constant} \end{cases} \quad (7)$$

A corresponding rule seems to hold for systems containing alkali chlorides.

b) *Conditions: Constant total concentration of dissolved cuprous chloride. Constant concentration of hydrochloric acid.* In a homogenous aqueous solution of cuprous chloride and hydrochloric acid the concentration of complex bound acetylene increases as illustrated by Fig. 2 a (from preliminary series). These absorption curves are not linear, the ratio $[C_2H_2]_{Cu}/P_A$ diminishes with rising P_A. A closer investigation of the pressure dependence of the absorption quotient in various systems has yielded the following results:

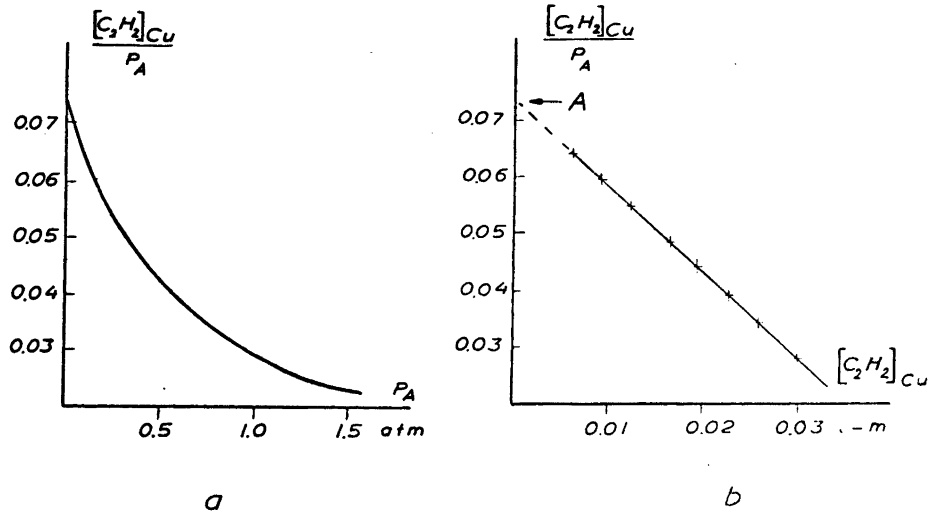


Fig. 3. The quotient $[C_2H_2]_{Cu}/P_A$ *as a function of* P_A *and of* $[C_2H_2]_{Cu}$
System: 0.05003 m CuCl; 1.000 m HCl, 25° C.

α) If $[C_2H_2]_{Cu}/P_A$ is plotted against P_A the resulting curve is of the type illustrated in Fig. 3 a.

β) The absorption ratio is a linear function of $[C_2H_2]_{Cu}$ in all cases investigated, *cf.* Fig. 3 b. In each individual case the correlation can thus be expressed by a formula of the type: $[C_2H_2]_{Cu}/P_A = A - B[C_2H_2]_{Cu}$.

γ) A depends on both $CuCl_t$ and HCl_t, B only on HCl_t. Experiments at different $CuCl_t$ but identical HCl_t thus give a group of parallel lines in a graph plotted as Fig. 3 b.

δ) Generally the ratio A/B has a numeric value which closely agrees with that of $CuCl_t$. In the experiment of Fig. 3, $CuCl_t$ is 0.05003; the diagram gives $A/B = 0.0501$. The function under *β*) can thus be written: $[C_2H_2]_{Cu}/P_A = A.(1 - [C_2H_2]_{Cu}/CuCl_t)$.

These observations lead to the introduction of the parameter π_A, defined as

$$\pi_A \equiv P_A\ (1 - [C_2H_2]_{Cu}/CuCl_t) \qquad (8)$$

and Tables 3 and 4 show the pressure independence of the quotient $[C_2H_2]_{Cu}/\pi_A$, in various systems. In Table 3 the quotient remains constant within ± 1% at acetylene pressures from 0.08 to 1.7 and 1.2 atm. resp. A definite trend can hardly be observed.

Condensed results from these and other experiments are found in Table 4, where are given highest and lowest figures for the quotient, observed within

Table 3. Relation between acetylene absorption and partial pressure in homogenous solutions with constant concentrations of cuprous chloride and hydrochloric acid. 25° C. $[C_2H_2]_{Cu}$ *computed as* $[C_2H_2]_t - aP_A$. *a-values from Table 1.*

P_A	$[C_2H_2]_t$	$\frac{[C_2H_2]_{Cu}}{P_A}$	$\frac{[C_2H_2]_{Cu}}{\pi_A}$
	4.00 m HCl_t and 0.5515 m $CuCl_t$		
0.0801	0.0232	0.252	0.262
.1616	.0456	.245	.263
.2428	.0661	.235	.262
.3269	.0862	.226	.261
.3853	.0997	.221	.262
.4820	.1206	.213	.261
.5710	.1392	.206	.262
.7074	.1650	.196	.261
.8192	.1847	.188	.261
.9531	.2071	.180	.261
1.1034	.2305	.171	.261
1.2908	.2576	.162	.261
1.4635	.2800	.154	.260
1.740	.3157	.144	.264
	10.00 m HCl_t and 0.975 m $CuCl_t$		
0.0410	0.0100	0.207	(0.210)
.0831	.0208	.212	.217
.1038	.0259	.212	.218
.1586	.0393	.211	.219
.2108	.0519	.209	.220
.2619	.0641	.207	.220
.3187	.0771	.205	.220
.3900	.0929	.201	.219
.4302	.1016	.199	.219
.5479	.1200	.195	.219
.6718	.1517	.189	.218
.8007	.1771	.184	.217
.9231	.2008	.180	.218
.9764	.2009	.178	.217
1.0561	.2237	.175	.216
1.1949	.2486	.171	.216

the interval 0.1—1.3 atm. The rather large variations in some series, particularly with 1 m HCl_t — 0.016 m $CuCl_t$, obviously depend on the circumstance that $[C_2H_2]_{Cu}$ cannot be determined with satisfactory accuracy since the value is smaller than the contribution from physically dissolved acetylene, especially at higher pressures. (According to the estimate in the experimental section

the ratio would in the extreme case quoted have an accuracy of ± 1 % at 0.1 atm and ± 4 % at 1 atm; the experiment gives a scattering around the mean of maximum 5 %.)

The scattering in the ratio values found in Table 3 can thus be regarded as representative; being more pronounced only where the measurements are of inferior accuracy. We thus obtain the rule

$$[C_2H_2]_{Cu}/\pi_A \text{ constant at } \begin{cases} CuCl_t \text{ constant} \\ HCl_t \text{ constant} \end{cases} \quad (9)$$

An analogous rule seems to hold in systems where the dissociating chloride mainly consists of an alkali chloride.

c) *Conditions:* $CuCl_t$ *altered in such a manner that* $CuCl_t - [C_2H_2]_{Cu}$ *is constant;* HCl_t *constant.* Absorption experiments in strict accordance with these conditions can hardly be performed. But the result from such covariation can easily be deduced starting from rule (9), according to which $[C_2H_2]_{Cu}/\pi_A$ can be regarded as a function of $CuCl_t$ and HCl_t, then studying how this quotient depends on $CuCl_t$ at a constant HCl_t. Such experiments are treated in detail in the next section and the results are condensed in equation (14). From this formula, which includes (9) also, it is evident that $[C_2H_2]_{Cu}$ is proportional to the product π_A. $CuCl_t$, which, according to (8) is identical with $P_A \cdot (CuCl_t - [C_2H_2]_{Cu})$. If the bracketed difference remains constant, the concentration of Cu^I-bound acetylene will increase proportionally to the acetylene pressure. Thus holds

$$[C_2H_2]_{Cu}/P_A \text{ constant at } \begin{cases} CuCl_t - [C_2H_2]_{Cu} = CuCl_{t,i} \text{ constant} \\ HCl_t \text{ constant} \end{cases} \quad (10)$$

where $CuCl_{t,i}$ denotes the initial concentration of cuprous chloride in the absence of acetylene. Rule (10) has been experimentally verified within the same concentration and pressure intervals as (9) and with approximately the same accuracy.

When $CuCl_{t,i}$ is equal to S (*i. e.* the solubility of CuCl in absence of acetylene) rule (10) will express the results from the earlier described experiments with *solid* cuprous chloride, though in other variables. For in such systems $CuCl_t$ increases equimolarily with $[C_2H_2]_{Cu}$, as has been confirmed by analyses. Already the derivation of (10) however, demonstrates that the $CuCl_t$-condition of the rule is the necessary condition for a constant absorption quotient at a given concentration of hydrochloric acid, and the saturated systems do not fall outside the interval investigated.

Of particular interest is the following conclusion: a system whose composition is changed according to the conditions of rule (10) remains in equilibrium

with solid cuprous chloride if $CuCl_{t,i} = S$. This holds independent of the hydrochloric acid concentration.

Rules (10) and (14) are verified (*cf.* Table 4) even at such low cuprous chloride concentrations that $[C_2H_2]_{Cu}$ amounts to only 1.5 % of HCl_t at 1 atm. It then follows that formula (3) can be applied with the following approximations: $[Cl^-]$ and activity coefficients constant, polynuclear complexes negligible, and CuCl-activity proportional to the concentration of "free" cuprous chloride. Then it follows that the activity of cuprous chloride must be independent of P_A if the conditions of rule (10) are maintained.

These conditions then fix the activity of cuprous chloride both at a maximum and at a low $CuCl_{t,i}$, and a different state at intermediate concentrations seems most improbable. (Potentiometric checks are difficult since the prescribed covariation cannot be experimentally arranged and a combination of separate measurements gives too low an accuracy.)

If the generalization

$$\{CuCl\} \text{ constant at } \begin{cases} CuCl_t - [C_2H_2]_{Cu} = CuCl_{t,i} \text{ constant} \\ HCl_t \text{ constant} \end{cases} \qquad (11)$$

is accepted for all $CuCl_{t,i}$ values the empirical rule (10) can be formulated

$$[C_2H_2]_{Cu}/P_A \text{ constant at } \begin{cases} \{CuCl\} \text{ constant} \\ HCl_t \text{ constant} \end{cases} \qquad (12)$$

Rule (7) valid for a cuprous chloride activity of unity thus is a special case, but directly experimentally verified.

d) *Conclusion on the composition of the acetylene complexes.* When applying the general equilibrium expression (3) to the results summarized in rule (12) it is necessary to estimate how the remaining variables: the concentration of free chloride ion and the quotients of the activity coefficients, change with the acetylene pressure.

The concentration of acetylene complexes normally is small in comparison with the total concentration of hydrochloric acid. In the experiments of Table 2, where solid cuprous chloride is present, $[C_2H_2]_{Cu}$ will reach values corresponding to 5—10 % of HCl_t. Experiments in a homogenous system as a rule give lower figures (unless the acetylene pressure exceeds 1 atm) and sometimes the maximum complex concentration does not reach 1 % of the hydrochloric acid concentration. The acetylene absorption can thus cause only comparatively small changes in the concentration of free chloride ion. For the same reasons the variations in the quotients of the activity coefficients remain small. The proportionality between $[C_2H_2]_{Cu}$ and P_A according to (12) shows that *the main part of the complex bound acetylene exists in compounds where the coefficient n equals 1, i. e.* containing one acetylene molecule only.

3. Variation of the activity of cuprous chloride

a) *Empirical relation between acetylene absorption and concentration of cuprous chloride at constant concentration of hydrochloric acid.* Table 4 contains results from absorption experiments with 1, 4, and 10 m hydrochloric acid as solvents. Most determinations refer to homogenous systems with a given total concentration of dissolved cuprous chloride; the results of the measurements are rendered by the quotient $[C_2H_2]_{Cu}/\pi_A$ which is independent of P_A under these conditions (9). Further the table includes some experiments on systems with solid cuprous chloride; the result then appears as the quotient $[C_2H_2]_{Cu}/P_A$, being in such cases the pressure-independent quantity (7).

In the series with 1 m and 4 m hydrochloric acid resp., each experiment gives on absorption curve, *i. e.* pairs of $[C_2H_2]_{Cu}$ and P_A values in analogy with Table 2, or $[C_2H_2]_{Cu}$ and π_A pairs as in Table 3. In Table 4 is given the mean of all (10—15) the quotients which correspond to acetylene pressures over 0.1 atm, and also the highest and lowest figures. In the series with 10 m acid the procedure has been the same at the lower copper concentrations but at a higher $CuCl_t$, where vinyl chloride forms with an appreciable velocity, only one, or some few, quotient values were determined in each experiment.

The quantity $([C_2H_2]_{Cu}/P_A)_0$ has been obtained by linear extrapolation to $[C_2H_2]_{Cu} = 0$, using a diagram where the values for $[C_2H_2]_{Cu}/P_A$ are plotted against $[C_2H_2]_{Cu}$. (*Cf.* Fig. 3 b, where the intercept is the extrapolated value.)

The two last columns contain the values of the following quantities:

$$k_c \equiv \frac{[C_2H_2]_{Cu}}{\pi_A} \cdot \frac{1}{CuCl_t} \text{ and } k \equiv \left(\frac{[C_2H_2]_{Cu}}{P_A}\right)_{CuCl(s)} \cdot \frac{1}{S} \qquad (13)$$

These experiments give the following results:

α) The quantity k_c is independent of the concentration of cuprous chloride and can thus be regarded as a constant at a given HCl_t. The only variations occurring can be attributed to the uncertainty in the $[C_2H_2]_{Cu}/\pi_A$ quotient, discussed under 2 b above. In the series with 1 m hydrochloric acid, which must be regarded as being at the limit of what can be studied with the technique described, k_c is changed by about 3 % when $CuCl_t$ is increased four times. In the two other series, where $CuCl_t$ is varied by approximately a power of ten, k_c remains constant within $\pm$ 1 %. We get:

$$k_c = [C_2H_2]_{Cu}/\pi_A \cdot CuCl_t \text{ constant (condition } HCl_t \text{ constant)} \qquad (14)$$

β) At a given HCl_t the quantity k_c has, within the experimental accuracy, the same numerical value as k; in the following we use the letter k for both quantities. Substitution of (8) in (13) shows that $CuCl_t\text{-}[C_2H_2]_{Cu} = S$, if the solution is saturated with cuprous chloride.

Table 4. Relation between acetylene absorption and concentration of cuprous chloride at constant concentration of hydrochloric acid. 25° C.

$CuCl_t$	$\left(\frac{[C_2H_2]_{Cu}}{P_A}\right)_0$	$[C_2H_2]_{Cu}/\pi_A$ Mean	Lowest value	Highest value	$\frac{[C_2H_2]_{Cu}}{P_A}$	k_c	k
a) 1.000 m hydrochloric acid							
0.01628	0.0240	0.0238	0.0226	0.0246	—	1.46	—
0.03531	0.0502	0.0498	0.0490	0.0508	—	1.41	—
0.05003	0.0708	0.0707	0.0704	0.0709	—	1.41	—
CuCl (s)	0.1210	—	—	—	0.1211	—	1.43
$S = 0.0848$							
b) 4.00 m hydrochloric acid							
0.0500	0.0240	0.0240	0.0237	0.0247	—	0.480	—
0.1503	0.0730	0.0727	0.0724	0.0730	—	0.484	—
0.2499	0.1210	0.1210	0.1206	0.1216	—	0.484	—
0.4490	0.215	0.215	0.214	0.216	—	0.479	—
0.5515	0.262	0.262	0.260	0.264	—	0.475	—
0.6500	0.308	0.308	0.307	0.310	—	0.474	—
0.750	0.358	0.357	0.354	0.360	—	0.476	—
CuCl (s)	0.354	—	—	—	0.354	—	0.476
$S = 0.744$							
c) 10.00 m hydrochloric acid							
0.506	0.115	0.116	0.114	0.117	—	0.229	—
0.975	0.218	0.218	0.216	0.220	—	0.224	—
1.497	0.337	0.336	0.334	0.338	—	0.225	—
1.998	—	0.455	—	—	—	0.228	—
2.308	—	0.531	—	—	—	0.230	—
2.501	—	0.574	—	—	—	0.228	—
2.978	—	0.678	—	—	—	0.228	—
3.112	—	0.710	—	—	—	0.228	—
3.271	—	0.747	—	—	—	0.228	—
3.512	—	0.806	—	—	—	0.229	—
CuCl (s)	0.776	—	—	—	0.780	—	0.229
$S = 3.410$							

γ) The extrapolated quotient $([C_2H_2]_{Cu}/P_A)_0$ agrees closely with the corresponding mean for $[C_2H_2]_{Cu}P_A$ (condition solid CuCl) and $[C_2H_2]_{Cu}/\pi_A$ (condition $CuCl_t$ constant) respectively, which can be foreseen from earlier results (formulas 7—9).

For several reasons it is valuable to know the limit value, which the quotient $[C_2H_2]_{Cu}/P_A$ approaches when $[C_2H_2]_{Cu}$ or P_A tends to zero under given

conditions. The value for $([C_2H_2]_{Cu}/P_A)_0$, obtained as described above, is accepted as limit, since in the diagram no such deviation is found as could indicate that the curve might bend at very low $[C_2H_2]_{Cu}$-values.

The limit value increases proportionally with the concentration of cuprous chloride, at a given concentration of HCl_t:

$$([C_2H_2]_{Cu}/P_A)_0 = k \cdot CuCl_t \tag{15}$$

The results so far can be summed up in the following formula, derived from (8) and (14)

$$[C_2H_2]_{Cu} = k \cdot P_A \cdot (CuCl_t - [C_2H_2]_{Cu}) \text{ or } [C_2H_2]_{Cu} = k \cdot P_A \cdot CuCl_t/(1 + k \cdot P_A) \tag{16}$$

where k is an empirical constant valid for each concentration of hydrochloric acid.

At a given hydrochloric acid concentration the absorption quotient $[C_2H_2]_{Cu}/P_A$ thus is proportional to the difference $CuCl_t - [C_2H_2]_{Cu}$ whichever of these terms is varied. Analogous results have been achieved by Gilliland and coworkers [11] from investigations of the absorption of ethylene and other olefinic hydrocarbons in cuprous chloride solutions. This difference has often been regarded as a measure of the concentration of "free" cuprous chloride; it is then assumed that each molecule of the hydrocarbon binds one molecule of cuprous chloride. Probably this is essentially correct, and anyway it enables a simple description of the functional relation. But it should be pointed out that the empirical relation can be determined without any assumptions as to the composition of the complexes.

From the explicit formulation of (16) it is evident that the concentration of complex-bound acetylene (and thus the total concentration of dissolved acetylene), as measured at a given acetylene pressure, is proportional to the concentration of cuprous chloride, the coefficient of proportionality depending on the pressure. *Cf.* Fig. 4.

Analogous relations seem to hold for cuprous chloride solutions containing alkali chlorides. *Cf.* Fig. 4 showing results from preliminary experiments, somewhat less accurate.

b) *Relation between activity and concentration of cuprous chloride at constant concentration of hydrochloric acid; acetylene absent.* It seems to be characteristic for systems consisting of cuprous chloride, a dissociating 1 : 1 chloride, and water, that the activity of the cuprous chloride is closely proportional to its concentration if the total concentration of dissociating chloride is kept constant. Earlier investigations by Szabo *et al.*[12] point in this direction. Their measurements show that the activity of Cu^+ (determined potentiometrically

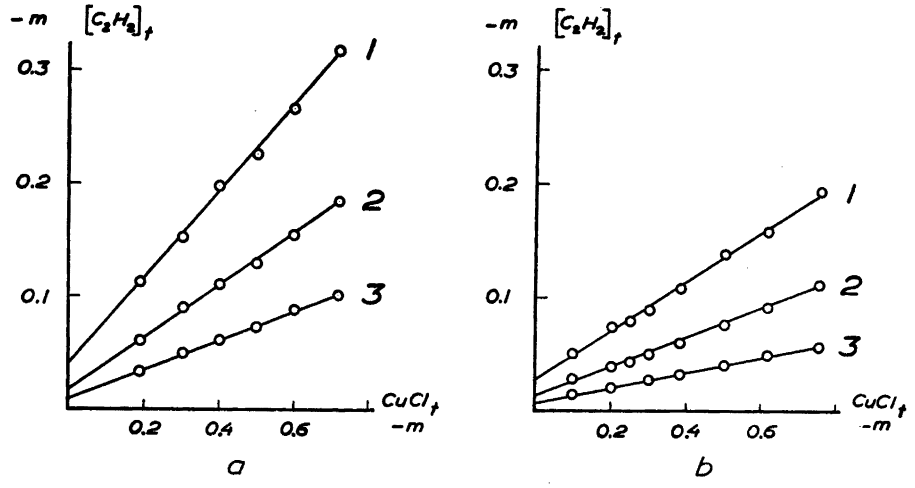


Fig. 4. Total acetylene absorption as a function of the cuprous chloride concentration at constant concentration of dissociating chloride, and constant pressure. 20°:

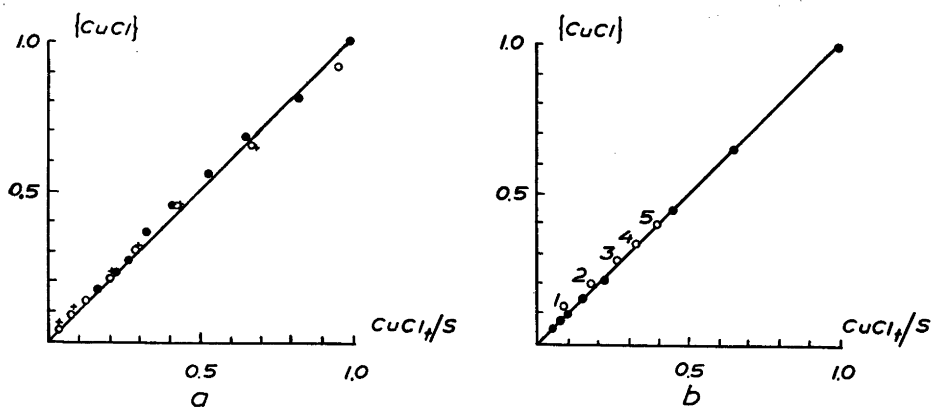
a. 4.00 m HCl *b. 3.80 m KCl; 0.20 m HCl*
1. 1.00 atm *2. 0.50 atm* *3. 0.25 atm*

in a cell with liquid junction) is approximately proportional to the concentration of cuprous chloride in solutions with approximately constant concentration of dissociating chloride.

Our own measurements [13] are also potentiometrical but so performed that the activity of the uncharged CuCl molecule is determined directly, utilizing Ag/AgCl and amalgamated copper as electrodes in a cell without liquid junction. The experiments were performed as dilution series starting from a solution saturated with cuprous chloride; as a rule the hydrochloric acid concentration was maintained constant. The standard potential of the electrode couple was determined in each series by measuring in the presence of solid cuprous chloride. Se further "Apparatus and Procedure".

At hydrochloric acid concentrations below 6 m the reproducibility is satisfactory even though the accuracy is hardly better than $\pm$ 1 mV corresponding to $\pm$ 4 % in $\{CuCl\}$. At higher acid concentrations the results become less satisfactory, particularly if the concentration of cuprous chloride is high also. (The disturbances seem to arise from the solubility of the silver chloride of the electrode.)

Fig. 5 a shows the relation between activity and concentration of cuprous chloride (concentration expressed by $CuCl_t/S$) in 4 m hydrochloric acid, according to three separate series. The deviations from the straight line are hardly



Fid. 5. The activity of cuprous chloride as a function of the saturation degree in hydrochloric acid solution (acetylene absent). Potentiometric measurement at 25°.

a: 4 m HCl, 3 series

b. ●—● *1 m HCl*
○—○ *$CuCl_t/HCl_t$ constant = 0.0306*
HCl_t: 10.0 m (point 1); 3.42 (2); 2.07 (3); 1.24 (4); 0.715 (5).

larger than corresponding to an uncertainty in the potential of $\pm$ 1 mV. (The linear scale at higher activities demands rather accurate measurements; the difference in $\{CuCl\}$ between the two uppermost points corresponds to about 2 mV.)

Results from analogous experiments in 1 m HCl are found in Fig. 5 b, where also are included measurements on a solution which has been stepwise diluted with water, the ratio $CuCl_t/HCl_t$ remaining constant. (The standard potential was determined at $HCl_t = 0.5$ after addition of solid cuprous chloride.)

The potentiometric measurements hitherto performed within the HCl_t-interval 1—10 m show no systematic deviation from a proportionality between activity and saturation when the hydrochloric acid concentration is below 6 m.

Thus in this range

$$\{CuCl\} = CuCl_t/S \qquad (17)$$

At higher concentrations of hydrochloric acid $d\{CuCl\}/d(CuCl_t)$ is constant over the main part of the $CuCl_t$ interval but probably diminishes in the vicinity of saturation. But these determinations are to uncertain to admit any safe conclusions.

It is to be expected that the quotient $\{CuCl\}/CuCl_t$ will keep constant when $CuCl_t$ is small; the only condition being that mononuclear complexes of cuprous

chloride exist. But it is astonishing, that in almost all systems measured the quotient has the same value, even where the concentration of dissolved cuprous chloride is appreciable. This question will be discussed later.

c) *Relation between acetylene absorption and activity of cuprous chloride at constant concentration of hydrochloric acid.* In a system where $CuCl_t$ and HCl_t are given, the absorption quotient will approach its limit value $([C_2H_2]_{Cu}/P_A)_0$ when $[C_2H_2]_{Cu}$ tends to zero. Simultaneously the cuprous chloride activity will approach the value $\{CuCl\}_0$ which holds in the absence of acetylene. According to (15) and (17) resp. both these limit values are proportional to $CuCl_t$ at a given HCl_t. Thus holds:

$$([C_2H_2]_{Cu}/P_A)_0 = k_a \{CuCl\}_0 \tag{18}$$

where k_a is an empirical constant, depending on HCl_t only. The expression is valid with an accuracy depending mainly on (17), *i. e.* on the potentiometric measurements.

Referring to the generalization (12), according to which the absorption quotient remains independent of $[C_2H_2]_{Cu}$ (and of P_A) when $\{CuCl\}$ and HCl_t are kept constant, this same expression can be applied to the absorption quotient when measured at a finite pressure:

$$[C_2H_2]_{Cu}/P_A = k_a \{CuCl\} \tag{19}$$

d) *Conclusions on the composition of the acetylene complexes.* Attempts to interpret these results using the general equilibrium expression (3) yields rather uninformative results. If the sum is expressed as a polynomial in $\{CuCl\}$ we get

$$\left(\frac{[C_2H_2]_{Cu}}{P_A}\right)_0 = \{CuCl\}\sum_q K_{11q}[Cl^-]^q \gamma^q_{Cl}/\gamma_{11q} + \{CuCl\}^2 \sum_q K_{12q}[Cl^-]^q \gamma^q_{Cl}/\gamma_{12q} + \cdots$$
$$= k_a\{CuCl\} \tag{20}$$

where the first term corresponds to mononuclear complexes, the second term to dinuclear complexes *etc.* We have simplified the formula, presuming that only complexes with n = 1 appear. Such a generalization of the conclusion in section 2. is evidently justified since the limit value holds when the pressure tends to zero. The use of the limit value also permits the disregard of the influence of the acetylene complexes on the composition of the solution.

The measurements apply to systems with constant HCl_t. When the activity of cuprous chloride is increased from zero to unity, the concentration of free chloride ion must decrease — if the solubility at all is due to the formation of complex compounds containing chloride ion. The decrease would be quite noticeable, particularly at higher acid concentrations. If we accept the assump-

tion, commonly held [12], that the main part of the cuprous chloride is solubilized as $CuCl_3^{2-}$, then in the series with 4 m HCl $[Cl^-]$ should fall from 4.0 to 2.5, and in the series with 1 m HCl from 1.0 to 0.83.

If we assume that only mononuclear complexes exist, the first Σ-function in expression (20) must remain constant, *i. e.* the activity coefficients must vary in such a way that the changes in $[Cl^-]$ are compensated for. But we might as well assume a not insignificant contribution from di- or polynuclear complexes. Whichever the case may be the linear relation must be explained as a result of accidental compensation.

As previously alluded to, a similar phenomenon occurs in the equilibria of the inorganic system $CuCl-Cl^-$. If the inorganic cuprous chloride complexes are denoted $(CuCl)_a(Cl^-)_b$, their equilibrium constants β_{ab}, and their activity coefficients γ_{ab} we get the following equation, comprising the empiric function (17):

$$\begin{aligned} CuCl_t &= \{CuCl\} \sum_b \beta_{1b} [Cl^-]^b \gamma_{Cl}^b/\gamma_{1b} + \{CuCl\}^2 \sum_b 2\beta_{2b} [Cl^-]^b \gamma_{Cl}^b/\gamma_{2b} + \cdots \\ &= S \cdot \{CuCl\} \end{aligned} \tag{21}$$

This formula is analogous with (20). Here also the assumption of the sole existence of mononuclear complexes would involve that the activity coefficients of the first term compensate for the changes in $[Cl^-]$. To illustrate how large this benevolent compensation would have to be, we assume that $CuCl_3^{2-}$ is the predominating complex, and that the activity coefficients remain constant. We then find that in 4 m HCl the quotient $CuCl_t/\{CuCl\}$ should increase from its saturation value 0.75, to a value of 1.74 in a 1/10-saturated solution. But the measurements have given 0.75 and 0.73 respectively, the difference not being significant.

It is possible that the two systems have analogous distributions between mono- and polynuclear complexes. But it seems more natural to try and trace the linear correlation to one and the same phenomenon, assuming that the chloride ion activity, *i. e.* the product $[Cl^-] \cdot \gamma_{Cl}$, is independent of $\{CuCl\}$ at a given HCl_t. This would mean that in both systems mononuclear complexes predominate, provided that their activity coefficients are constant at given HCl_t. But as long as the inorganic system is not fully understood it seems fruitless to attempt a complete analysis of the composition of the acetylene system.

We are however entitled to conclude that mononuclear complexes *exist*. The linear relations (15) and (17), thus (18) also, are experimentally verified at low cuprous chloride activities where the concentration of dissolved cuprous chloride is small in comparison with the concentration of hydrochloric acid, and where the concentration of free chloride ion and the activity coefficients

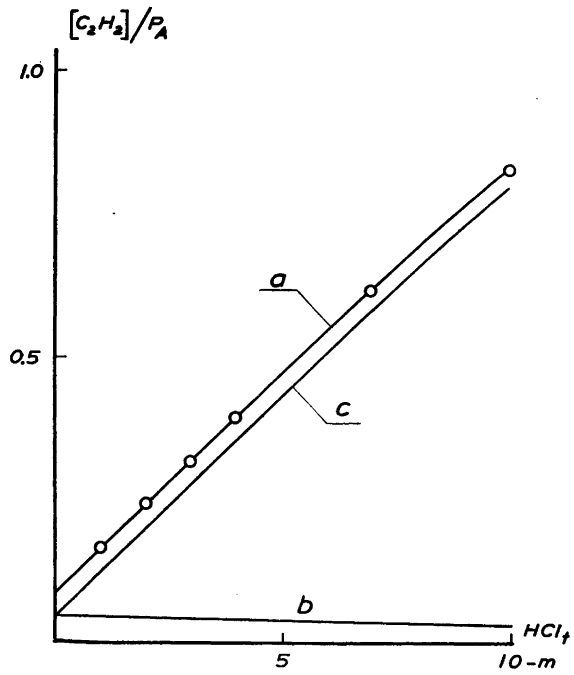


Fig. 6. Acetylene absorption as a function of the concentration of hydrochloric acid in the presence of solid cuprous chloride. 25° C.

a: with cuprous chloride
b: cuprous chloride absent — "physical" solubility
c: difference a—b, i.e. $[C_2H_2]_{Cu}/P_A$

consequently should remain approximately constant. As shown by Table 4 acceptable measurements of the acetylene absorption can be performed even in solutions where $CuCl_t$ only amounts to a few percent of HCl_t. But to what extent complexes of other composition appear at higher activities of cuprous chloride cannot be definitely established.

4. Variation of the hydrochloric acid concentration

a) *Condition: solid cuprous chloride present.* Fig. 6 shows how the quotients $[C_2H_2]_t/P_A$ and $[C_2H_2]_{Cu}/P_A$ depend on the concentration of hydrochloric acid in systems containing solid cuprous chloride, where these two quotients are independent of P_A. The points of curve *a* are means from experiments with P_A-variations between 0.1 and 1.0 atm; treated as in Table 2.

The relation between $[C_2H_2]_{Cu}/P_A$ and HCl_t thus is linear; possibly a small deflection, concave towards the HCl-axis, can be observed in the uppermost part of the *c*-curve. If only the interval 1—7 m hydrochloric acid is taken into account the result can best be expressed by the empirical formula

$$[C_2H_2]_{Cu}/P_A = A + B \cdot HCl_t \qquad (\text{Condition } \{CuCl\} = 1)$$
$$A = 0.046 \text{ m/atm} \qquad B = 0.0759 \text{ atm}^- \qquad (22)$$

Computed and measured values agree within $\pm$ 1 %. If the same expression is applied to 10 m hydrochloric acid the computed value is 3 % higher than the measured one. The deviation appears in the interval where the formation of vinyl chloride is noticeable, and where the measurements have been performed slightly differently. Therefore it is difficult to judge whether the difference is real. Anyway the deviation is so small that we can regard (22) as approximately valid for the whole interval 1—10 *M* hydrochloric acid.

The same expression applies also for the limit $([C_2H_2]_{Cu}/P_A)_0$.

b) *General function of the variables* P_A, $\{CuCl\}$ *and* HCl_t. The combination of (18) and (22) yields

$$([C_2H_2]_{Cu}/P_A)_0 = \{CuCl\} \cdot (A + B \cdot HCl_t) \qquad (23)$$

The expression has been experimentally verified only within that limited interval for HCl_t, and with that limited accuracy, under which (17) and (18) are valid. Only at the extreme: $\{CuCl\} = 1$ does the expression directly follow from (22).

If the generalization formulated in (12) is applied here, the same expression will hold also for the absorption quotient at a finite pressure:

$$[C_2H_2]_{Cu} = P_A \cdot \{CuCl\} \cdot (A + B \cdot HCl_t) \qquad (24)$$

At a constant cuprous chloride activity the absorption quotient thus is a linear function of the hydrochloric acid concentration, with a constant term corresponding to the ordinate intercept of the extrapolated *c*-curve in Fig. 6.

c) *Conclusions on the composition of the acetylene complexes.* From the linear HCl_t function one gets the immediate impression that two complexes predominate, one with q = 1, *i. e.* the anion $C_2H_2 \cdot CuCl_2^-$ whose concentration rises proportionally with HCl_t, and the other with q = 0, *i. e.* the uncharged compound $C_2H_2 \cdot CuCl$ whose concentration is independent of HCl_t. It seems most likely that this interpretation is correct, but the evidence is only circumstantial. But if the discussion is limited to those cases where $\{CuCl\}$ is so low that the concentration of inorganic copper complexes and of acetylene complexes can be neglected in comparison with HCl_t, and where complexes with

p = 1 can be expected to predominate, we get from (3) and (24) the following equation

$$\frac{[C_2H_2]_{Cu}}{P_A} \cdot \frac{1}{\{CuCl\}} = \sum K_{11q} \cdot HCl_t^q \cdot \gamma_{Cl}^q / \gamma_{11q} = A + B \cdot HCl_t.$$
$$\text{(Condition: } \{CuCl\} \ll 1) \qquad (25)$$

Thus a possible explanation is that only the complexes mentioned, with q = 0 and + 1 resp., do exist, and that the following expressions hold

$$\begin{cases} \text{For } C_2H_2 \cdot CuCl: & K_{110}/\gamma_{110} = A = 0.046 \text{ m/atm} \\ \text{For } C_2H_2 \cdot CuCl_2^-: & K_{111} \cdot \gamma_{Cl}/\gamma_{111} = B = 0.0759 \text{ atm}^{-1} \end{cases} \qquad (26)$$

This would mean that the uncharged complex has an activity coefficient which is independent of the hydrochloric acid concentration, and that the activity coefficient of the anion complex remains proportional to that of the chloride ion when HCl_t is varied. These two assumptions might both be plausible. But in this system the variations of the activity coefficients of the ions present are quite large. (The mean activity coefficient of HCl [14] is 0.81 in 1 m and 10.5 in 10 m solution.) Other interpretations are thus feasible, even though they seem less probable.

To attain reliable conclusions on the chloride content of the complexes it is desirable to study systems where the chloride ion concentration can be altered without major displacements of the activity coefficients. We have achieved good results with mixtures of hydrochloric and perchloric acid. These experiments, which will be described in a forthcoming paper, give good evidence that the complexes mentioned, $C_2H_2 \cdot CuCl$ and $C_2H_2 \cdot CuCl_2^-$, predominate. It is therefore likely that this holds also for the system with hydrochloric acid only.

The conclusion is: the measurements are compatible with the assumption that the complexes $C_2H_2 \cdot CuCl$ and $C_2H_2 \cdot CuCl_2^-$ predominate at a low cuprous chloride activity. Measurements at higher activities allow no definite conclusions, although the same empirical relations hold.

5. Comprehensive discussion of the equilibrium experiments

The absorption of acetylene in an aqueous solution of cuprous chloride and a dissociating 1 : 1 chloride depends on the composition of the solution as can be described by simple empirical functions. In systems where hydrochloric acid is the dissociating chloride the concentration of Cu^I-bound acetylene can

be expressed as a function, derived from (22), (16) and (13), of the variables P_A, $CuCl_t$ and HCl_t.

$$[C_2H_2]_{Cu} = P_A \cdot \frac{CuCl_t - [C_2H_2]_{Cu}}{S} \cdot (A + B \cdot HCl_t) \tag{27}$$

where the contants (25° C) are $A = 0.046$ m/atm, $B = 0.0759$ atm^{-1} (28)

The corresponding explicit expression is

$$[C_2H_2]_{Cu} = \frac{P_A \cdot CuCl_t \cdot (A + B \cdot HCl_t)}{S + P_A \cdot (A + B \cdot HCl_t)} \tag{28}$$

S denotes the solubility of cuprous chloride in hydrochloric acid in the absence of acetylene, and is regarded as an empirical function of HCl_t. The expressions are derived from measurements, where the hydrochloric acid concentration has been varied within the interval 1—10 m, and each of the other variables over the largest interval practicable (about one power of ten). Only at the upper boundary of the HCl_t-interval the results might indicate a systematic deviation from these expressions, amounting however to some few percent only. Otherwise the expressions are valid with an accuracy of ± 1 %, which approximately corresponds to the experimental error.

Using the activity of cuprous chloride as a variable we get a simpler expression:

$$[C_2H_2]_{Cu}/P_A = \{CuCl\} \cdot (A + B \cdot HCl_t) \tag{24}$$

This also is an empirical function, at least when applied to the limit value $([C_2H_2]_{Cu}/P_A)_0$, otherwise a generalization according to (11) and (12) must be introduced. This formula, founded on potentiometric measurements also, has a lesser accuracy and a smaller interval of validity. But it is supported by measurements on systems with solid cuprous chloride, which result in:

$$[C_2H_2]_{Cu} = P_A \cdot (A + B \cdot HCl_t) \qquad (\text{Condition } \{CuCl\} = 1) \tag{22}$$

where the empirical constants are the same as above.

In order to determine the composition of the acetylene complexes $(C_2H_2)_n(CuCl)_p(Cl^-)_q$ we have to compare (24) and the thermodynamic expression (3):

$$P_A \cdot \{CuCl\} \cdot (A + B \cdot HCl_t) = \sum n.K_{n,p,q} \cdot P_A^{\,n} \cdot \{CuCl\}^p \cdot HCl_t^{\,q} \cdot F_{n,p,q} \tag{29}$$

where $F_{n,p,q}$, which we regard as functions of P_A, $\{CuCl\}$, and HCl_t are defined according to

$$F_{n,p,q} \equiv \frac{[Cl^-]^q \cdot \gamma_{Cl}{}^q}{HCl_t{}^q \cdot \gamma_{n,p,q}} \tag{30}$$

We do not know how the $F_{n,p,q}$ functions depend on the three variables. But under certain conditions we have reason to assume approximately constant $F_{n,p,q}$ values, when altering one of the variables. Therefore we have drawn definite conclusions on the composition of the complexes only for such conditions. The result is that the acetylene content (n) of the complexes can de determined with fair certainty, the copper content (p) less accurately, and regards the chloride content (q), the premises for definite conclusions are not very substantial.

Since the simple empirical functions are valid over the whole range, we are forced to consider the assumption that the $F_{n,p,q}$ values for the existing complexes are *entirely independent* of the three variables, and that the complexes $C_2H_2 \cdot CuCl$ and $C_2H_2 \cdot CuCl_2^-$ predominate. The assumption would imply

$$\begin{cases} \text{For } C_2H_2 \cdot CuCl: & F_{1,1,0} = \gamma_{1,1,0} \text{ constant} & (31\text{ a}) \\ \text{For } C_2H_2 \cdot CuCl_2^-: & F_{1,1,1} = [Cl^-] \cdot \gamma_{Cl}/HCl_t \cdot \gamma_{1,1,1} \text{ constant} & (31\text{ b}) \end{cases}$$

It is reasonable to assume a constant activity coefficient for the uncharged complex, also $F_{1,1,1}$ being independent of HCl_t, which would imply a proportionality between the activity coefficients of the complex anion and of the chloride ion at variations of HCl_t. But it is more difficult to understand how $F_{1,1,1}$ could possibly remain constant when the cuprous chloride activity is varied. *Cf.* the previous discussion of this question where it is pointed out that an assumption of a constant product $[Cl^-] \cdot \gamma_{Cl}$, *i.e.* a hydrochloric acid activity independent of $\{CuCl\}$ and P_A, might be feasible.

To judge the possibility of these hypotheses further investigations are necessary, particularly concerning the inorganic $CuCl-Cl^-$ system. But it should be stressed that the simple empirical relations indicate a system of simple complexes, and that the activity coefficients, in spite of (or perhaps due to) the high electrolyte concentration, depend on the composition of the system in a simple manner.

Continued investigations aim at an analysis of the composition of the acetylene complexes in other systems and at an explanation of the empirical relations in the equilibrium experiments described here.

APPARATUS AND PROCEDURE

For the *absorption experiments* has been used the simple apparatus of Fig. 7. The vessel *a* is exchangeable, its size is so chosen that the amount of liquid suits the absorption capacity of the solution. The relation between liquid and total volume of the vessel is so adjusted that 20—40 % of the acetylene introduced remains in the gas-phase.

The distribution between liquid and gas remains practically unaltered at the successive additions of acetylene, since the absorption in these experiments is almost propor-

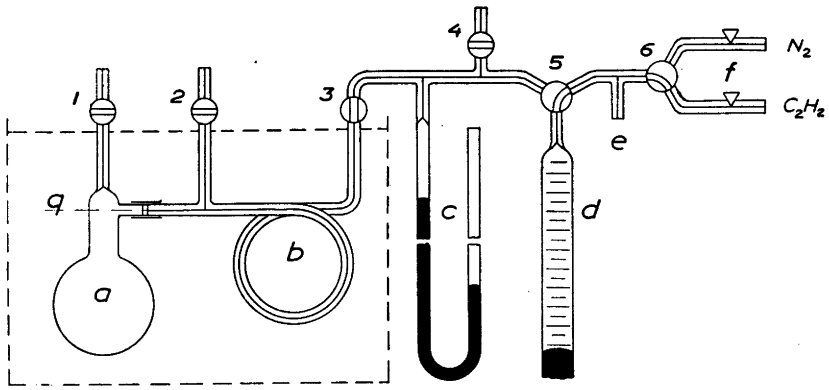


Fig. 7. Apparatus for absorption measurements.

a) Reaction flask. Sways with line q as axis.
b) Diffusion capillary, length 600 mm, int. diam. 1 mm
c) Mercury manometer
d) Burette, water-jacketed
e) To differential manometer for burette pressure
f) Mercury seal

tional to the acetylene pressure. Impurities in the acetylene (about 0.4 vol. per cent) thus cause a constant relative error in P_A. If 40 % of the acetylene remains in the gaseous phase and the impurities be insoluble in the liquid, an enrichment occurs which gives a P_A-error of 1 %. If the impurities are partly soluble the relative error is smaller, but still remains approximately constant.

The flask is connected by rubber tubing whose impermeability for the gases used has been checked. The capillary *b* facilitates measurements at higher temperatures; no condensation will occur in the manometer or other cold parts, if the experiment is so performed that the gas is flowing *towards* the flask only.

The volume of the gas phase is determined in each experiment by calibration with nitrogen. The deflections of the manometer will cause minor changes in the volume of the system; the quotient between nitrogen introduced (V_N) and the partial pressure (P_N) increasing linearily with P_N, which may be used for a graphical check.

Type Experiment

Determination of acetylene absorption in 4 M HCl; CuCl (s); 25.0°. In a 100 ml flask is introduced 6.0 g of fine-crystalline cuprous chloride. The side tube is sealed and the flask is evacuated (c. 0.01 mm Hg) for half an hour with intermittent shaking. 4.00 m hydrochloric acid, prepared from constant-boiling acid, is freed from dissolved oxygen by bubbling with nitrogen in a burette-like device with a fritted disk in the bottom and a liquid seal at the top. The burette valve is connected via a three-way stopcock to the closed evacuated flask. When this passage has been evacuated a suitable quantity of

acid is sucked into the flask; in this case 21.28 g of 4.00 *M* hydrochloric acid, containing 18.57 g water.

The flask, filled with nitrogen through *1*, is then connected to the nitrogen-filled apparatus; during this operation a rapid stream of nitrogen is flowing out through the side tube, after connection venting through *2*.

The whole system is evacuated to determine the vapour pressure of the solvent. Unnecessary evaporation is avoided by using a closed vessel (volume about 200 ml) which is alternatively evacuated and connected to *1* until a constant pressure is achieved, in this experiment 19.6 mm.

After complete evacuation above *3*, the apparatus is calibrated by introducing portions of nitrogen, the flask remaining immobile. The operation gives the following values for V_N/P_N and P_N (units: ml at 16.3°, 759.8 mm Hg uncorrected): 0.1112—193.4; 0.1150—395.6; 0.1226—783.2; 0.1310—1239.2.

After a second evacuation to vapour pressure (complete above *3*) portions of acetylene are introduced under brisk shaking of the flask.

As shown in Table 5 constant pressure readings are obtained after 1—3 minutes. But when the pressure has reached about 1 atm the solid addition compound begins to crystallize from the supersaturated solution, and the pressure suddenly drops. From this particular experiment we have utilized even the introductory readings from the last acetylene addition ($V_t = 244.6$) since the pressure remained constant for about 5 min. before it dropped.

The accuracy of the experimental data can be estimated in the following way:

The partial pressure value is a difference between 2 readings (4 meniscuses) and the accuracy in P_A can be estimated to $\pm 3 \times 10^{-4}$ atm. The gas volumes are read in a 100 ml burette, graduated in 0.1 ml. The absolute error would be $\pm$ 0.15 ml when V_t is less than 100 ml, and for volumes over 100 ml (more than one burette) we assume a relative error of $\pm$ 0.15 %. V_g is known with the numerical accuracy of the tabulated values. The relative error in $[C_2H_2]_t$ is computed from the relative error in V_t, using the factor $V_t/(V_t - V_g)$, in this experiment approximately equal to 1.5. The first four values of $[C_2H_2]_t$ thus have an uncertainty of $\pm$ 3 units in the fourth place, other values about $\pm$ 0.2 %.

Potentiometric measurements. Amalgamated copper wire, and silver wire with electrolytically deposited silver chloride have been used as electrodes. To eliminate disturbances arising from the dissolution of the silver chloride a fresh covering is obtained by electrolyzing in the test solution (silver electrode as anode) using a low current density (1 mA/cm^2) for 2 minutes. The polarization potential quickly disappears and the potential then remains constant for a satisfactory period, 15—30 min. This electrolysis does not influence the composition of the solution. At the first measurement the vessel contains 4 m hydrochloric acid, saturated with cuprous chloride; this measurement thus yielding the standard potential, E_0, of the electrode couple. The solution is then diluted with acid, and the resulting molality of cuprous chloride is calculated, and checked by analysis. The experiment is concluded by the addition of cuprous chloride in excess to check the constancy of the standard potential.

The vessel, immersed in an oil-thermostat at 25.0°, is closed and has a slight overpressure of nitrogen, and is connected with a burette permitting the addition of oxygen-free solvent. The potentiometer is a PHM 3i (Radiometer, Copenhagen) so adjusted that the values of $f(E) = E \cdot F/RT \cdot 2.303$ at 25.00° is read directly. The difference $f(E_0) - f(E)$ gives log $\{CuCl\}$.

Table 5. Readings and computed data from an absorption experiment: 4 m hydrochloric acid, containing solid cuprous chloride. 25° C.

V_t	Minutes	P_A in mm	V_g	$[C_2H_2]_t$	$[C_2H_2]_{Cu}/P_A$ (atm.)
18.4	1	55.2			
	2	55.2	6.0	0.02825	0.351
43.9	1	130.9			
	2	130.7			
	3	130.7	14.0	0.06720	0.3545
76.0	2	225.2			
	4	225.0	25.2	0.1157	0.3537
95.9	1	283.6			
	2	283.4			
	4	283.4	32.0	0.1456	0.3529
123.0	3	361.9			
	4	361.9			
	5	361.9	41.4	0.1859	0.3533
155.0	1	453.2			
	2	453.3	52.6	0.2333	0.3541
188.4	2	548.2			
	3	547.9			
	4	547.9	64.7	0.2818	0.3540
203.6	3	591.0			
	3	591.0	70.2	0.3039	0.3538
218.3	1	633.2			
	3	633.1	75.7	0.3248	0.3527
228.8	2	662.4			
	3	662.4			
	7	662.3	79.5	0.3401	0.3534
244.6	2	706.3			
	4	706.1	85.4	0.3627	0.3524
	6	695.2			
	10	602.1			
	20	465			
	30	375.4			
	40	330.2			
	50	310.6			
	60	305.3			

V_t Amount of acetylene introduced, in ml (16.3°, 759.8 mm Hg).

V_g Amount of acetylene is gas phase, in ml (conditions as above). Computed from calibration described in text.

$[C_2H_2]_t$ Concentration (molality) of acetylene absorbed, computed from $V_t - V_g$ and quantity of water (18.57 g).

$[C_2H_2]_{Cu}$ Computed from $[C_2H_2]_t - aP_A$ with a-value from table 1.

$[C_2H_2]_{Cu}/P_A$ Absorption quotient in molality/atm.

Chemicals

Cuprous chloride of high purity, entirely free from cupric and metallic copper [15].

Acetylene of good purity in tubes with normal filling but without acetone.

Oxygen-free nitrogen has been prepared according to v. Wartenberg [16]. Analyses show the oxygen content to be below 0.001 % by volume.

Other chemicals of reagent grade.

SUMMARY

1. In an aqueous chloride solution of monovalent copper two types of dissolved acetylene compounds appear: yellow acetylidic compounds, where copper is substituted for the hydrogen atoms, and colourless addition compounds, where the hydrocarbon retains its hydrogen. As a rule the main part of the acetylene absorbed is present in addition complexes.

2. For systems consisting of cuprous chloride and hydrochloric acid empirical relations have been determined, connecting the total concentration of acetylene in addition complexes and the three variables: acetylene pressure, total concentration of dissolved cuprous chloride, and total concentration of hydrochloric acid. If the solubility of cuprous chloride is introduced as a parameter a simple algebraic function results (27, 28). Preliminary experiments in systems containing alkali chlorides instead of hydrochloric acid suggest analogous relations.

3. With cuprous chloride activity, acetylene pressure, and total hydrochloric acid concentration as variables a function (24) is obtained, according to which the total concentration of addition complexes is proportional to the two first variables and a linear function (with a constant term) of the third one.

4. From equilibrium experiments only the following conclusions can be drawn with certainty on the composition of the addition complexes:

a) complexes with *one* acetylene molecule predominate
b) complexes with *one* atom of copper exist
c) complexes with *one* and *two* atoms of chlorine can be assumed.

But the simple relations provoke some radical assumptions concerning activity coefficients *etc.* If these be accepted the experiments show that the complexes $C_2H_2 \cdot CuCl$ and $C_2H_2 \cdot CuCl_2^-$ predominate in 1—10 m hydrochloric acid.

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Studies on the Proteolytic and Antiproteolytic Activity of Blood Serum

I. Activation of Plasminogen with Trypsin

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Denys and Marbaix [1] showed that normal blood serum can be rendered proteolytic by treatment with chloroform. Somewhat later Delezenne and Pozerski [2] made the same observation. They also showed that the trypsin inhibiting effect of serum is diminished by shaking with chloroform. It has also long been known that the addition of certain tissue cells can render serum proteolytic. (For references see Permin [3].)

Tillett and Garner [4] showed that certain β-hemolytic streptococci produce a substance that can break down fibrin clots. Milstone [5] found that this substance does not break down fibrin clots prepared from highly purified fibrinogen and that a serum factor and a streptococcus factor are needed for the break-down of fibrin clots. He also showed that this serum factor is to be found in the euglobulin fraction of serum.

According to Christensen [6] the serum factor is an enzymogen, which can be enzymatically activated to a proteolytic enzyme. Christensen and MacLeod [7] named the precursor plasminogen, the active enzyme plasmin, and the streptococcus factor streptokinase. Ratnoff [8], however, claims that plasminogen reacts stoichiometrically with streptokinase to give plasmin.

Christensen [9] confirmed Delezenne and Pozerski's experiment and also showed that after the trypsin inhibitor has been destroyed by chloroform the activation curve for plasmin is of an autocatalytic type.

Recently Lewis and Ferguson [10] claimed to have prepared a plasminogen activator from blood serum.

The experiments reported below show that on the addition of trypsin to a crude plasminogen fraction part of the trypsin added is immediately inhibited, that the remaining free trypsin has the power to activate plasminogen, and that the rate of activation is dependent on the remaining trypsin activity.

EXPERIMENTAL

Temperature. All experiments were performed at 25° C ±0.1° C.

Buffer solutions. Buffer A was prepared from sodium acetate (final concentration 0.029 *M*) and sodium diethyl-barbiturate (final concentration 0.032 *M*) and HCl to give a pH of 7.5. Buffer B = buffer A but contains also $CaCl_2$ in a final concentration of 0.27 *M*.

Trypsin. Crystalline trypsin (Worthington lab.) was dissolved in 0.0025 *N* HCl and diluted in buffer B ten minutes before use. Trypsin is stabilized by the presence of Ca-ions as shown by Bier and Nord [11].

Plasminogen. 40 ml human blood serum was diluted with distilled water to a specific resistance of 700 ohms and adjusted to pH 5.2 with *N* acetic acid. It was allowed to stand for 1 hour at +4°C and was then centrifuged after which the precipitate was dissolved in 20 ml buffer B. To this solution 6 ml ether was added and the test tube was placed in an ethanol-carbon dioxide bath at −30 − 35° C from which it was removed at short intervals and turned upside down to secure a satisfactory mixture during freezing. The test tube was kept at this temperature for about 15 minutes and then placed in a refrigerator where it was allowed to thaw slowly at +4° C. The ether layer was then sucked off and discarded. The aqueous layer was again frozen and the ether was evaporated with the aid of a water-suction pump.

Streptokinase. The hemolytic streptococcus H 46 A * was grown on a synthetic substrate. The streptokinase was then highly purified but was still not quite free from plasmin inhibitor.

Fibrinogen was prepared from oxalated pig's plasma according to the freezing method of Ware, Guest and Seegers [12] and dissolved in buffer A to a concentration of 0.17%.

Thrombin Topical (Parke & Davis) was dissolved in buffer A. The sample used contained a trypsin inhibitor that inhibited about 5 γ trypsin/ml.

Determination of plasmin activity. To 0.5 ml fibrinogen was added 0.1 ml buffer A and 0.1 ml buffer B and 0.2 ml of the solution to be tested and within 0.07 minutes 0.1 ml thrombin, which clotted the fibrinogen within 0.10 minutes. Immediately after the addition of thrombin the tube was shaken. In this way air bubbles were included in the clot and the lysis time was thus easy to record as the time when the air bubbles had risen to the surface.

For the concentrations of plasmin used in these experiments there is a fairly good proportionality between the inverted lysis time and concentration. Concentrations were, however, calculated from a standard curve relating concentration to lysis time. The fibrinolytic unit for plasmin was taken so that one unit will lyse the clot in 10 minutes under the conditions used. Because of spontaneous lysis less than 0.02 fibrinolytic units cannot be measured. Details of the standardization of plasmin and the influence of ions and of coagulation time will be published later in this series.

Determination of trypsin activity was made essentially according to Anson's [13] hemoglobin method but with hemoglobin in buffer A instead of in phosphate buffer. The extinction values of the trichloroacetic acid filtrate were measured spectrophotometrically at 2 800 Å. The trypsin concentration was checked according to Kunitz [14] with 5 times crystallized soy bean trypsin inhibitor (Worthington lab.).

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* Obtained by courtesy from Dr Christensen, New York.

Interaction of trypsin and plasmin. In order to measure the trypsin and the plasmin activity separately in a mixture of the two enzymes, it is desirable to have either different substrates, which are attacked only by one of the enzymes or to have potent inhibitors that act selectively on the two enzymes. Grob [15] showed that plasmin does not attack hemoglobin in strong urea solution. This was confirmed in the present experiments using Anson's hemoglobin method for determining trypsin. Streptokinase-activated plasmin containing 20 fibrinolytic units/ml had no demonstrable activity against hemoglobin. Thus trypsin can be determined separately when mixed with plasmin.

No substrate is known that is attacked by plasmin but not by trypsin, neither is an inhibitor available that inhibits trypsin but not plasmin. The results of earlier experiments, however, showed that fibrinogen prepared according to the method used here seems to be contaminated with an inhibitor which reacts rapidly with trypsin but only slightly with plasmin (Jacobsson, unpublished experiments).

This might explain why under the experimental conditions used the highest amount of free trypsin present possessed only a relatively slight fibrinolytic activity as compared with the fibrinolytic activity of the activated plasmin. Thus a solution containing 20 γ trypsin/ml had a fibrinolytic effect equal to 0.6 fibrinolytic units/ml.

Activation experiments. To each of a set of test tubes containing 2 ml plasminogen was added 2 ml of trypsin of varying concentration. 0.1 ml aliquots were drawn from each tube immediately after mixing and 2 hours later and tested for trypsin activity.

Table 1. shows that the same amount of trypsin was immediately inhibited in each tube, and that afterwards the trypsin activity remained unchanged for at least 2 hours.

Table 1.

γ Trypsin / ml			
	Free trypsin		
Total trypsin	immediately	after 2 hours	Trypsin inhibited
37.0	19.5	19.4	17.5
33.3	16.2	16.2	17.1
29.6	11.8	11.6	17.8
25.9	7.9	7.7	18.0
22.2	4.4	4.4	17.8
18.5	1.1	1.2	17.4

From each tube also 0.2 ml aliquots were withdrawn immediately and after varying intervals and tested for plasmin activity. The plasmin activity rose with time in all tubes (Fig. 1). The rate of plasminogen activation was very slow in the tube containing only 1.1 γ free trypsin/ml and the plasmin activity was not measurable until after 3 hours. The maximum activities of the tubes with 4.4 and 7.9 γ free trypsin/ml were 6 and 7 fibrinolytic units obtained after 6 and 2 hours respectively. Plasminogen in two control tubes without trypsin did not become spontaneously active even after 24 hours.

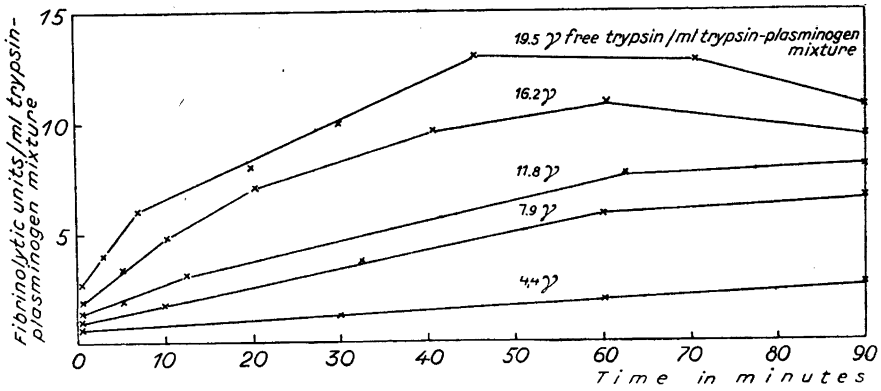

Fig. 1. The plasmin activity in fibrinolytic units/ml trypsin-plasminogen mixture as a function of time.

DISCUSSION

The interceptions of the time-activity curves with zero time lie at somewhat higher values than those expected from the values of free trypsin. The reason for this is not clear.

The rate of activation as measured by the slope of the initial part of the activation curves rises with increasing concentration of free trypsin in the trypsin-plasminogen mixture. The rate of activation is, however, not quite proportional to the concentration of free trypsin. High concentrations of free trypsin seem to produce a proportionally greater plasminogen activating effect than low concentrations. This is at least partly due to an unavoidable experimental error: the activation proceeds during the time of lysis. Thus the moment corresponding to the measured fibrinolytic activity is not the moment when the trypsin-plasminogen mixture is added to fibrinogen but at a time somewhat later, which cannot be exactly determined. The trypsin inhibitors present in fibrinogen and thrombin counteract this effect relatively more with those trypsin-plasminogen mixtures containing lower concentrations of free trypsin than with those containing higher concentrations of free trypsin. Thus this experimental error has its greatest effect on the curves representing the highest concentrations of trypsin.

As seen from the upper curves on Fig. 1 plasmin is not stable in the presence of trypsin. This may possibly explain that the final plasmin activity is lower, when the activation rate is slow, and higher when the activation is more rapid. The highest plasmin activity obtainable with trypsin as an activator was 13 fibrinolytic units/ml as compared to 17 units when streptokinase was used as an activator.

The enzyme precursor, that can be activated to a fibrinolytic enzyme by streptokinase has been named plasminogen by Christensen and MacLeod. The enzyme precursor activated by trypsin in the experiments above is obtained from the same serum fraction as plasminogen. As the trypsin-activated enzyme is similar to plasmin in respect to its action on fibrin and on hemoglobin in urea it may be assumed to be plasmin. Of course these experiments do not prove conclusively that it is the same precursor which is activated by streptokinase and by trypsin.

SUMMARY

When varying amounts of trypsin are added to a constant amount of a crude plasminogen fraction, a constant amount of trypsin is immediately inhibited. Then the remaining free trypsin activates plasminogen to plasmin. The rate of activation increases with increasing concentration of free trypsin.

Addendum: After this article had gone to press Kocholaty, W., Ellis, W.W., and Jensen, H. *Blood* **7** (1952) 882 have also shown that trypsin can activate plasminogen to plasmin.

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A Study of Yeast Catalase

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The enzyme catalase has been prepared in crystalline form from a number of animal sources and from the bacterium *Micrococcus lysodeikticus* (Herbert and Pinsent [1]); and a highly purified preparation from spinach leaf has recently been described (Galston, Bonnichsen and Arnon [2]). Little is known about the nature of catalase from sources other than the above due to the very low concentrations of the enzyme in most cells and tissues. Von Euler, Fink and Hellström [3] have demonstrated the presence of catalase activity in yeasts and have shown that the catalase and cytochrome-c contents vary in parallel.

The present paper describes the purification, and a study of the properties of catalase obtained from yeast cells.

EXPERIMENTAL

A. Estimation

Assays of catalase activity were made by the rapid titration method of Bonnichsen, Chance and Theorell[4], and monomolecular k values calculated from the titration data according to the equation:

$$k = \frac{1}{t} \log_{10} \frac{x_0}{x_t}$$

where x_0 is the titre at zero time and x_t is the titre t minutes after the reaction has begun. The Kat.f. value of von Euler and Josephson [5] was used to express the purity of samples where

$$\text{Kat.f.} = \frac{k}{\text{g enzyme used in test}}$$

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As solutions of the partially purified material appeared to be unstable and to lose a considerable amount of activity during dialysis, the k value was measured before dialysing the sample free of salt to estimate the dry weight, and protein determinations were made before and after dialysis to observe the dilution factor during this process. The dry weight per ml of the sample after dialysis for 24 hours against a total of 4 litres of distilled water was measured after drying a suitable volume on a tared watch-glass at 105° C for 12 hours. The protein determinations were carried out by adding appropriate amounts of the solution to 1 ml of 0.6 M ammonium sulphate, adding distilled water to 9.5 ml and 0.5 ml of 1.5 M trichloroacetic acid, then shaking and reading optical density at 350 mμ within a few minutes.

Observations were carried out on sufficient diluted, dialysed samples to give a standard curve for the sample. The weight of the preparation used in the Kat.f. determination was then calculated from the dry weight observation and the dialysis dilution factor.

B. Extraction

Preliminary experiments were carried out to find the most advantageous method of extracting catalase from yeast cells. Extracting air-dried or acetone-ether dried baker's yeast with two volumes of aqueous $M/15$ secondary sodium phosphate or $M/15$ sodium acetate gave extracts containing approximately 70 g of solids per kilogram of dried yeast, with Kat.f. values between 6 and 7. Preliminary extraction of the yeast with n-butanol did not appreciably affect this result. Autolysis under toluene and extraction with water according to Meyerhof [6] resulted in preparations with Kat.f. values between 7 and 8 but with no increase in total activity extracted per kilogram dry weight above that obtained from dried yeasts. From these figures it can be seen that the amount of extractable catalase in Swedish baker's yeast is very small, about 14 mg per kilogram of dried yeast if pure yeast catalase is assumed to have a Kat.f. of 36 000 as estimated below.

C. Purification of the Enzyme

20 kg of commercial dried yeast (supplied in this form by Svenska Jästbolaget) were stirred into 50 litres of $M/15$ sodium acetate and the mixture centrifuged at 2 000 r.p.m. for 1 hour after standing for 2 hours. 65 ml of $M/2$ basic lead acetate was added per litre of the cloudy yellow supernatant and the resultant suspension was centrifuged for 20 minutes at 2 000 r.p.m., the precipitate being discarded. 400 g of solid ammonium sulphate per litre were dissolved in the supernatant and the suspension left for 12 hours at 4° C and then centrifuged at 2 000 r.p.m. for 90 minutes. The supernatant was discarded and the precipitate dissolved in 450 ml water and 1/5 volume of 1.6 % calcium phosphate gel, prepared according to Keilin and Hartree [7], added. After standing at room temperature for 30 minutes the suspension was centrifuged and the precipitate discarded. The supernatant was made $M/15$ with respect to secondary sodium phosphate and cooled to 0° C. A $\frac{1}{2}$-volume of cold (−15° C) acetone was added slowly with vigorous stirring while the temperature of the mixture was lowered to −5° C and the precipitate discarded. The acetone concentration of the supernatant was then brought to 50 % while the temperature was lowered to −8° C. The precipitate was centrifuged down at −8° C, stirred with 200 ml of ice-cold distilled water, and dialysed against distilled water at 4° C for 12 hours. (No loss of activity was apparent during this dialysis if insoluble denatured material was left in suspension in the dialysis tube). The suspension was centrifuged clear

and the supernatant made 0.02 *M* with respect to acetate buffer pH 5.7 and the solution made 40 % with respect to ethyl alcohol, the temperature being lowered to −12° C during the slow addition of the alcohol. The suspension was centrifuged at −12° C and the precipitate dissolved in 75 ml of cold distilled water. An equal quantity of 1.6 % calcium phosphate gel was added at 0° C and the mixture was centrifuged after 1 hour. The precipitate was washed four times, each with 1 litre of cold distilled water. The enzyme was eluted by decomposing the calcium phosphate precipitate with 25 ml of a cold aqueous solution which was 0.5 *M* with respect to potassium oxalate and 0.5 *M* with respect to potassium hydrogen phosphate, and centrifuging down the resulting calcium oxalate which was washed with a further 5 ml of the potassium oxalate solution and 15 ml water. 52 g/100 ml of solid ammonium sulphate was added to the combined supernatant and washings and the resultant precipitate was centrifuged down. This precipitate was dissolved in sufficient 0.1 *M* acetate buffer, pH 5.0, to give an optical density of 1 at 405 mμ after centrifuging clear, and 24.3 g of solid ammonium sulphate/100 ml added, and the resulting precipitate was centrifuged down and discarded. A further 9.6 g of ammonium sulphate/100 ml were then added and the precipitate centrifuged down and stored at −15° C.

The Kat.f. of samples purified in this way varied between 5 000 and 8 000, about a 1 000-fold purification being achieved by the above method with yields varying between 16 % and 24 %. Electrophoresis at pH 7.0 in the Tiselius apparatus showed the presence of three components all moving to the anode. Attempts to purify the enzyme further by electrophoresis were unsuccessful due to the instability of solutions of the partially purified enzyme. Attempts to crystalline the partially purified material with ammonium sulphate and alcohol at low temperature met with no success.

D. Properties of the Purified Enzyme Preparation

1. Stability. Yeast catalase appears to be very much less stable than most of the catalases so far studied in a purified state. Precipitation of yeast catalase preparations at pH 7.0 with 60 % alcohol at temperatures above −5° C results in almost complete disappearance of activity, whereas fractionation with alcohol at room temperature has been a common step in the preparation of most of the other catalases so far studied. The action of acetone on yeast catalase appears to be less severe, approximately 50 % loss in activity occurring when it is precipitated at 4° C with 50 % acetone. A clarified solution of the purified material at pH 7 lost about 20 % of its activity on standing at 4° C for 3 hours. At pH 4.5 this percentage of activity was lost within 15 minutes.

2. Absorption Spectrum. The absorption spectra of a purified preparation with a Kat.f. value of 8 000 and its cyanide complex are shown in Fig.1. The spectra were taken of solutions in *M*/15 phosphate buffer pH 6.8, and show all the absorption peaks characteristic of catalases and their cyanide complexes. The peak at 340 mμ in the spectrum of the cyanide complex was probably due to the presence of a flavoprotein impurity which is masked by the Soret band in the spectrum of the original preparation. The ratio E_{280}/E_{405} for this preparation was 4.17.

3. Nature of the Prosthetic Group. An aqueous solution of the purified preparation was treated with three times its volume of cold 1 % HCl in acetone. The resulting precipitate of denatured protein was removed by centrifugation and the acetone removed from the supernatant by a stream of air. A brown precipitate appeared in the remaining colourless aqueous layer and was separated by centrifuging and dissolved in a small amount of

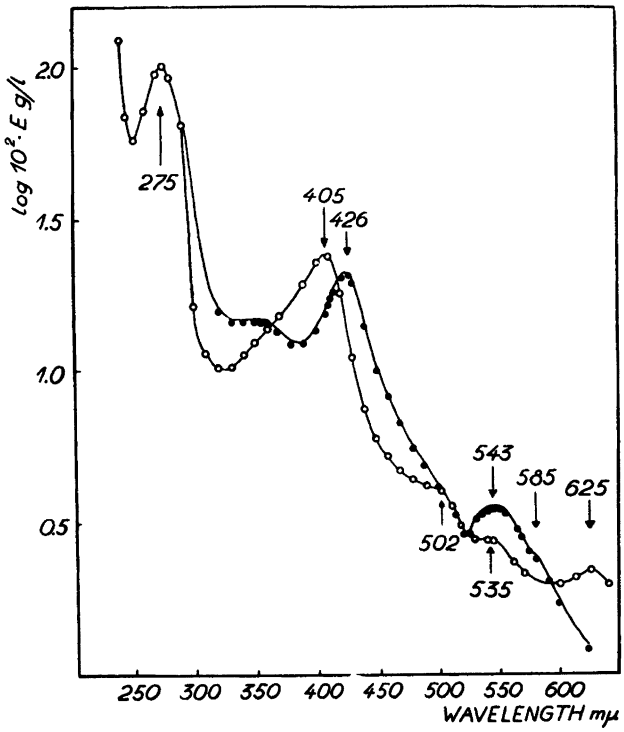

Fig. 1. The absorbtion spectra of purified yeast catalase and its cyanide complex. Yeast catalase: O——O——O *Yeast catalase cyanide:* ●——●——●

pyridine. After the addition of a quarter-volume of *N* NaOH and a few crystals of $Na_2S_2O_4$, a sharp absorption peak with a maximum at 556 mμ was observed with a Beckman spectrophotometer. This observation, and the fact that the aqueous layer after removal of acetone was colourless, indicates that the prosthetic group of yeast catalase is protohaematin and that no detectable bile pigment haematin is present.

4. Iron Content. After digestion with sulphuric acid in the presence of H_2O_2, the preparation was washed into a 10 ml volumetric flask and 0.2 ml of 20 % sulphosalicylic acid added. Ammonia was then added slowly until the solution became yellow when it was made up to volume and the absorption at 424 mμ measured in a Beckman spectrophotometer. The sample with a Kat.f. value of 8 000 contained 0.021 % iron.

5. Inhibition Studies. The effects of potassium cyanide and sodium azide on the activity of yeast catalase are shown in Fig. 2. A NaN_3 concentration of $1.4. \times 10^{-6}$ *M* produces a 50 % inhibition of the enzyme activity, and the same percentage inhibition is produced by a 3.8×10^{-5} *M* concentration of KCN.

6. Electrophoretic Behaviour. Although it was not possible to purify the preparation any further by electrophoresis due to the large loss of activity that occurred during the electrophoresis and the preliminary dialysis, useful information concerning the composition of the preparation was obtained by observations on a sample with a Kat.f. value of

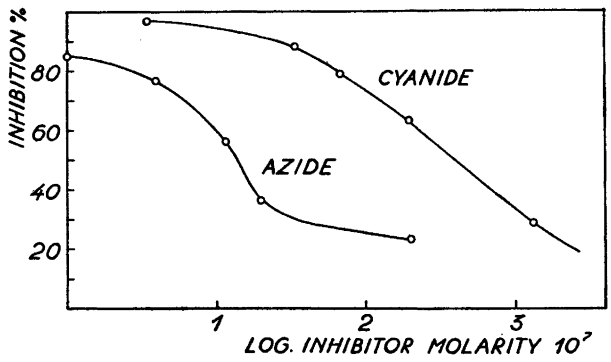

Fig. 2. The effect of potassium cyanide and sodium azide on the activity of purified yeast catalase.

7 200 and an iron content of 0.019 %. After 3 hours of electrophoresis at 25 mA, and 200 V of a 0.5 % solution in phosphate buffer, pH 7.05 of ionic strength 0.1, three distinct components A, B and C were observed all moving to the anode. A, the fastest moving component, was colourless, B had a deep yellow colour, and C a pale yellow colour. Samples were pipetted using a hollow needle from between the A and B boundaries, the B and C boundaries, and behind the C boundary in the anodic limb of the U-tube, and activity and iron determinations were carried out on each sample. The first two samples did not contain any detectable iron or enzyme activity, but the sample taken from behind the C boundary contained all the iron and the residual enzyme activity of the solution. It seems, therefore, that the component C was the partially inactivated catalase. From the areas enclosed under the peaks representing each boundary, and by assuming that the refractive index increments of the three components were the same, it was calculated that this preparation, with Kat.f. 7 200, contained 28 % of component A, 52 % of B, and 20 % of C.

DISCUSSION

Although the complete purification and crystallisation of yeast catalase has been so far prevented by its instability, it has been possible to characterise it sufficiently to show its similarity to other purified catalases. Assuming that the preparation with a Kat.f. value of 7 200, and an iron content of 0.019 %, was 20 % pure, as indicated by the electrophoresis data, pure yeast catalase would have a Kat.f. value of 36 000 and an iron content of 0.095 % as compared with the values of 65 000 and 0.095 % obtained by Bonnichsen [8] for horse erythrocyte catalase. If 0.095 % is accepted as the correct value for the iron content of pure yeast catalase, then the purest preparation with Kat.f. of 8 000 and iron content 0.021 % would have a purity of 23 %, and the Kat.f. of the pure catalase in this preparation would be 36 500. The value of E_{405}/E_{280} would then be 1.08 as compared with Bonnichsen's value of 1.26 for horse

erythrocyte catalase [8]. It appears, then, that the above facts are consistent with yeast catalase being a four haematin catalase with a Kat.f. value of 36 000.

The instability of yeast catalase is not so surprising in the light of the recent observation of Deutsch [9] who has shown the existence in erythrocyte catalase preparations of components with high Kat.f. values which rapidly decrease on dilution or standing to the values usually obtained. This instability may explain the low Kat.f. value estimated for yeast catalase in this work.

SUMMARY

A catalase from yeast has been purified over 1 000-fold and its properties studied. It has been shown that its properties are similar to those of catalases purified from other sources, except in its large degree of instability, and these properties are consistent with it being a four haematin catalase with a Kat.f. of 36 000.

The author is greatly indebted to Professor Hugo Theorell for his advice and the hospitality of his laboratory, and to Dr. R. K. Bonnichsen for advice and for suggesting this work.

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Die Zuckerarten in *Allium cepa*

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Die Angaben über die Art und Menge der Zucker in den Zwiebeln von *Allium cepa* L. sind nicht übereinstimmend. Ich habe darum Monosaccharide und Disaccharide in den Zwiebeln von *Allium cepa*, Sorte Blutrot Holländisch untersucht. Die Aldosen wurden nach Willstätter-Schudel, in der Modifikation von Auerbach und Bodländer [1], bestimmt und die Fructose nach Nijn, in der Modifikation von Zerban und Sattler [2]. Ausserdem wurden die Versuchslösungen nach der papierchromatographischen Methode untersucht.

Von den älteren Arbeiten ist die von Wächter [3] zu erwähnen. Er fand einen Gesamtzuckergehalt nach der Inversion von 6,7—7,0 %, berechnet auf das Frischgewicht der Zwiebel. Kayser [4] findet als erster in den Zwiebelknollen Rohrzucker; doch der eigentliche Beweis, dass der nichtreduzierende Zucker tatsächlich Saccharose ist, geht nicht aus der Arbeit hervor. Schulze und Frankfurt [5] finden zwar grosse Mengen eines nicht reduzierenden Zuckers, doch meinten sie den Nachweis zu erbringen, dass dieser Zucker nicht mit Saccharose identisch ist. Grafe [6] bestätigt, dass keine Saccharose vorhanden ist, meint aber, das Vorkommen von Maltose mit Hilfe der mikrochemischen Phenylhydrazinmethode beweisen zu können. Cooke [7] hat die oberirdischen grünen Blätter von *Allium cepa* untersucht und fand darin Saccharose. Yanovsky und Kingsbury [8] konnten in *Allium nuttallii* S. Wats. ein Polyfructosan nachweisen. Wilson [9] und Zeller [10] haben als gegeben angenommen, dass aller nichtreduzierende Zucker Saccharose ist, aber ein beträchtlicher Teil davon sind Polyfructosane, die durch gewöhnliche Hydrolyseverfahren in Fructose übergehen.

Methodisches: Die Zwiebeln wurden am 10. Nov. 1952 fein zerschnitten und mehrere Portionen zu je 20 g Frischgewicht abgewogen. Die einzelnen Portionen wurden mit 2 g $BaCO_3$ und 150 ml 85 % Äthanol auf dem Wasserbad kurz aufgekocht, eine Woche im Dunkeln verwahrt und dann mit soviel 85 % Äthanol nachgefüllt, dass die einzelnen Kolben genau 200 g Flüssigkeit enthielten. Nach guter Umrührung, abermaligem Stehen

in 24 Stunden und Filtrieren wurden 150 g Flüssigkeit einer Portion in Arbeit genommen. Äthanol wurde auf dem Wasserbad abgedampft und der Rückstand in einen 25 ml Kolben überführt. 10 ml dieser Zuckerlösung wurden mit 5 ml 10 % neutralisiertem Bleiacetat gefällt, in einem Kolben mit Wasser zu 25 ml aufgefüllt und das Ganze filtriert. In dem klaren Filtrat wurden die Zucker bestimmt.

Zur Inversion wurden die übrigen 15 ml der ursprünglichen Zuckerlösung 1 Stunde auf dem Wasserbad mit 10 ml 10 % Citronensäure gekocht, mit 5 ml 11,4 % NaOH neutralisiert, mit 10 ml 10 % neutralisiertem Bleiacetat gefällt und filtriert. In diesem Filtrat wurden die Zucker bestimmt. Die papierchromatographische Methode wurde in der von Partridge[11] beschriebenen Form gehandhabt. Als Lösungsmittel kam Butanol-Essigsäure in Anwendung und das Entwickeln geschah teils mit Anilinphthalat teils mit Naphthoresorcin.

Ergebnisse. Die Papierchromatographie ergab den Beweis für das Vorkommen von Saccharose und Fructose in den nichtinvertierten Zuckerlösungen — mit Naphthoresorcin entwickelt — ausserdem fanden sich Stoffe mit niedrigeren R_F-Werten als Saccharose. Die Identifizierung dieser Stoffe wurde nicht vorgenommen. Es müssen aber verschiedene Polyfructosane sein; denn nach der Hydrolyse mit Citronensäure wurde mehr Fructose gefunden, als durch Hydrolyse von Saccharose gebildet werden konnte. Aus Papierchromatogrammen von nichtinvertierten Zuckerlösungen, mit Anilinphthalat entwickelt, konnte mit Sicherheit geschlossen werden, dass das von Grafe hervorgehobene Vorkommen von Maltose in den Zwiebeln von *Allium cepa* nicht nachweisbar war. Das Vorkommen von Glucose und Fructose konnte sowohl vor als auch nach der Inversion bestätigt werden.

Die quantitativen Untersuchungen nach den obengenannten Reduktionsmethoden von 6 der bereiteten Portionen ergaben im Durchschnitt 411 mg Glucose und 179 mg Fructose pro 20 g Frischgewicht von *Allium cepa*, beides vor Inversion der Zuckerlösung bestimmt. Das Verhältnis Glucose : Fructose vor der Inversion ist ungefähr 2 : 1, ein anscheinend häufig vorkommendes Phänomen in unterirdischen Vorratsorganen. Es ist auch von Middelboe und Müller[12] in Kohlrabi *(Brassica napus napobrassica)* und Weissrüben *(Brassica campestris rapifera)* gefunden worden. Nach der Inversion ergab die Bestimmung 751 mg Glucose und 840 mg Fructose, ebenfalls pro 20 g Frischgewicht von *Allium cepa*. Bei der Inversion wurden also 340 mg Glucose und 661 mg Fructose gebildet. Daraus errechnet sich der Gehalt an Saccharose zu 646 mg pro 20 g Frischgewicht. Die übrigen 321 mg Fructose, gebildet bei der Inversion, müssen bei der Hydrolyse von in 85 % Äthanol lösligen Polyfructosanen entstanden sein. Die Resultate umgerechnet in g, bezogen auf 100 g Frischgewicht und 100 g Trockengewicht sind in der Tabelle 1 zusammengestellt.

Tabelle 1. Zwiebeln von Allium cepa.

100 g Frischgewicht enthalten	% der Trockensubstanz
2,05 g Glucose	17,34
0,90 g Fructose	7,55
3,23 g Saccharose	27,26
1,45 g Polyfructosan	12,19
11,85 g Trockensubstanz	
	64,34 %

ZUSAMMENFASSUNG

Die Zuckerarten in *Allium cepa* wurden papierchromatographisch, nach Willstätter-Schudel und nach Nijn bestimmt. Die Chromatographie ergab den Beweis für das Vorkommen von Glucose, Fructose, Saccharose, unbekannte Polyfructosane und das Nicht-Vorkommen von Maltose. Die quantitativen Bestimmungen ergaben die in der Tabelle 1 zusammengestellten Resultate.

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Short Communications

The Assay of Horse Radish Peroxidase (HRP) with Mesidine

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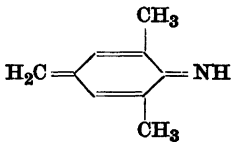
Several procedures have been suggested for the assay of HRP (for references see [1]), of which the purpurogallin test is the most commonly used. There is a common experience of this method that it frequently gives erratic results, which have been attributed to impurities in the pyrogallol or in the distilled water. Byproducts can also sometimes appear in the reaction.

Chapman and Saunders [2], studying the HRP-catalyzed oxidation of mesidine (2,4,6-trimethylaniline) by hydrogen peroxide, found that mesidine gave rise only to one compound (2,6-dimethyl-benzoquinone-4-(2′,4′,6′)anil, "purple compound"), which could be isolated in 95 % yield. One mole of ammonia is liberated during the reaction. They also suggested as a hypothesis that an intermediary compound could be formed with the subsequent



release of the methylene group as formaldehyde.

We have confirmed their hypothesis and found the over-all reaction formula to be 2 mesidine + 3 H_2O_2 = 1 purple compound + 1 NH_3 + 1 HCHO + 4 H_2O;

The formaldehyde was identified as methylene dimedone (80 % yield, m.p. 189–192° after 3 crystallizations).

When the reaction is carried out at 0° and pH 4.7–4.8 (5 mM H_2O_2, 15 mM mesidine, total volume 2 ml) and stopped by the addition of one volume of acid alcohol, the formation of the purple compounds is linearily proportional to the time after a certain "lag" phase. This permits the calculation of the amount of HRP from the slope of $\log \frac{I_0}{I}$ at 490 mμ vs. time according to

$$[\mathrm{HRP}] = k \times \frac{\log \left(\frac{I_0}{I}\right)_2 - \log \left(\frac{I_0}{I}\right)_1}{t_2 - t_1};$$

[HRP] and reaction time must be chosen so that $\log \left(\frac{I_0}{I}\right)_1 > 0.175$, and the time to reach or pass this value less than 1.2 min. Practically this corresponds to [HRP] = 15–75 mμM. For the specimen of HRP (twice crystallized) employed in our determinations, HRP is obtained in mμM when $k_1 = 89.3$. Experiments with other preparations of HRP are being done to test the reproducibility of the numerical values of k.

The reaction is very little, if at all, sensitive to ferric and cupric ions.

We express our thanks to Dr. E. B. Saunders for suggesting mesidine as a suitable hydrogen donor for the method.

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Ultraviolet Absorption Spectra of Creatinine

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Grinbaum and Marchlewski [1] have reported that the ultraviolet absorption curve of creatinine in water exhibits a maximum at 234.5 mμ and a minimum at 228.7 mμ, though the curve published by them shows the minimum to lie approximately 10 mμ lower. These authors have further reported that the maximum at 234.5 mμ is enhanced in acid and abolished in alkali. According to the data presented below the latter findings are both erroneous. Bandow's statement [2] that the ultraviolet absorption spectrum of creatinine in water shows little which is worth of interest is likewise misleading.

Fig. 1 shows the absorption of ultraviolet light by creatinine between 210 and 290 mμ at various ranges of pH. The absorption measurements were taken with a Hilger Uvispek photoelectric spectrophotometer. The creatinine was a Kebo, Stockholm, product and was recrystallized from alcohol. A concentration of 10^{-4} molar in buffer, HCl, KOH, or water was used, with the solvent serving as the blank. The temperature was 22° C. Curve 2 of the figure is the absorption curve of creatinine in water and in buffer solutions and dilute alkali of pH 6.5—12.3. The position and intensity of the absorption maximum (λ = 234 mμ; ε = 6.9 × 10^3) agree well with the values given by Grinbaum and Marchlewski [1] for creatinine in "acid". The curve has a minimum at 216 mμ. Addition of acid does not enhance the absorption at 234 mμ, as was claimed by Grinbaum and Marchlewski, but diminishes it and causes a shift of the maximum to lower wave lengths. The hypsochromic effect is not surprising and may be attributed to fixation of the distribution of the π-electrons by the positive charge conferred upon the molecule in acid. At pH 3 and below the ultraviolet absorption spectrum of creatinine is represented by curve 1, which has a maximum at 217 mμ (ε = 4.5 × 10^3). An increase in pH above 12.3 produces at first a shift of the maximum of curve 2 to lower wavelengths with a simultaneous increase of the maximum. In 0.9—1.5 *N* hydroxide curve 3 is obtained, the maximum of which (ε = 11.1 × 10^3) lies at 225 mμ. On increasing the alkali concentration above 1.5 *N* the absorption peak is shifted back to higher wave lengths. Thus in 10 *N* KOH it lies at 229 mμ and in 16 *N* KOH at 231.5 mμ. Creatine, which under these conditions is generated from creatinine at appreciable rates, exhibits no comparable absorption in this ultraviolet region.

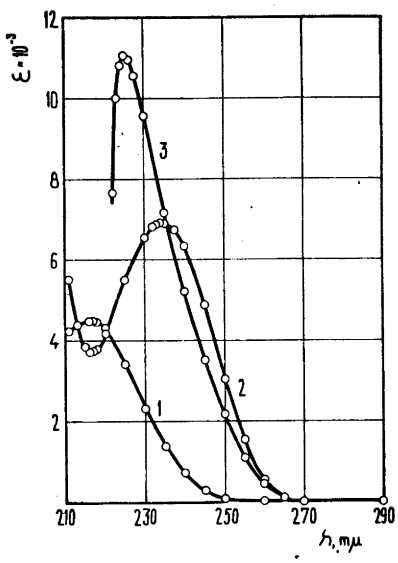

Fig. 1

* Fellow of the Life Insurance Medical Research Fund, New York, U.S.A.

Absorption curve 1 is obviously that of the creatininium ion and curve 2 that of the neutral molecule, while curve 3 represents chiefly the ion with a single negative charge. The bathochromic shift on increasing the alkali concentration above 1.5 N corresponds probably to the acquisition of a second negative charge. The existence of equilibria between the different ionic and molecular species is demonstrated by the fact that the described changes in the ultraviolet absorption spectrum with change in pH are all reverrible. From the spectrophotometric data the apparent constant of the basic ionization equilibrium of the neutral molecule was calculated, the pK'_a obtained being 5.02. The pK'_a of the acidic ionization is in the neighborhood of 13.4.

For analytical applications the absorption curve of the neutral creatinine has the advantage that it occurs over a wide range of pH and that its λ_{max} lies well within the limits of the recording instruments commonly in use. The absorption at this wave length was found to obey Beer's law over a concentration range of $8 \times 10^{-6} - 2 \times 10^{-4}$ M. Making use of this absorption we have determined creatinine and compounds convertible into creatinine (creatine and phosphocreatine) in biological material, following their separation from other chromogenic substances. This is being reported elsewhere. A more detailed account of the present work, which is also to include evidence concerning the specific chromophores responsible for the various absorption bands shown, will be presented at a later date.

I wish to thank Dr. O. Snellman for his interest and for kindly having made available the facilities of the laboratory.

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Occurrence of Betulinic Acid in *Menyanthes trifoliata* L.

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During the last years the triterpene betulinic acid, first found in nature by Robertson, Soliman and Owen (1939) [1], has been isolated from a series of plants, and various previously isolated substances have been shown to be identical with this acid. Betulinic acid may be very difficult to obtain in a crystalline state, and recrystallization of the crude acid does not always remove all impurities. Thus it is possible that this substance in many instances has been overlooked and may prove to be more common than is thought today.

It can be seen from Table 1 that betulinic acid has a rather scattered distribution throughout the plant kingdom, and does not seem to be characteristic of certain families. Remarkable is the report that, in *Alyxia*, betulinic acid is found to occur in specimen from low-rainfall areas away from the coast, whereas in coastal forms of the same plant it is reported to be replaced by ursolic acid and oleanolic acid [2].

Betulinic acid has been found in the leaves of *Alyxia* and in the leaves and the stems of *Nuytsia* [2], in the seeds of *Zizyphus* [3], and in the rhizomes of *Menyanthes* (this paper). In the other plants listed in the table it has been isolated from the bark.

As first pointed out by Barton and Jones [6], it seems highly probable that gratiolone, isolated from the drug *Herba Gratiola officinalis (Scrophulariaceae)* by Retzlaff [7], is identical with betulinic acid. Different substances isolated from *Platanus spp.* (lit. quoted by [4]) are probably also betulinic acid, as may be true for a substance isolated from *Cornus sanguinea* L. by Zellner and Fajner [8].

Absorption curve 1 is obviously that of the creatininium ion and curve 2 that of the neutral molecule, while curve 3 represents chiefly the ion with a single negative charge. The bathochromic shift on increasing the alkali concentration above 1.5 N corresponds probably to the acquisition of a second negative charge. The existence of equilibria between the different ionic and molecular species is demonstrated by the fact that the described changes in the ultraviolet absorption spectrum with change in pH are all reverrible. From the spectrophotometric data the apparent constant of the basic ionization equilibrium of the neutral molecule was calculated, the pK'_a obtained being 5.02. The pK'_a of the acidic ionization is in the neighborhood of 13.4.

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Table 1.

Loranthaceae:
Nuytsia floribunda (Labill.) R.Br. [2]
Platanaceae:
Platanus acerifolia Willd. [4]
Myrtaceae:
Melaleuca rhaphiophylla Schau. [2]
M. cuticularis Labill. [2]
M. viminea Lindl. [2]
M. leucadendron L. [2]
M. parviflora Lindl. [2]
M. pubescens Schau. [2]
Syncarpia laurifolia Tenn. [5]
Rhamnaceae:
Zizyphus vulgaris Lamark *var. spinosus* Bunge [3].
Cornaceae:
Cornus florida L. [1]
Gentianaceae:
Menyanthes trifoliata L.
Apocyanaceae:
Alyxia buxifolia R. Br. [2]

Experimental. (All melting points are corrected.) Fresh rhizomes of *Menyanthes trifoliata*, collected in shallow waters in a lake near Trondheim in October, 1949, were continuously extracted with hot ethanol in a Soxhlet extractor. A green gelatinous mass precipitated from the extract. It was filtered off and washed thoroughly, first with water and then, after drying, with petroleum ether (b.r. 60–90°), which removed most of the chlorophyll. The residue was recrystallized from methanol (charcoal) until colourless. Yield about 1 g per kg of fresh rhizome.

The isolated substance crystallised as fine needles, m.p. 304–7°; $[\alpha]_D^{20}$ + 10° (ethanol; *C*, 0.62; *l*, 2 dm). *M* (by titration) 459, 467. Calculated for $C_{30}H_{48}O_3$: *M* 457.

Acetylation, by the method of [1], gave the mixed anhydride of acetylbetulinic acid and acetic acid. The crude crystals melted at 193–94°. After 3 recrystallisations from diluted ethanol, acetylbetulinic acid, m.p. 293–94°, was obtained. Saponification gave betulinic acid, m.p. 313°.

For comparison acetylbetulinic acid was prepared from betulin by the method of Ruzicka, Lamberton and Christie [9]. M.p. 292–93°. Mixed m.p. 292.5–93.5°. The X-ray powder diagrams were identical.

The evaporated alcoholic extract from *Menyanthes* amounts to about 10 per cent of the fresh weight, and consists to a great extent of saponins. Whether these contain betulinic acid has not yet been determined.

I wish to express my thanks to Professor N. A. Sörensen for his continuous interest during this investigation, and to Dr. H. Sörum for preparing the powder diagrams. I am indebted to *Norges Tekniske Högskole* for a research fellowship.

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Paper Strip Identification of Phenyl Thiohydantoins

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Department of Physiological Chemistry, University of Lund, Lund, Sweden

In Edman's method [1] for determining the amino acid sequence in peptides phenyl thiohydantoins are formed from the amino acids. For identification, the former are hydrolysed by hot alkali to the corresponding amino acids. However, certain amino acids are decomposed by this treatment, making the identification ambiguous or impossible. This applies to serine, threonine, cystine, arginine, asparagine, and glutamine. This difficulty could be avoided by a direct identification of the phenyl thiohydantoins. A procedure to this purpose using paper chromatography is reported here.

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Experimental. The descending technique was used throughout. For localisation of the spots on the paper a modification [2] of the iodine-azide reaction described by Feigl [3] was used. This reaction is said to be specific for divalent sulphur. The test is highly sensitive and care should be taken to avoid contamination by extraneous sulphur (*e. g.* rubber).

Pretreatment of paper. Whatman No. 1 filter paper was used. It was found advantageous to impregnate the paper with the starch prior to use. Water soluble starch was freed from metal ions by extracting it first with 2.5 % ethanolic 8-hydroxyquinoline and then several times with ethanol. The filter paper was soaked in a 0.5 % aqueous solution of this starch preparation and then dried at 40—50° C for about one hour.

Solvents. A) 70 ml heptane (dist., b.p. 98° C) and 30 ml pyridine (dried over pellets of KOH and dist.). B) 40 ml heptane, 20 ml *n*-butanol (dried over CaO and dist.) and 40 ml 90 % formic acid (Baker's Analyzed) were shaken in a separatory funnel. The upper layer was used in the trough and the lower for saturating the atmosphere. C) 40 ml heptane, 40 ml *n*-butanol and 20 ml 90 % formic acid.

For a satisfactory and reproducible result it was necessary to equilibrate the paper with a saturated atmosphere of the solvent before starting the chromatography. This was particularly important with the solvents B and C. In order to reduce the equilibration time it is recommended to hang in the chromatographic chamber a piece of cloth soaked with the solvent.

Localisation of the spots. The paper was dried at 90—100° C for 5—10 minutes. The iodine-azide reagent was then applied in a finely divided spray. The reagent was made up immediately before use by mixing equal volumes of an iodine solution (*M*/100 J_2, *M*/2 KJ in water) and an azide solution (*M*/2 NaN_3 in water). The hydantoins revealed themselves as bleached areas on a dark blue background. As little as 0.5 μg could easily be detected.

The table shows the R_F-values of a number of phenyl thiohydantoins prepared from naturally occurring amino acids *. It can be seen that one—dimensional runs in these solvents permit the unambiguous identification of every hydantoin. The small difference in R_F-values between the leucine and isoleucine hydantoins was found to be consistent.

Phenyl thiohydantoin of	R_F-value in solvent A	B	C
Arginine	0	0	0.46
Aspartic acid	0.05	0.05	0.71
Histidine	0.05	0	0.41
Asparagine	0.06	0.02	0.57
Glutamine	0.06	0.02	0.61
Glutamic acid	0.10	0.06	0.75
Lysine **	0.16	0.04	0.86
Tyrosine	0.19	0.15	0.81
Tryptophan	0.24	0.41	0.83
Hydroxyproline	0.24	0.14	0.73
Glycine	0.26	0.20	0.69
Alanine	0.37	0.40	0.80
Phenylalanine	0.40	0.63	0.87
Methionine	0.40	0.54	0.85
Threonine	0.47	0.63	0.87
Proline	0.48	0.58	0.87
Valine	0.51	0.67	0.88
Isoleucine	0.59	0.74	0.90
Leucine	0.61	0.75	0.91

* The phenyl thiohydantoins from serine and cystine are not included since pure specimens have not been available.

** ε-phenyl thioureido-.

Acknowledgement. I wish to express my gratitude to Dr. P. Edman for encouragement and suggestions throughout the course of this work. This investigation was supported by a grant from the *Medical Faculty of the University of Lund.*

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Studies on Pyrazolones

V. Reaction Products from Pyrazole Blue and Alcohol

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In a previous paper [1] it was shown that pyrazole blue reacts with the methylene group of 1-phenyl-3-methyl-5-pyrazolone with the formation of 1,1′,1″-triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione. In the present investigation the great reactivity of the double bond system of pyrazole blue is further demonstrated by the easy preparation of addition compounds from pyrazole blue and alcohols. (That alcohols can be added to α,β-unsaturated carbonyl compounds is well known [2].)

Alkaline ethanol readily dissolves pyrazole blue (I) at room temperature with the formation of a colourless compound which is very soluble is ethanol, ether, ethyl acetate and benzene. The same compound in formed slowly without alkali at room temperature (*cf.* p. 452). When heated on a water-bath the reaction product decomposes into ethanol and pyrazole blue. It does not form pyrazole blue on treatment with nitrous acid (*cf.* p. 451).

Analyses show that the addition compound contains one mole of ethanol per mole of pyrazole blue. This fact in conjunction with the acidic qualities of the compound (equiv. wt. 390) indicates that the hydrogen atom from the hydroxyl group of the alcohol has been attached to the carbon atom 4 or a tautomeric position, probably the oxygen atom, of one of the pyrazolone nuclei and the ethoxyl group to the other. For the ethoxyl group a 4-position seems most reasonable. Consequently the compound is probably 1,1′-diphenyl-3,3′-dimethyl-4-ethoxy-[4,4′-bi-2-pyrazoline]-5,5′-dione (II). The light absorbtion curve of the substance, which lies between the corresponding curves of 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione and 1-phenyl-3,4-dimethyl-4-hydroxy-5-pyrazolone [3] in the range $2\,300 > \lambda > 3\,000$ Å (Fig. 1) favours this formulation.

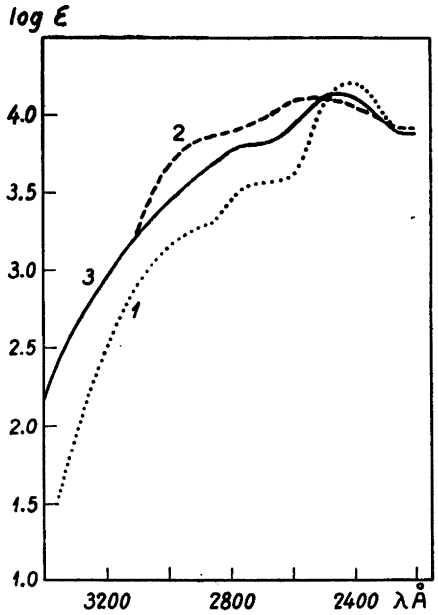
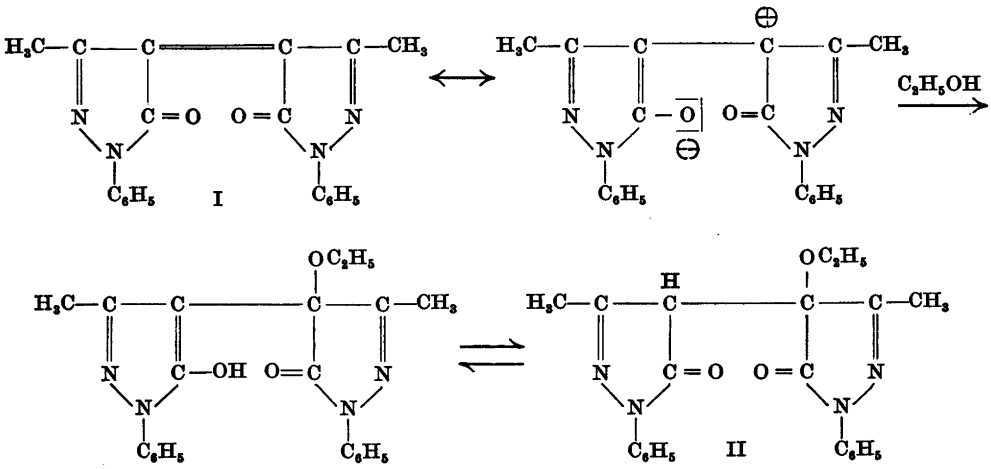


Fig. 1. 1) 1-Phenyl-3,4-dimethyl-4-hydroxy-5-pyrazolone in ethanol ($5.8 \cdot 10^{-3}$, $5.8 \cdot 10^{-4}$ and $5.8 \cdot 10^{-5}$ M solutions). 2) 1,1'-Diphenyl-3,3'-dimethyl-[4,4'-bi-2-pyrazoline]-5,5'-dione in ethanol ($4.0 \cdot 10^{-5}$ M solution). 3) 1,1'-Diphenyl-3,3'-dimethyl-4-ethoxy (or propoxy)-[4,4'-bi-2-pyrazoline]-5,5'-dione in ethanol ($3.8 \cdot 10^{-3}$, $3.8 \cdot 10^{-4}$ and $3.8 \cdot 10^{-5}$ M solutions). (The concentration, c, of the equation $\log \varepsilon = \log \log \frac{I_0}{I} - \log l \cdot c$ has been counted in pyrazolone units per liter solution.)



1,1′-Diphenyl-3,3′-dimethyl-4-methoxy-[4,4′-bi-2-pyrazoline]-5,5′-dione and 1,1′-diphenyl-3,3′-dimethyl-4-*n*-propoxy-[4,4′-bi-2-pyrazoline]-5,5′-dione have been prepared in the same way as the ethoxyl compound.

Knorr[4, p. 173] suggests that 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione is formed when pyrazole blue is boiled with alcohol. This experiment has been repeated, but the bispyrazolone of Knorr was not obtained. Instead the main product of the reaction was 1,1′,1″-triphenyl-3,3′,3″-trimethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione (64 % yield) identified by equivalent weight, decomposition temperature and the formation of pyrazole blue with nitrous acid. Traces of 1,1′-diphenyl-3,3′-dimethyl-4-ethoxy-[4,4′-bi-2-pyrazoline]-5,5′-dione, together with unisolated decomposition products, were also formed. 1,1′-Diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione is so insoluble that it should separate if it were a reaction product. No precipitate was observed.

$$Py = Py^{*} \longrightarrow 2\ PyH_2;$$

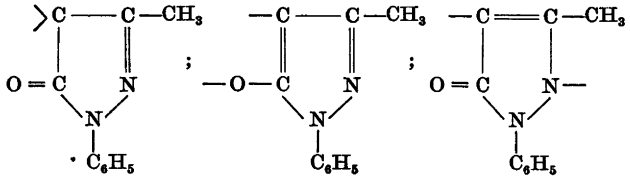
$$Py = Py + C_2H_5OH \rightarrow PyHPyOC_2H_5;\quad Py = Py + PyH_2 \rightarrow PyHPyPyH.$$

EXPERIMENTAL

1,1′-Diphenyl-3,3′-dimethyl-4-ethoxy-[4,4′-bi-2-pyrazoline]-5,5′-dione. Pyrazole blue (0.765 g) was shaken with ethanol (10 ml) and dilute sodium hydroxide (2 ml of 2.5 *N* solution) until all Py = Py had dissolved (3 min.). Addition of water (200 ml) and dilute sulphuric acid (2 ml of 5 *N* solution) to the yellow solution precipitated a colourless substance that was filtered by suction, washed with water and air-dried. Yield 0.80 g (92 %). (Found: C 67.7; H 5.5; N 14.28; equiv. wt. 390 on titration with 0.1 *N* barium hydroxide solution against phenolphthalein. Calc. for $C_{22}H_{22}O_3N_4$ (390.4): C 67.7; H 5.7; N 14.35; equiv. wt. 390.) The compound decomposed without melting but shrank considerably at 94° C. On decomposing the substance at about 100° C in a Reihlen-Weinbrenner oven and combusting the volatile parts, 49.6 per cent carbon and 13.1 per cent hydrogen were found. Calc. for C_2H_5OH: C 52.1; H 13.1. Pyrazole blue remained (87.8 %. Calc. for $C_{22}H_{22}O_3N_4$: 88.2 %.)

1,1′-Diphenyl-3,3′-dimethyl-4-methoxy-[4,4′-bi-2-pyrazoline]-5,5′-dione. This compound was prepared by dissolving pyrazole blue (1.0 g) in methanol (50 ml) containing sodium hydroxide (5 ml of 2.5 *N* solution). Water (350 ml) and then dilute sulphuric acid (3 ml) were added to the yellow solution. The colourless precipitate that formed (1.05 g, 96 %) was filtered by suction, washed with water and dried at room temperature. It decomposed without melting to give pyrazole blue. (Found: C 67.2; H 5.3; N 14.86;

* Py represents the radicals



equiv.wt. 372. Calc. for $C_{21}H_{20}O_3N_4$ (376.4): C 67.0; H 5.4; N 14.89; equiv.wt. 376.) The compound is soluble in alcohol, slightly soluble in ether.

1,1'-Diphenyl-3,3'-dimethyl-4-n-propoxy-[4,4'-bi-2-pyrazoline]-5,5'-dione. PyHPyOCH$_2$-CH$_2$CH$_3$ was prepared by dissolving pyrazole blue (2.0 g) in 1-propanol (50 ml) and dilute sodium hydroxide (3 ml). A dark yellow solution was obtained. Water (350 ml) and then dilute sulphuric acid (2 ml) were added. A grayish precipitate was formed. It was easily soluble in ethanol at room temperature, but it precipitated from the solution in a stabler crystal form in a few minutes. The precipitate was white, and from the mother liquor more white crystals could be obtained by dilution with water and seeding with the crystals already obtained. The product is only slightly soluble in ether. Heat causes the compound to decompose without melting. (Found: C 68.3; H 5.9; N 13.83; equiv. wt. 401. Calc. for $C_{23}H_{24}O_3N_4$ (404.5): C 68.3; H 6.0; N 13.85; equiv. wt. 404.5.)

Reaction between Pyrazole Blue and Ethanol at Room Temperature. Pyrazole blue (1.23 g) was shaken with ethanol (100 ml) and chloroform (100 ml) until decoloration occurred (10 days). The solvents were evaporated at room temperature. The crude, yellow product (1.45 g) was very soluble in alcohol. It gave pyrazole blue when heated at 100° C, but not when treated with nitrous acid. These facts show that 1,1'-diphenyl-3,3'-dimethyl-4-ethoxy-[4,4'-bi-2-pyrazoline]-5,5'-dione had been formed, and also that neither 1,1'-diphenyl-3,3'-dimethyl-[4,4'-bi-2-pyrazoline]-5,5'-dione nor 1,1',1''-triphenyl-3,3',3''-trimethyl-[4,4',4''-ter-2-pyrazoline]-5,5',5''-trione had been. (Found: equiv. wt. of the crude product 409. Calc. for $C_{22}H_{22}O_3N_4$ (390.4): equiv. wt. 390.)

Reaction between Pyrazole Blue and Alcohol at Boiling Temperature. Pyrazole blue (1.39 g) was boiled with 96 % ethanol (50 ml) during 9 hours. The yellow solution was precipitated with water and a few ml of 5 *N* sulphuric acid. The precipitate was filtered by suction and washed with water. The dry product (1.21 g) was extracted with ether. The ether-insoluble part (0.895 g, 64 %) was 1,1',1''-triphenyl-3,3',3''-trimethyl-[4,4',4''-ter-2-pyrazoline]-5,5',5''-trione [1], m.p. 200° C with decomposition after crystallization from alcohol. (Found: equiv. wt. 264. Calc. for $C_{30}H_{26}O_3N_6$: equiv. wt. 259.) The product was slightly contaminated with 1,1'-diphenyl-3,3'-dimethyl-4-ethoxy-[4,4'-bi-2-pyrazoline]-5,5'-dione (strongly adsorbed by the surface-active terpyrazolone crystals) identified by the formation of small amounts of pyrazole blue on heating to 100° C. The ether solution also contained traces of 1,1'-diphenyl-3,3'-dimethyl-4-ethoxy-[4,4'-bi-2-pyrazoline]-5,5'-dione.

SUMMARY

Pyrazole blue reacts with ethanol at room temperature to give 1,1'-diphenyl-3,3'-dimethyl-4-ethoxy-[4,4'-bi-2-pyrazoline]-5,5'-dione. The reaction is strongly catalyzed by alkali. Analogous products are obtained with methanol and 1-propanol. Pyrazole blue reacts with boiling ethanol to give 1,1',1''-triphenyl-3,3',3''-trimethyl-[4,4',4''-ter-2-pyrazoline]-5,5',5''-trione.

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Studies on Pyrazolones

VI. 1,1′,1″-Tri-*p*-tolyl-3,3′,3″-trimethylfurlone and 1,1′,1″-Tri-*o*-tolyl-3,3′,3″-trimethylfurlone

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The present paper describes the synthesis of two new furlone yellows, 1,1′,1″-tri-*p*-tolyl-3,3′,3″-trimethylfurlone and 1,1′,1″-tri-*o*-tolyl-3,3′,3″-trimethylfurlone. They were prepared from 1-*p*- and 1-*o*-tolyl-3-methyl-4-bromo-5-pyrazolone, respectively, by the procedure used for furlone yellow [1] itself except that the reaction medium was acetone instead of alcohol. This change improves the yield of furlone yellows, as shown by an experiment in which the yield of 1,1′,1″-triphenyl-3,3′,3″-trimethylfurlone was increased from 65 per cent to 87 per cent by substituting acetone for alcohol. The cause of lower yields in alcoholic media is that pyrazole blues are transient intermediates, and these, as was recently shown [2], can react with alcohols to give other products.

The light absorption spectra of the two new furlone yellows are shown in Fig. 1. The spectrum of the *ortho* compound is shifted to lower wave-lengths and has smaller extinction coefficients than that of the *para* compound, which is similar to spectra of known unsubstituted and *para*-substituted furlone yellows [1]. The light absorption spectrum of 1-*o*-tolyl-3-methyl-4-bromo-5-pyrazolone differs similarly from the spectra of 1-phenyl-3-methyl-4-bromo-5-pyrazolone [3] and 1-*p*-tolyl-3-methyl-4-bromo-5-pyrazolone (Fig. 2).

EXPERIMENTAL

1,1′,1″-Triphenyl-3,3′,3″-trimethylfurlone. 1-Phenyl-3-methyl-4-bromo-5-pyrazolone (1.36 g) was dissolved in acetone (90 ml), and acetic acid (30 ml of 1.8 *N* solution), sodium acetate (30 ml of 1.8 *N* solution) and copper sulphate (1.6 ml of 0.01 % $CuSO_4 \cdot 5H_2O$ solution) were added. The next day the yellow crystals formed (0.70 g) were filtered by

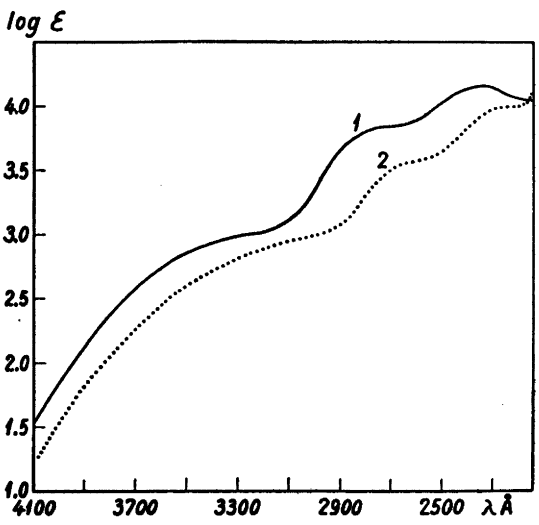

Fig. 1. 1) 1,1′,1″-Tri-p-*tolyl-3,3′,3″-trimethylfurlone in ethanol (2.0 · 10⁻³, 2.0 · 10⁻⁴ and 2.0 · 10⁻⁵ M solutions). 2) 1,1′,1″-Tri*-o-*tolyl-3,3′,3″-trimethylfurlone in ethanol (1.7 · 10⁻³, 1.7 · 10⁻⁴ and 1.7 · 10⁻⁵ M solutions).*

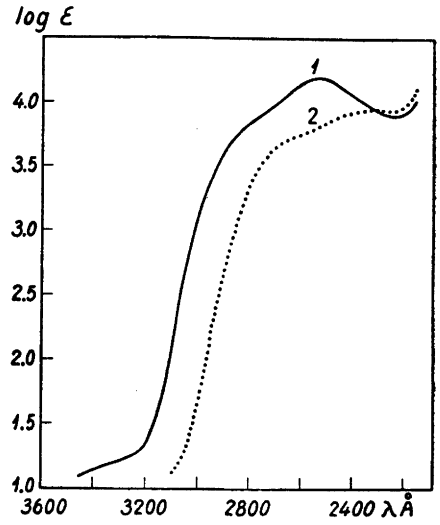

*Fig. 2. 1) 1-*p-*Tolyl-3-methyl-4-bromo-5-pyrazolone in ethanol (8.5 · 10⁻³, 8.5 · 10⁻⁴ and 8.5 · 10⁻⁵ M solutions). 2) 1-*o-*Tolyl-3-methyl-4-bromo-5-pyrazolone in ethanol (5.7 · 10⁻³, 5.7 · 10⁻⁴ and 5.7 · 10⁻⁵ M solutions).*

suction and washed with water and alcohol. From the combined filtrate and washings an additional 0.11 g of furlone yellow was obtained by precipitation with water, filtration, washing with water and recrystallization from alcohol. Yield 87 %. M .p. 158° C with decomposition. (Found: C 69.7; H 4.7. Calc. for $(C_{10}H_8ON_2)_3$ (516.6): C 69.7; H 4.7.)

1,1′,1″-Tri-p-*tolyl-3,3′,3″-trimethylfurlone.* 1-*p*-Tolyl-3-methyl-4-bromo-5-pyrazolone (1.91 g) in acetone (100 ml) was buffered with acetic acid (30 ml of 1.8 *N* solution) and sodium acetate (30 ml of 1.8 *N* solution). Cupric catalyst (2 ml of 0.01 % $CuSO_4 \cdot 5H_2O$ solution) was added. The solution deposited 1.05 g of yellow crystals in the course of 12 hours at room temperature. These were filtered by suction, washed with water and alcohol, and air-dried. From the combined filtrate and washings another 0.05 g was obtained by precipitation with water and recrystallization from alcohol. Yield 83 %. M.p. 172° C (decomposes) after crystallization from alcohol. (Found: C 70.8; H 5.3; N 15.02. Calc. for $(C_{11}H_{10}ON_2)_3$ (558.6): C 70.95; H 5.4; N 15.05.)

1,1′,1″-Tri-o-*tolyl-3,3′,3″-trimethylfurlone.* 1-*o*-Tolyl-3-methyl-4-bromo-5-pyrazolone (1.02 g) in acetone (50 ml) was buffered with 30 ml of a solution 0.9 *N* in acetic acid and sodium acetate. Copper catalyst (3 ml of 0.01 % $CuSO_4 \cdot 5H_2O$ solution) was added, and the solution was left at room temperature over night. Faintly-yellow crystals deposited. They were purified as described in the preceding synthesis. Yield 0.36 g (51 %). M.p. 162.5—163.5° C (decomposes) after crystallization from alcohol. (Found: C 70.7; H 5.3; N 15.00. Calc. for $(C_{11}H_{10}ON_2)_3$ (558.6): C 70.95; H 5.4; N 15.05.)

Light Absorption Measurements. In the diagrams $\log \varepsilon = \log \log \frac{I_0}{I} - \log l \cdot c$ is plotted against the wave-length λ. The concentration, c, is counted in pyrazolone units per liter solution.

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Studies on Pyrazolones

VII. Brominating Action of 1-Phenyl-3-methyl-4,4-dibromo-5-pyrazolone and 1-Phenyl-3,4-dimethyl-4-bromo-5-pyrazolone

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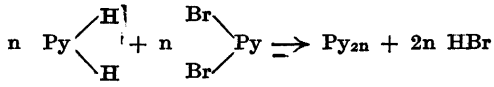
During investigations of the couplings of 4-bromo-5-pyrazolones, some anomalous reactions were encountered. Thus furlone yellow [1] can be prepared from a buffered solution of 1-phenyl-3-methyl-5-pyrazolone and 1-phenyl-3-methyl-4,4-dibromo-5-pyrazolone. This reaction seems to contradict the formulation of furlone yellow as a Py_3*-compound, for a condensation between the pyrazolone and the dibromopyrazolone ought to give rise to a Py_{2n}-product according to the equation:



Further, 1-*p*-bromophenyl-3-methyl-5-pyrazolone reacts with 1-phenyl-3 methyl-4,4-dibromo-5-pyrazolone to give a mixture containing 1,1′-diphenyl

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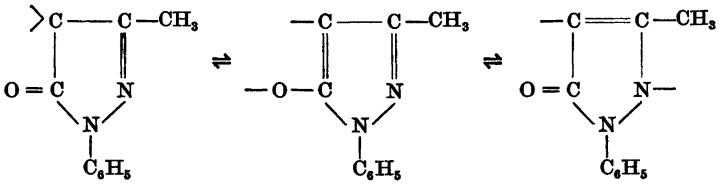
* Py is used to represent the radicals



3,3′-dimethyl-[$\Delta^{4,4'}$-bi-2-pyrazoline]-5,5′-dione and 1,1′-di-*p*-bromophenyl- 3,3′-dimethyl-[$\Delta^{4,4'}$-bi-2-pyrazoline]-5,5′-dione.

Furthermore, attempts to prepare 1,1′,1″-triphenyl-3,3′,3″,4,4″-pentamethyl-[4,4′,4″-ter-2-pyrazoline]-5,5′,5″-trione from 1-phenyl-3-methyl-5-pyrazolone and 1-phenyl-3,4-dimethyl-4-bromo-5-pyrazolone [2] or from 1-phenyl-3,4-dimethyl-5-pyrazolone and 1-phenyl-3-methyl-4,4-dibromo-5-pyrazolone have not been successful. In the first case pyrazole blue or 1,1′-diphenyl-3,3′, 4,4′-tetramethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione has been obtained, in the second furlone yellow.

All these reactions can be explained by assuming that the bromo-compounds act as brominating agents:

1) $3\ PyH_2 + 3\ PyBr_2 \rightarrow 6\ PyHBr \rightarrow 2\ Py_3$

2) $2\ PyBr_2 + 2\ Py_{p\text{-}Br}H_2 \rightarrow 2\ PyHBr + 2\ Py_{p\text{-}Br}HBr$
$\downarrow \qquad\qquad \downarrow$
$Py{=}Py \qquad Py_{p\text{-}Br}{=}Py_{p\text{-}Br}$

3) $2\ PyH_2 + 2\ Py\ (CH_3)Br \rightarrow 2\ PyHBr + 2\ PyHCH_3$
$\downarrow \qquad\quad \downarrow Py(CH_3)Br$
$Py{=}Py \qquad Py(CH_3)PyCH_3$

4) $3\ PyHCH_3 + 3\ PyBr_2 \rightarrow 3\ PyHBr + 3\ Py(CH_3)Br$
$\downarrow$
Py_3

It is known that replacement of a hydrogen of an acidic methylene group by bromine may give a product that can act as a brominating agent. Dibromobarbituric acid [3], ethyl *a*-bromoacetoacetate [4] and dibromomalodinitrile [5] are examples of such products.

The formation of the suggested intermediates in reactions 1—3 is demonstrated by light absorption measurements on ethanol solutions of the reactants (Figs. 1 and 2; buffer and catalyst are excluded to eliminate the condensation reactions) and also by titration with sodium hydroxide solution, the right sides of the equations containing twice as many equivalents of acid as the left ones. The titrations accelerate the reactions by consuming the acids and consequently show rapid and complete brominations.

The above is in accordance with the electron-seeking character of the bromine atoms in 1-phenyl-3-methyl-4,4-dihalo-5-pyrazolones and 1-phenyl-3,4-dimethyl-4-halo-5-pyrazolones indicated in a previous paper [2].

Light Absorption Spectra. In the diagrams $\log \varepsilon = \log \log \frac{I_0}{I} - \log l \cdot c$ is plotted against the wave length λ. When several light absorbing substances are present in the same solution, c is the total concentration of pyrazolone.

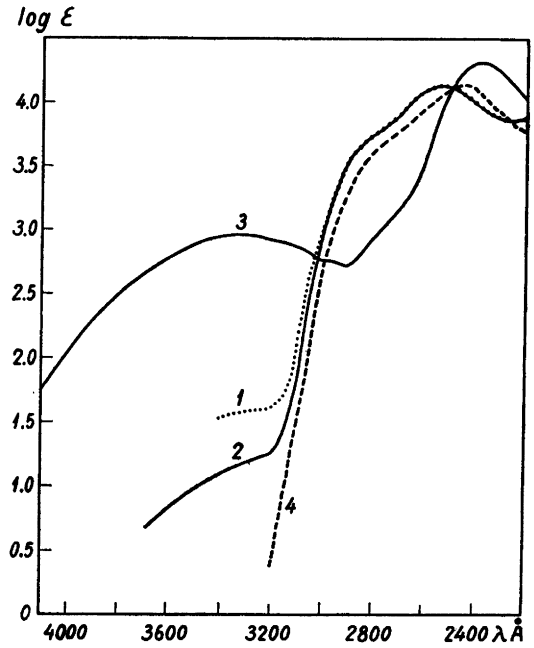


Fig. 1. 1) 1-Phenyl-3-methyl-5-pyrazolone and 1-phenyl-3-methyl-4,4-dibromo-5-pyrazolone in ethanol (both in 1.9 · 10⁻³, 1.9 · 10⁻⁴ and 1.9 · 10⁻⁵ M solutions) measured 1½ hours after the preparation of the solution. 2) 1-Phenyl-3-methyl-4-bromo-5-pyrazolone in ethanol (4.0 · 10⁻³, 4.0 · 10⁻⁴ and 4.0·10⁻⁵ M solutions). 3) 1-Phenyl-3-methyl-4,4-dibromo-5-pyrazolone in ethanol (4.0 · 10⁻³, 4.0 · 10⁻⁴ and 4.0 · 10⁻⁵ M solutions). 4) 1-Phenyl-3-methyl-5-pyrazolone in ethanol (7.3 · 10⁻³, 7.3 · 10⁻⁴ and 7.3 · 10⁻⁵ M solutions).

EXPERIMENTAL

Reactions between 1-Phenyl-3-methyl-5-pyrazolone and 1-Phenyl-3-methyl-4,4-dibromo-5-pyrazolone

1. *Formation of Furlone Yellow.* $PyBr_2$ (1.31 g) and PyH_2 (0.72 g) were dissolved in alcohol (65 ml). Sodium acetate (20 ml of 1.8 *N* solution), acetic acid (20 ml of 1.8 *N* solution) and catalyst (0.8 ml of a 0.01 % solution of $CuSO_4 \cdot 5H_2O$) were added. The next day the yellow crystals which had deposited were filtered by suction and washed with 60 % alcohol. After one recrystallization from alcohol they melted at 157° C with decomposition; yield 0.80 g (59 %). (Found: C 69.6; H 4.7. Calc. for $(C_{10}H_8ON_2)_3$: C 69.7; H 4.7.) The light absorption spectra are identical for this product and furlone yellow, prepared from 1-phenyl-3-methyl-4-bromo-5-pyrazolone.

2. *Titration of an Ethanol Solution of the System $PyH_2 + PyBr_2 \rightarrow 2\ PyHBr$.* An ethanol solution prepared from 1.00 millimole of PyH_2 and 1.00 millimole of $PyBr_2$ consumed 2.00 milliequivalents of sodium hydroxide on titration against thymolphthalein. No hydrogen bromide had been liberated. Thus PyHBr had been formed quantitatively.

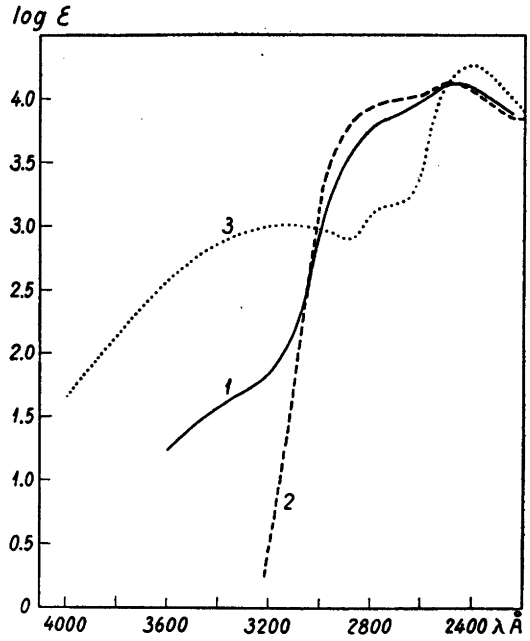


Fig. 2. 1) 1-Phenyl-3-methyl-5-pyrazolone (2.4 · 10⁻³, 2.4 · 10⁻⁴ and 2.4 · 10⁻⁵ M solutions) and 1-phenyl-3,4-dimethyl-4-bromo-5-pyrazolone (2.3 · 10⁻³, 2.3 · 10⁻⁴ and 2.3 · 10⁻⁵ M solutions) in 55 % ethanol measured 25 hours after the preparation of the solution. 2) 1-Phenyl-3,4-dimethyl-5-pyrazolone in ethanol (2.1 · 10⁻², 4.5 · 10⁻³, 4.5 · 10⁻⁴ and 4.5 · 10⁻⁵ M solutions). 3) 1-Phenyl-3,4-dimethyl-4-bromo-5-pyrazolone in ethanol (4.0 · 10⁻³, 4.0 · 10⁻⁴ and 4.0 · 10⁻⁵ M solutions).

3. *Light Absorption.* According to the light absorption measurements, 1-phenyl-3-methyl-4-bromo-5-pyrazolone had been formed in 95 % yield after 90 minutes when the initial concentrations of 1-phenyl-3-methyl-5-pyrazolone and 1-phenyl-3-methyl-4,4-dibromo-5-pyrazolone were $1.9 \cdot 10^{-3}$ moles per liter of ethanol (calculated on the assumption that Beer's law is valid from the values at 3 300 Å: $\log \frac{I_0}{I} = 0.147$, $\varepsilon_{PyHBr} = 15.4$, $\varepsilon_{PyBr_2} = 922$ and ε_{PyH_2} negligible).

Reactions between 1-Phenyl-3-methyl-5-pyrazolone and 1-Phenyl-3,4-dimethyl-4-bromo-5-pyrazolone

1. *Formation of Pyrazole Blue.* PyH_2 and excess $Py(CH_3)Br$ were dissolved in alcohol. When a few ml of a 3 % solution of $CuSO_4 \cdot 5H_2O$ was added, pyrazole blue precipitated.

2. *Formation of 1,1'-Diphenyl-3,3',4,4'-tetramethyl-[4,4'-bi-2-pyrazoline]-5,5'-dione.* PyH_2 (0.8 g) and $Py(CH_3)Br$ (2.5 g) were dissolved in alcohol (100 ml), and sodium acetate (33 ml of 1.8 *N* solution), acetic acid (33 ml of 1.8 *N* solution) and catalyst (1.3 ml of a 0.01 % solution of $CuSO_4 \cdot 5H_2O$) were added. White crystals (0.67 g) precipitated in the

course of three hours. After several crystallizations from acetic acid they melted at 165° C alone and mixed with 1,1′-diphenyl-3,3′,4,4′-tetramethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione. The light absorption curves of the two products were identical.

3. *Titration of an Ethanol Solution of the System* $PyH_2 + Py(CH_3)Br \rightarrow PyHBr + PyHCH_3$. An alcohol solution of 1.00 millimole of both PyH_2 and $Py(CH_3)Br$ consumed 2.00 milliequivalents of sodium hydroxide on titration with thymolphthalein as indicator. No hydrogen bromide had been split off. Thus PyHBr and $PyHCH_3$ were formed quantitatively during the titration.

4. *Light Absorption Measurement.* The reaction $PyH_2 + Py(CH_3)Br \rightarrow PyHBr + PyHCH_3$ is slow in ethanol solution but can be accelerated by the addition of water. This is the reason why the light absorption has been measured in dilute alcohol (Fig. 2). In spite of the long reaction time no hydrogen bromide was split off. The first part of equation 3 was realized to 90 per cent after 25 hours in the 2.3—2.4 *M* solution of the reactants in 55 per cent alcohol (at λ = 3 300 Å: $\log \frac{I_0}{I} = 0.235$, $\varepsilon_{PyBrCH_3} = 875$, $\varepsilon_{PyHBr} = 15.4$, ε_{PyH_2} and ε_{PyHCH_3} negligible).

Reaction between 1-Phenyl-3-methyl-4-alkyl-5-pyrazolone and 1-Phenyl-3-methyl-4,4-dibromo-5-pyrazolone

To a solution of $PyHC_2H_5$ or $PyHCH_3$ (1.2 g) and $PyBr_2$ (1.7 g) in alcohol (75 ml) were added 25 ml of 1.8 *N* acetic acid, 25 ml of 1.8 *N* sodium acetate solution and 2.5 ml of a 0.01 % $CuSO_4 \cdot 5H_2O$ solution. The next day yellow crystals (0.4 g) were collected by filtration and recrystallized from alcohol; m.p. 157° C with decomposition. (Found: C 69.4; H 4.6. Calc. for $(C_{10}H_8ON_2)_3$ (516.6): C 69.7; H 4.7.) Light absorption curves are identical for this product and furlone yellow.

SUMMARY

1-Phenyl-3-methyl-4,4-dibromo-5-pyrazolone and 1-phenyl-3,4-dimethyl-4-bromo-5-pyrazolone act as brominating agents.

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An Attempt to Explain the Interaction of Auxin and Antiauxin in Root Growth by an Adsorption Mechanism

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The effects of auxins and their antagonists in root growth can be shown in diagrams, (*cf.* Åberg [1]) in which the molar concentration of the chemical compound in decadical logarithms is plotted against the observed root growth per cent of the root growth under normal conditions. S-shaped curves usually result, *e. g.* with 2,4-dichloro-phenoxyacetic acids; *cf.* also the full line curve in Fig. 1 of this paper.

Ass. professor Börje Åberg of this College drew the author's attention to this fact and pointed out that no attempts to explain the curve-form in mathematical terms have been published. This prompted the investigation described below.

It is not the author's intention to discuss the chemical processes connected with the retardation or acceleration of growth, but to describe the phenomenon in mathematical terms on the basis of the results obtained by Åberg.

The curves, given by Åberg for the phenoxy-acid mentioned above and other acids greatly resemble the curves for the dissociation of a weak acid; when pH is plotted against the fraction of acid dissociated. In this case $\alpha/(1-\alpha) = C_H + /k_s$ where k_s is the dissociation constant.

Another point, however, caught the author's interest. The equation above may be written

$$\frac{1}{\alpha} = \frac{k}{m} + 1 \qquad (1)$$

It is identical with the Langmuir adsorption isotherm, α being the fraction of areas occupied in the adsorption pattern, m the concentration in solution.

The curve, Fig. 1 (full line), is actually the curve for the variables log m/k against α according to (1). As $m \to \infty$, $\alpha = 1$ *i. e.* saturation is reached.

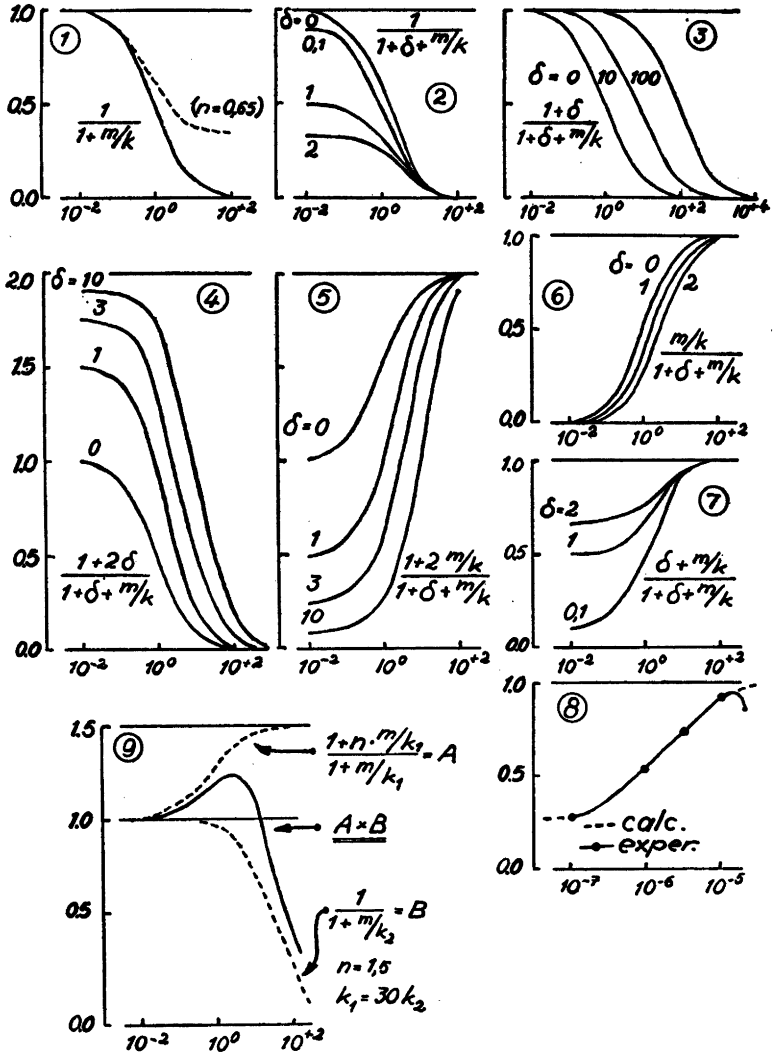

Fig. 1—9. The effects according to Table 1 and discussions in the text, of one component, Fig. 1, and combinations of two components, Fig. 2—8, in a single pattern p. 464 and in two patterns, Fig. 9, p. 467. Abscissa: m/k.

When $1/\alpha = 2$, semi-saturation is attained and at this point $m = k$ mole/lit. Thus the constant k is the concentration corresponding to $\alpha = 1/2$. Formally the constant corresponds to the concentration as a characteristic of the substance. It may be observed that the constant k is the only parameter of the

equation and predicts the situation of the curve along the $1/m$-axis. The constant is referred to below as the constant of effect. For practical reasons its decadical logarithm, pk_v, is used.

Comparing the curve, Fig. 1 (full line), with those communicated by Åberg [1] for 2,4-dichloro-phenoxyacetic acid and α-indolylacetic acid the agreement in general is evident. Further the jump from 90 to 10 % effect, that is a jump in α from 0.9 to 0.1 occurs within two powers of ten in the concentration, that is also in m/k. This fact indicates a quantitative agreement. (Åberg's curves approach a value of 0.05 or 0.10 as $m \to \infty$, but the curve in Fig.1 tends to 0. This may be due to the fact that the root growth is not entirely regulated by the chemical used; *cf.* dotted curve in Fig. 1, referring to 65 per cent of the area available for adsorption.)

Thus, it seems possible to relate the retarding effect to an adsorption process. A corresponding process can be assumed for the accelerating effect and in principle a series of S-shaped curves should describe the growth process. Whether or not a chemical takes part in the biochemical processes, therefore, depends upon its concentration and constant of effect. Evidently an active chemical cannot produce any action if m/k does not rise to values markedly different from zero.

This rough picture can be submitted to further tests, for which some deductions from the Langmuir isotherm are needed, and are given below:

A system with one adsorbate gives:

$$\alpha_1 = \frac{m_1/k_1}{1 + m_1/k_1} = \frac{m_1/k_1}{M}$$

A system with two adsorbates gives:

$$\alpha_1 = \frac{m_1/k_1}{1 + m_1/k_1 + m_2/k_2} = \frac{m_1/k_1}{D}$$

$$\alpha_2 = \frac{m_2/k_2}{1 + m_1/k_1 + m_2/k_2} = \frac{m_2/k_2}{D}$$

A system with three adsorbates gives:

$$\alpha_1 = \frac{m_1/k_1}{1 + m_1/k_1 + m_2/k_2 + m_3/k_3} = \frac{m_1/k_1}{T}$$

$$\alpha_2 = \frac{m_2/k_2}{1 + m_1/k_1 + m_2/k_2 + m_3/k_3} = \frac{m_2/k_2}{T}$$

$$\alpha_3 = \frac{m_3/k_3}{1 + m_1/k_1 + m_2/k_2 + m_3/k_3} = \frac{m_3/k_3}{T}$$

Table 1. Combinations in systems with one and two components with activity (+) or none (0) in the free area and in the area occupied by the component.

Combination No.	Components	Activity in area	
		free	occupied
1 : a	1	+	0
2 : aa	1 2	+	0 0
2 : ab	1 2	+	0 +
1 : b	1	0	+
2 : ba	1 2	0	+ 0
2 : bb	1 2	0	+ +

The equations deduced can be connected with each other in many ways. Table 1 gives a summary of some combinations, whereby the free area is assumed to be active (+), or inactive (0).

In order to use the equations an assumption must be made regarding the connection between the state of adsorption and the growth. The most simple one is to put the growth (control L, experiment l) proportional to the free area, α. Thus $L \cdot c = 1$ and $l \cdot c = 1-\alpha$.

1 : a gives: $\frac{l}{L} = \frac{(1-\alpha)}{1} = \frac{1}{1 + m/k} = \frac{1}{M}$,

i. e. the full line curve Fig. 1.

1 : b gives: $\frac{l}{L} = \alpha = \frac{m_1/k_1}{1 + m_1/k_1}$, *i. e.* a mirror-image of the curve case 1:a.

2 : aa gives: $\frac{l}{L} = \frac{1-\alpha_1-\alpha_2}{1} = \frac{1}{D}$; m_1/k_1 may be written δ, thus $\frac{l}{L} = \frac{1}{1 + \delta + m_2/k_2}$, *i. e.* curves in Fig. 2.

2 : ab gives: $\frac{l}{L} = \frac{1-\alpha_1-\alpha_2+\alpha_2}{1-\alpha_0} = M_0 \cdot \frac{1 + m_2/k_2}{D}$. (The subscript 0 refers to L.)

If m_2 is kept constant this equation may be written $\frac{l}{L} = M_0 \cdot \frac{1+\delta}{1+\delta+m_1/k_1}$. The curve for the last factor is given in Fig. 3. Assuming that the area occupied by the component 2 (Table 1) is twice as active as the free area, the curve in Fig. 4 is obtained. If the ratio between the constants of effect is 10, the limit is 0 when $m_1 \to \infty$, and $(1 + 10\delta) / (1 + \delta)$ when $m_1 \to 0$.

If m_1 is kept constant one obtains $\frac{l}{L} = M_0 \frac{1 + m_2/k_2}{1 + \delta + m_2/k_2}$, (where $\delta = m_1/k_1$) and the curves in Fig. 5, assuming that the area occupied by 2 is twice as active as the free area.

2 : ba gives: $\frac{l}{L} = \frac{\alpha_1}{\alpha_2} = \frac{M_0}{m_0/k_0} \cdot \frac{m_1/k_1}{D}$.

If m_1 is kept constant the last factor has the form $\delta/(1 + \delta + m_2/k_2)$ giving curves of the form shown in Fig. 2.

If m_2 is kept constant the last factor is $(m_1/k_1) / (1 + \delta + m_1/k_1)$ giving the curves in Fig. 6.

2 : bb gives: $\frac{l}{L} = \frac{\alpha_1 + \alpha_2}{\alpha_0} = \frac{M_0}{m_0/k_0} \cdot \frac{m_1/k_1 + m_2/k_2}{D}$;

resulting in the curves in Fig. 7.

Application of the derived equations. The simple cases in Fig. 1, combination 1 : a and 1 : b, refer to chemicals with retarding and accelerating effects. In these cases proportionality is supposed between the fraction of free area and action; with retarding effect the occupied area is supposed to be inactive or much less active than the free area; with accelerating effect the reverse is supposed to be the case.

The simultaneous effect of two chemicals or one chemical with complicated effect, *e. g.* 3-iodo-benzoic acid [1], needs further discussion. In principle there are two possibilities. The first is, that one adsorption pattern is available and the chemicals compete for the available area, the other, that two adsorption patterns are available, each pattern with its separate effect upon the main result produced. The last case may refer for example to 1-naphtylsulfide-acetic acid, which gives an accelerating effect in low concentrations, in high a retardation (Fig. 9, full line curve). Patterns of this kind, of course, may or may not be present in or on the same adsorbent; the theoretical postulates are nevertheless valid.

Evidently the two possibilities do not exclude each other, but in order to prove the assumptions, they must, of course, be discussed separately.

Single pattern. Åberg found that 2,4-dichloro-phenoxyacetic acid in the presence of 1-naphtylmethyl-sulfide-β-propionic acid gave a normal curve, somewhat displaced at high values, comparable with the curves in Fig. 3.

With the value $m_1 = 2 \cdot 10^{-7}$, estimated from Åberg [3], and $k_1 = 4 \cdot 10^{-8}$ for the phenoxy acid from the equation valid for the curves in Fig. 3 (this paper) the value $\delta = 4$ is obtained. In this case, $m_2 = 1 \cdot 10^{-5}$, pk_v is 5.60. The concentration $m_2 = 2 \cdot 10^{-5}$ gives $m_1 = 4 \cdot 10^{-7}$ and p$k_v = 5.66$. Both experiments give the same value for p$k_v = 5.6$.

Another test may be performed using the G-values in Åberg's publication [3] (Table 11). The curve for $(1 + m/k) / (1 + \delta + m/k)$ being drawn for $\delta = 10^{-7}/4 \cdot 10^{-8} = 2.5$ and then being moved along the concentration axis until it most closely coincides with the experimental curve, we obtain with naphtylmethyl-sulfide-acetic acid Fig. 8, where the full line represents the experimental curve, the dotted line the calculated curve. The point $m/k = 10^0$ on the calculated curve is marked on the experimental curve by a point on the m/k-axis; this point, 6.35, is of course the pk_v value for the acid. Values determined in this way are given in Table 2 (I). In these calculations, as evident from the discussion, the retarding effect of the sulfide and selenide acids were disregarded, only the accelerating effect being taken into account.

Further, equation $(1 + m/k) / (1 + \delta + m/k) = l/L$ may be used for graphic representation. Using β for l/L one gets the expression log

Table 2. The decadical logarithm of the constant of effect, pk_v, *for some acids, calculated in two ways, (I) and (II), see text.*

No.	Substance	pk_v	
		(I)	(II)
a1	1-Naphtyl-methyl-sulfide-acetic acid	6.35	6.3
a2	2- » » » » »	6.5	6.3
b1	1- » » » -β-propionic acid	5.5	5.6_5
b2	2- » » » » »	6.1	6.0_5
c1	1- » » » -*a*-*iso*-butyric acid	*	(5.2) **
c2	2- » » » » » »	*	(6.4) **
d1	1- » » -selenide-acetic acid	6.7	6.6
d2	2- » » » » »	*	(6.3) **

* The curve given by Åberg [3] is a little obscure because of the strong retarding effect.

** Only one G-value could be used because of the strong retarding effect.

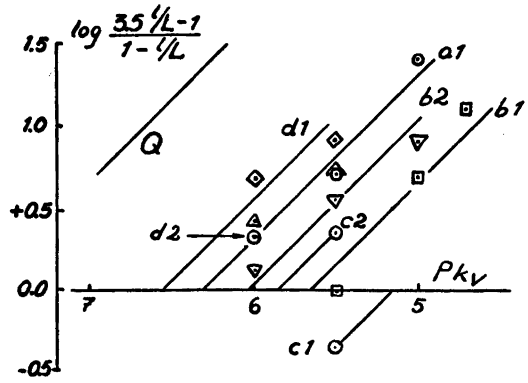

Fig. 10. Graphical evaluation of the pk_v *values for the acids, a1:* △ *, a2 (the same line as for a1): O, b1:* □ *, b2:* ▽ *, c1: O, c2: O, d1:* ◇ *and d2: (→) quoted in Table 2 and the theoretical slope (Q) according to deduction in text, p. 466—7.*

$(3.5\ \beta-1)\ /\ (1-\beta) + \log k = \log m$, where m and k refer to the sulfide- and selenide-acid respectively, and a straight line with the intercept pk_v on the log m-axis should be obtained. Of course only the G-values unaffected by the retarding effect can be used. Bearing this in mind we obtain Fig. 10, and the results may be taken as a verification of the postulates. The intercepts, *i. e.* pk_v values, are given in Table 2 (II).

The acids mentioned accelerate the growth above that of the controls, $l/L > 1$. This may be due to the fact that the activity of the areas occupied by them is higher than the activity of free areas (*cf.* Fig. 5). Such an assumption introduces a new parameter. Other deductions could also be made with the aid of the relations given in Table 1. In this case it seems necessary to discuss the presence of natural auxins and antiauxins, systems with three of even four actual components. In such a case so many parameters appear in the formulas that the discussion becomes rather unprofitable. The author therefore, concludes the discussion at this stage.

Multi patterns. A discussion of adsorptions in more than one pattern must be founded on some assumption concerning the manner in which they interact. If a single chemical promotes the growth at one concentration and represses it at another, finally causing complete inhibition, it seems obvious that the interaction cannot be expressed in terms of a sum. An interaction in the form of a product, on the other hand, seems more likely.

If this assumption is applied to the case where the accelerating and retarding effect is caused by a single chemical the following expression can be derived according to the equations given above: $\frac{1+n\cdot m/k_1}{1+m/k_1}\cdot\frac{1}{1+m/k_2}$ Fig. 9

gives the curves for each factor and for the product. The parameters chosen were $n = 1.5$ and $k_1 = 30\ k_2$. Other parameters, of course, give other curves, and obviously it is possible to construct curves valid for the experimental facts and the postulates. The curve in Fig. 9 undoubtedly gives satisfactory agreement with those given by Åberg [3].

Ultimately, combining the dotted line curve, Fig. 1, with a retardation in another pattern, the triiodo-benzoic acid curve given by Åberg [1] may be understood and discussed in a qualitative manner.

SUMMARY

The interaction of chemicals on root growth is difficult to interpret. It is shown that some simple mathematical deductions give surprisingly good agreements with experimental data.

The deductions may be founded on kinetical or adsorptionchemical conceptions. The latter especially were applied with the aid of the Langmuir adsorption theory. Adsorption in one pattern as well as in several (two) patterns have been discussed, and also the manner in which two (or more) patterns interact to produce the final result, here the root growth.

Applications should be made with caution, because with each assumption new parameters appear in the equations. Therefore no attempts have been made in this paper to discuss the presence of natural auxin or antiauxin.

Though caution is necessary, the author considers the ideas may be of value in this field as well as in the discussion of bacterial growth [4,5], enzyme action [6] and antagonism of drugs [7].

The author is indebted to ass. professor B. Åberg for stimulating discussions.

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On the Application of the Mass Action Law to Cation Exchange Equilibria

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During the last ten years several investigations concerning the applicability of the mass action law to cation exchange equilibria especially on synthetic organic exchangers have been reported in the literature. Some of the investigators have found that the mass law product (which we will call k here analogous to the notations below) is very nearly a mass action constant. Thus Boyd, Schubert, and Adamson [1] studied the exchange $Na^+ - H^+$ on Amberlite IR-1 and arrived at the result that k was approximately constant even at great variations of the mole fractions of the resin phase. A similar result was obtained by Kressman and Kitchener [2] at a great number of exchanges.

Other authors, however, have found that sometimes the mass law product k is rather dependent upon the mole fractions of the resin phase. Such results are for instance reported by Samuelson [3], and Högfeldt, Ekedahl, and Sillén [4]. From the three last-mentioned authors' investigation of the exchange $Ag^+ - H^+$ on Dowex 50 it is evident that at mole fractions < 0.03 of silver in the resin phase k rapidly increases with decreasing silver load, while at mole fractions between 0.03 and 0.40 k is approximately constant.

In three previous papers by the present author (Fronæus [5–7]) concerning the investigation of complex equilibria by the use of cation exchangers, the mass law products k_0 and k_1 of the exchange equilibria: $M^{2+} - Na^+$ and $MA^+ - Na^+$ ($M^{2+} = Cu^{2+}, Ni^{2+}$; $A^- = Ac^-, SCN^-$) on Amberlite IR-105 were also studied. At these investigations the following facts were established.

1. For both cupric and nickel ions and at $p[H^+] \approx 5$ the mass law product k_0 increased rapidly with decreasing values of the cupric respective nickel load C_{MR} on the cation exchanger, though C_{MR} was kept very low in comparison with the exchange capacity of the ion exchanger, and the ionic strength of the

outer solution was kept constant (I = 1 C) by addition of sodium perchlorate. This behaviour is very surprising, for under the conditions mentioned the resin phase is to be regarded as a sodium sulphonate solution of high concentration, and at minute exchanges of sodium ions for cupric or nickel ions we should expect the *rational* activity coefficients in both phases not to be changed appreciably and thus k_0 to be a constant.

2. At the same value of p[H^+] and with A^- = acetate ion it was proved that at a fixed value of C_{MR} both k_0 and k_1 were constant and independent of the distribution of C_{MR} between M^{2+} and MA^+. Thus it seems that at small C_{MR} these cations in spite of their different charges had the same influence on the *stoichiometric* activity coefficients within the resin phase.

3. The measurements on the nickel thiocyanate system showed that at p[H^+] $\approx$ 3 k_0 and k_1 were but slightly dependent upon the nickel load C_{MR}.

These findings indicate that the variations of the stoichiometric activity coefficients must sure be caused by the presence of *small* amounts of coordinating groups, attached to the resin used and operating at p[H^+] = 5 but blocked up at p[H^+] = 3. It will be shown below that in this way the variations of k_0 can be perfectly explained.

To test the theory in the present investigation the exchange Cu^{2+}—Na^+ at different p[H^+]-values has been studied on Amberlite IR-105 and Dowex 50.

THEORETICAL TREATMENT

We presuppose that the solutions contain a divalent cation M^{2+} and a monovalent anion A^- forming mononuclear complexes. Then M^{2+} and the first complex MA^+ can be taken up by the cation exchanger in the sodium form. Furthermore we presuppose that the exchanger contains small amounts of a group B^-, attached to the resin and capable of forming complexes with both M^{2+} and MA^+. Thus we have within the exchanger: M_R^{2+}, MA_R^+, MB_R^+, and MAB_R. (The index R only marks the resin phase.)

Now we apply the mass law to the following exchange equilibria between the "free" ions:

$$M^{2+} + 2\ Na_R^+ \rightleftharpoons M_R^{2+} + 2\ Na^+$$
$$MA^+ + Na_R^+ \rightleftharpoons MA_R^+ + Na^+$$

and get:

$$\frac{[M^{2+}]_R}{[M^{2+}]} = k_0' \cdot \frac{[Na^+]_R^2}{[Na^+]^2}\ ;\ \frac{[MA^+]_R}{[MA^+]} = k_1' \cdot \frac{[Na^+]_R}{[Na^+]} \qquad (1)$$

In the previous papers [5–7] we have not distinguished between M_R^{2+} and MB_R^+ and between MA_R^+ and MAB_R. Thus if in eq. (1) $[M^{2+}]_R$ is exchanged for

$[M^{2+}]_R + [MB^+]_R$, we get the quantity k_0 instead of k_0' (*cf.* Fronaeus [5, p. 861]). On the other hand, if we define a complexity constant β of the complex MB^+ by the relation $[MB^+]_R = \beta \cdot [M^{2+}]_R \cdot [B^-]_R$ we have: $[M^{2+}]_R + [MB^+]_R = [M^{2+}]_R (1 + \beta [B^-]_R)$ and then the relation between k_0' and k_0 is obtained:

$$k_0 = k_0' (1 + \beta [B^-]_R) \tag{2}$$

Analogously we define a complexity constant γ of MAB_R: $[MAB]_R = \gamma \cdot [MA^+]_R \cdot [B^-]_R$ and get the relation between k_1' and the previous mass law product k_1:

$$k_1 = k_1' (1 + \gamma [B^-]_R) \tag{3}$$

For the total concentrations C_{BR} and C_{MR} in the resin phase we have the following expressions:

$$C_{BR} = [HB]_R + [B^-]_R + [MB^+]_R + [MAB]_R \tag{4}$$

$$C_{MR} = [M^{2+}]_R + [MA^+]_R + [MB^+]_R + [MAB]_R \tag{5}$$

Let us denote by k_c the dissociation constant of the acid HB corresponding to the group B^-. Then, using the equations of β and γ, given above, we can transform the expressions (4) and (5) into the following ones:

$$C_{BR} - (1 + [H^+]_R \cdot k_c^{-1}) \cdot [B^-]_R = [B^-]_R (\beta [M^{2+}]_R + \gamma [MA^+]_R) \tag{6}$$

$$C_{MR} = [M^{2+}]_R (1 + \beta [B^-]_R) + [MA^+]_R (1 + \gamma [B^-]_R) \tag{7}$$

Under certain conditions it is possible to obtain very simple relations between the load C_{MR} on the exchanger and the mass law products. Thus it is suitable to distinguish between three different cases.

a) The load C_{MR} is kept so small that $C_{MR} < C_{BR}$, and the complexity constants β and γ have great values. Then $[M^{2+}]_R$ and $[MA^+]_R$ can be neglected in eq. (5) and we get: $C_{BR} - (1 + [H^+]_R \cdot k_c^{-1}) \cdot [B^-]_R = C_{MR}$ and the expression for k_0 becomes:

$$k_0 = k_0' \left(1 + \frac{\beta (C_{BR} - C_{MR})}{1 + [H^+]_R \cdot k_c^{-1}}\right) \tag{8}$$

For k_1 we get a quite analogous expression from eq. (3).

If C_{MR} is kept very small in comparison with the exchange capacity, and the ionic strength of the solutions is constant, the rational activity coefficients involved in k_0' can be presumed to be nearly constant. The value of C_{BR} is fixed for a given exchanger, and we see that at a constant value of $[H^+]$ (and thus of $[H^+]_R$) k_0 is a linearly decreasing function of C_{MR} *solely*.

b) C_{MR} is of about the same magnitude as C_{BR}, or β and γ have not great values. Then we can get simple relations only if $\beta \approx \gamma$. In that case the following equation is at once obtained from eq. (6) and (7):

$$\frac{C_{BR}-(1+[H^+]_R \cdot k_c^{-1})[B^-]_R}{C_{MR}} = \frac{\beta [B^-]_R}{1+\beta [B^-]_R} \qquad (9)$$

From eq. (9) it is easily seen that $[B^-]_R$ and consequently k_0 and k_1 decrease at increasing values of C_{MR}.

A possible complex formation between M^{2+} and sulphonate groups cannot cause a perceptible dependence of k_0 and k_1 upon C_{MR}, for in this case ($B^- = -SO_3^-$) C_{BR} has a great value, equal to the exchange capacity, and thus $C_{MR} \ll C_{BR}$ and $[B^-]_R \approx C_{BR}/(1+[H^+]_R \cdot k_c^{-1})$. Besides, if the ion M^{2+} in reality is not "free" but is present as MSO_3^+-, and we have a second fixed coordinating group B^-, it is easily seen that our eq. (8) and (9) are still valid if k_0' relates to MSO_3^+-. In this case the fulfilment of the condition $\beta \approx \gamma$ for the validity of eq. (9) is plausible, for MSO_3^+- and MA^+ are of the same type with equal net charge and we should expect them to have about the same affinity for B^-, provided there is no steric hindrance to the coordination of the two fixed groups to the same central ion.

c) $C_{MR} > C_{BR}$ and $\beta \approx \gamma$ has a great value. Then in eq. (4) we can neglect $[B^-]_R$ and $[HB]_R$ (at not very low p$[H^+]$-values). From eq. (9) we obtain $C_{BR}/(C_{MR}-C_{BR}) = \beta[B^-]_R$ and:

$$\frac{k_0}{k_0'} = \frac{k_1}{k_1'} = 1 + \frac{C_{BR}}{C_{MR}-C_{BR}} \qquad (10)$$

As a summary of the different cases we can say that at p$[H^+]$-values not far away from pk_c and at a great value of $\beta \approx \gamma$ the relation between the mass law products and C_{MR} can be represented by a curve of the type given in Fig. 1. At decreasing values of $\beta \approx \gamma$ the rectilinear part will be less pronounced. Of course C_{MR} must be much smaller than the exchange capacity, otherwise variations in the rational activity coefficients will occur.

In all the cases discussed it is valid that at decreasing p$[H^+]$-values $[HB]_R \to C_{BR}$ (that is $[B^-]_R \to 0$) if HB is a weak acid. Then at sufficient low p$[H^+]$-values the dependence of k_0 and k_1 upon C_{MR} disappears, and we obtain: $k_0 = k_0'$, $k_1 = k_1'$.

Finally it should be emphasized that a direct experimental determination of the relation between k_0 and C_{MR} is possible only if no diffusible ligand A^- is present. Then all our equations for k_0 are of course valid without the restrictive condition $\beta \approx \gamma$.

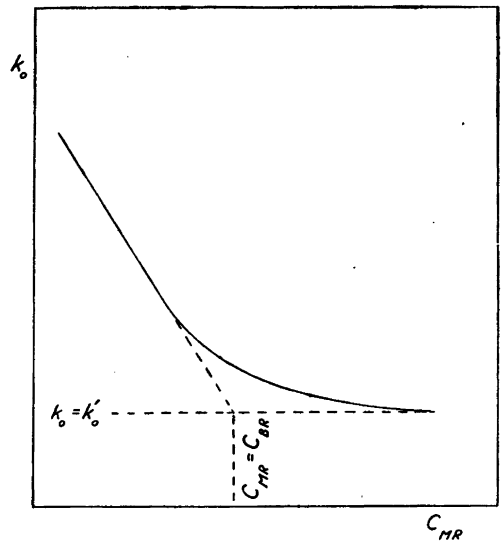

Fig. 1. The fulldrawn curve represents the predicted relation between k_0 and C_{MR} at a great value of $\beta \approx \gamma$.

MEASUREMENTS

Chemicals used. The cation exchangers used were Amberlite IR-105 and Dowex 50. They were transferred into the sodium forms and air-dried. Their exchange capacities were determined at $p[H^+] = 5.0$ in the manner described in a previous paper [7]. The other chemicals were prepared as before [8].

For the determination of corresponding values of k_0 and C_{MR} $v(= 0.01)$ liters of a non-complex solution of the composition: C'_M mC $Cu(ClO_4)_2 + C'_H$mC $HClO_4 + (1000 - C'_H - 3C'_M)$ mC $NaClO_4$ were shaken at 20.0° C with m $(= 0.4)$ grams of the dried exchanger, until an exchange equilibrium was attained. Then the resin was separated and the equilibrium copper concentration C_M and $p[H^+]$ of the solution were measured. The copper determination was performed extinctiometrically with the use of ammonia at the wave length 6 200 Å or for the lowest C_M-values with the use of dithizone (*cf.* Liebhafsky and Winslow [9]) at 5 080 Å. As to experimental details at the determination the reader is referred to a previous paper [5].

The value of $p[H^+]$ was measured potentiometrically with the quinhydrone electrode. At these measurements the reference electrode contained perchloric acid and sodium perchlorate.

When the solutions contain no ligand A^-, we have $C_M = [M^{2+}]$, and the expression (5) takes the form: $C_{MR} = [M^{2+}]_R + [MB^+]_R$. Then according to our definition of k_0 we have:

$$\frac{C_{MR}}{C_M} = k_0 \frac{[Na^+]_R^2}{[Na^+]^2} \tag{11}$$

C_{MR} is obtained from the relation: $C_{MR} = \frac{v}{m}(C'_M - C_M \cdot \delta)$. The value of the factor δ, depending upon the swelling of the resins in the solutions, was measured on a solution with $C'_M = C'_H = 0$. Its total salt concentrations before and after the treatment with the exchanger were determined in the ordinary way with a cation exchanger column in the hydrogen form. The quotient between these concentrations gave us the value of δ. It was 0.985 for Amberlite IR-105 and 0.990 for Dowex 50 at the value of v/m used.

For $[Na^+]$ and $[Na^+]_R$ the following expressions are valid:

$$[Na^+] = \frac{1000 - C'_M}{\delta} - 2\ C_M - [H^+]\ \text{mC}$$

$$[Na^+]_R = a - 2\ C_{MR} - [H^+]_R\ \text{mmoles/gram}$$

At the calculation of $[Na^+]/[Na^+]_R$ the correction terms $[H^+]$ and $[H^+]_R$ can be neglected at p$[H^+]$-values $\geqq 2$. a is the exchange capacity (= sulphonate ion concentration) of the dried exchanger in the sodium form and has the values 2.31 (Amberlite IR-105) and 3.80 (Dowex 50) meq.per gram.

Table 1. Determination of the mass law product k_0 for the exchange $Cu^{2+} - Na^+$ on Amberlite IR-105 at different values of p$[H^+]$ and C_{MR}.

p$[H^+]$	C'_M mC	C_M mC	$C_{MR} \cdot 10^2$ mmole · g^{-1}	$k_0 \cdot 10^{-3}$ l^{-1} · g
2.9	2.46	1.48	2.50	3.27
2.9	4.98	3.10	4.83	3.09
2.9	9.97	6.60	8.70	2.75
2.9	15.00	10.33	12.10	2.55
2.9	20.00	14.17	15.15	2.40
2.0	5.01	3.68	3.48	1.80
2.0	10.01	7.40	6.83	1.83
2.0	15.00	11.18	10.00	1.82
2.0	20.00	15.00	13.10	1.84

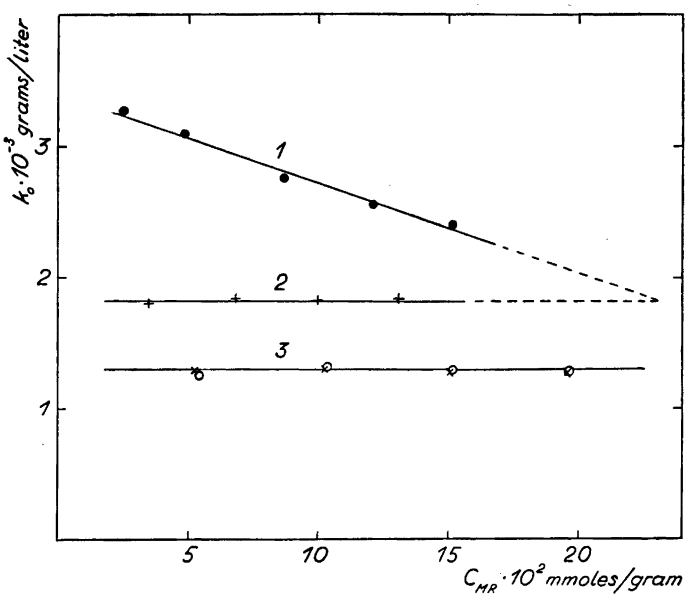

Fig. 2. The relation between k_0 and C_{MR} for the exchange $Cu^{2+}-Na^+$ on different exchangers and at different $p[H^+]$-values.

1. and 2. Amberlite IR–105 at $p[H^+] = 2.9$ and 2.0 respectively;
3. Dowex 50 at $p[H^+] = 4.2$ (○) and 3.1 (×).

Table 1 contains the values obtained at the measurements of the exchange $Cu^{2+}-Na^+$ on Amberlite IR-105 at $p[H^+] = 2.9$ and 2.0. From Fig. 2 it is evident that at $p[H^+] = 2.9$ k_0 decreases approximately linearly with increasing C_{MR} at these low values of the cupric load. From this we conclude that β has a rather great value and that $C_{MR} < C_{BR}$ (case *a*) above). At $p[H^+] = 2.0$ on the other hand k_0 is independent of C_{MR}. At this lower value of $p[H^+]$ the groups B^- evidently are blocked up by hydrogen ions, and thus we have here $k_0 = k_0' = 1.82 \cdot 10^3$ grams/liter. Eq. (8) indicates that if the straight lines 1 and 2 in Fig. 2 are extrapolated, the C_{MR}-value of the intersection gives the value of the concentration C_{BR} (*cf.* Fig. 1). Thus we get here $C_{BR} = 0.23$ mmoles/gram.

In Fig. 3 the connection between k_0 and C_{MR} at $p[H^+] = 4.9$ is represented. The values have been calculated from distribution measuremenets in a previous paper [5]. The measurements, that were performed at varying concentrations of an acetate buffer, have been extrapolated to acetate-free solutions. At $p[H^+] = 4.9$ the dependence of k_0 upon C_{MR} is much greater than at $p[H^+]$

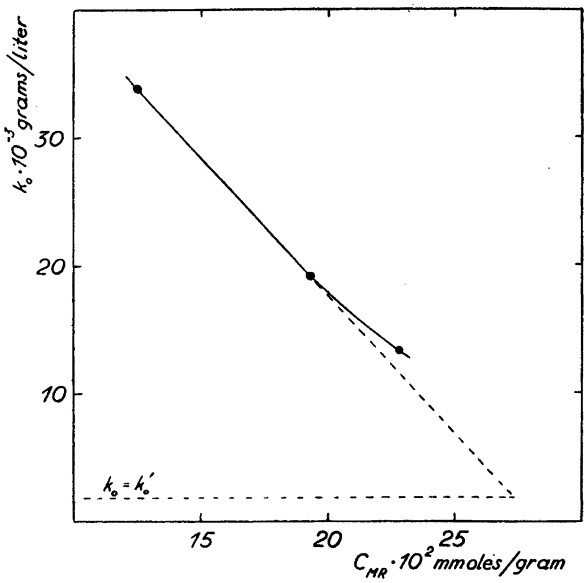

Fig. 3. The fulldrawn curve represents the relation between k_0 and C_{MR} for the exchange $Cu^{2+}-Na^+$ on Amberlite IR-105 at $p[H^+] = 4.9$.

= 2.9. The quotient between the slopes of the two lines is about 30 and this is in accordance with eq. (8) if the dissociation constant of HB has the order of magnitude 10^{-5} C.

In order to determine the concentration C_{BR} anew we have drawn the line $k_0 = k_0'$ in Fig. 3. The intersection of the dashed lines gives us here C_{BR} = 0.27 mmoles/gram. The agreement with the value above is satisfactory.

In Table 2 are collected the values obtained at measurements of the exchange $Cu^{2+}-Na^+$ on the resin Dowex 50. We see that with this ion exchanger k_0 is quite independent of both C_{MR} and $p[H^+]$. As emphasized by Boyd [10] possibly a few carboxyl groups are introduced into a resin like Dowex 50 by the oxidation of chain-ends, but if there is in the preparation used here any fixed group B^- in small concentration, forming a complex with the cupric ion, C_{BR} evidently must be < 0.05 mmoles/gram or less than 1.3 % of the exchange capacity. The value of $k_0 = k_0'$ is here $1.29 \cdot 10^3$ grams/liter.

Fig. 4 represents the connection between k_0 and C_{MR} for the exchange $Ni^{2+}-Na^+$ on Amberlite IR-105 (*cf.* Fronæus [6,7]). At $p[H^+] = 4.9$ the value of k_0 is clearly dependent upon C_{MR}, but the variation is much smaller than that of k_0 for the exchange $Cu^{2+}-Na^+$ at the same $p[H^+]$. Though C_{MR} has been kept lower than the value of C_{BR} determined above, the connection is

Table 2. Determination of the mass law product k_0 for the exchange $Cu^{2+}-Na^+$ on Dowex 50 at different values of $p[H^+]$ and C_{MR}.

$p[H^+]$	C'_M mC	C_M mC	$C_{MR} \cdot 10^2$ mmole · g^{-1}	$k_0 \cdot 10^{-3}$ $l^{-1} \cdot g$
4.2	5.28	3.16	5.38	1.25
4.2	9.99	5.92	10.33	1.32
4.2	15.05	9.08	15.18	1.30
4.2	20.00	12.26	19.65	1.28
3.1	5.03	2.96	5.25	1.29
3.1	10.06	6.00	10.30	1.30
3.1	15.07	9.12	15.10	1.29
3.1	20.00	12.28	19.60	1.28

not linear, showing (case *b*) in the theoretical treatment) that the affinity of the nickel ion for the group B^- is much weaker than that of the cupric ion. This is in agreement with the results from previous investigations [5,6] of the cupric and nickel acetate systems, if B^- is the group $-COO^-$. At $p[H^+] = 3.0$

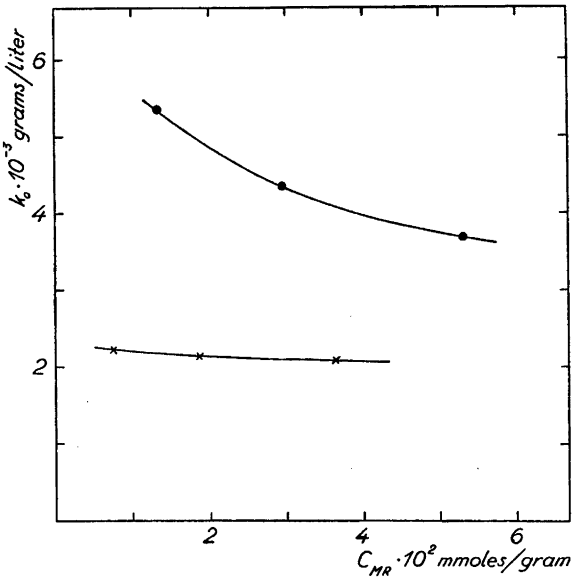

Fig. 4. The relation between k_0 and C_{MR} for the exchange $Ni^{2+}-Na^+$ on Amberlite IR-105 at $p[H^+] = 4.9$ (●) and 3.0 (×).

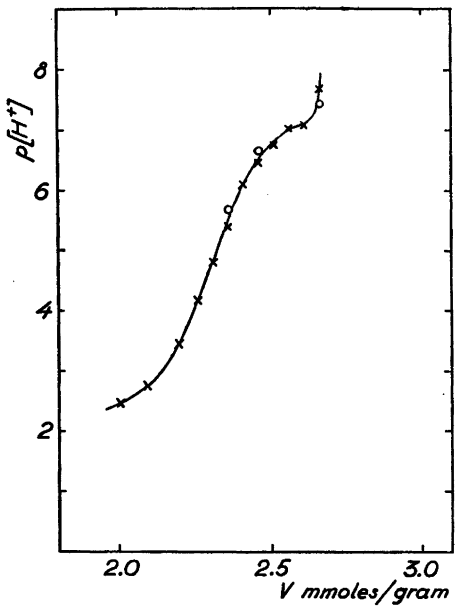

Fig. 5. p[H$^+$] of the outer solution as a function of the amount V of sodium hydroxide added per gram of air-dried Amberlite IR—105 in the hydrogen form.

the B^--groups are practically blocked up by hydrogen ions, and the variation in k_0 is very slight.

In order to get a further proof of the existence of the group B^- in Amberlite IR-105 a potentiometric p[H$^+$]-titration of the exchanger in the hydrogen form was performed.

To 0.5 g of the air-dried exchanger 15.0 ml of 1 C sodium perchlorate solution and varying amounts of sodium hydroxide solutions were added. The solution was shaken with the exchanger for twenty-four hours, and then the solution was separated and its p[H$^+$]-value was measured potentiometrically with the quinhydrone electrode. The reference electrode had the composition mentioned above. Some of the measurements were repeated with a glass electrode.

In Fig. 5 p[H$^+$] of the solution is represented as a function of the amount V of sodium hydroxide added per gram of the dried exchanger. Within a V-range surrounding the steepest part of the curve the quotient $[Na^+]_R/[Na^+]$ is almost constant. Then the quotient $[H^+]_R/[H^+]$ and the difference p[H$^+$] $-$p$[H^+]_R$ must also be approximately constant, and the p[H$^+$]-curve gives us a picture of the form of the p$[H^+]_R$-curve.

At p[H$^+$] $\approx$ 7 the slope has a relative minimum, indicating the presence of a weak acid group within the exchanger in the hydrogen form. At this point

of inflection of the curve $[Na^+]$ is about 0.5 C, whereas $[Na^+]_R$ is very high, and thus $p[H^+]_R$ ($< p[H^+]$) certainly has a value between 5 and 6. This makes it probable that HB is the carboxyl group, and from Fig. 5 it is evident that its concentration is very small in comparison with that of the sulphonic acid group.

Similar curves for the titration of other cation exchangers have been obtained by Gregor and Bregman [11].

DISCUSSION

On account of the very good agreement between the theory given and different experimental findings we can consider it proved that the dependence of k_0 upon C_{MR} sometimes is caused by the presence of small amounts of complex-forming structurally bound groups in the exchanger. Of course the equations are applicable also to the case that the groups have no marked proton affinity ($k_c = \infty$). Thus *e.g.* in a resin like Dowex 50 double bonds in chain-ends possibly could be such groups showing great affinity for certain cations over the whole $p[H^+]$-range.

SUMMARY

The mass action law, applied to cation exchange equilibria, sometimes gives mass law products that are very dependent on the ionic composition of the exchanger, even when variations in the rational activity coefficients cannot possibly be appreciable.

On the basis of previous measurements by the present author [5–7] a theory of the variation of the mass law products is given, founded on the presumtion that *small* amounts of a fixed group with great affinity for certain cations are present in the exchanger.

The results from measurements of the exchanges Cu^{2+}—Na^+ and Ni^{2+}—Na^+ on Amberlite IR-105 are in complete agreement with the theory and show that the groups have a great proton affinity. The presence of the groups has been proved independently by pH-titrations. Presumably they are carboxylate groups.

Measurements of the exchange Cu^{2+}—Na^+ on Dowex 50 show that in the preparation used of this exchanger there are no such groups with a great affinity for the cupric ion, or else their concentration is less than 1.3 % of the exchange capacity.

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The Preparation of Some Carboxylabelled Bile Acids

Bile Acids and Steroids 2

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Lithocholic acid labelled with deuterium in position 3, 4 and 5 has been prepared by Pearlman *et al.*[1] and labelled cholic acid has been prepared biosynthetically by administering tritium water [2] or ^{14}C-labelled acetate [3] to rats and isolating cholic acid from the bile.

We have now prepared cholic, desoxycholic, chenodesoxycholic, lithocholic and cholanic acid labelled in the carboxylgroup with ^{14}C or ^{13}C for use in metabolic studies in progress in this institute.

They have all been prepared by bromine degradation of the silver salt of the acetylated acids, using the modified conditions described by Rottenberg [4]. The properties of the resulting norbromides are listed in Tables 1 and 2. Of these bromides only the one corresponding to desoxycholic acid has been prepared earlier by Brinck, Clark and Wallis [5]. The optical data in Table 2 show that the differences between the molecular rotation (M_D) of an acetylated norbromide and the corresponding acid is reasonably constant, varying between + 60° and + 82°.

The bromides have then been reconverted into the corresponding acids *via* a nitrile synthesis with labelled potassium cyanide prepared according to Belleau and Heard [6]. The yields calculated on the labelled cyanide have been 80—90 per cent.

The yield from the amorphous triacetoxynorbromide corresponding to cholic acid is as good as from the other crystalline bromides. An earlier report [4] that this bromide failed to yield a nitrile has been found to be a mistake.

From the labelled acids described here, the different, partially oxidized acids are easily available by existing methods (*cf.* esp. Fieser *et al.*[7]).

EXPERIMENTAL

23-Bromo-norcholane. 11.4 G of silver cholanate and 6.7 g of silver acetate were dried, finely powdered and treated with 3.4 ml of bromine in boiling ethyl bromide and worked up as described earlier [4]. The neutral product obtained weighed 9.3 g, *i. e.* 96.5 per cent crude yield. A small amount was sublimed at 130°/0.1 mm and recrystallized from ethanol, m.p. 93—94°. The main part (7.5 g) in light petroleum was filtered through a column of active alumina (30 g). Exhaustive extraction with petrol ether gave 6.9 g of bright yellow, wax-like material. A specimen, after two recrystallizations from acetone, melted at 93—94°. Analysis: see Table 1.

Table 1.

			M. p.	% C		% H		% Br	
				Calc.	Found	Calc.	Found	Calc.	Found
I	23-Bromonorcholane	$C_{23}H_{39}Br$	93—94	69.85	69.3	9.95	9.83	20.2	19.4
II	3(α)Acetoxy »	$C_{25}H_{41}O_2Br$	186—186.5	66.21	66.0	9.11	9.14	17.62	17.5
III	3(α)7(α)Diacetoxycholane	$C_{27}H_{43}O_4Br$	183—185	63.39	63.0	8.47	8.35	15.62	15.9
IV	3(α)12(α)Diacetoxycholane	$C_{27}H_{43}O_4Br$	133—134	63.39	63.2	8.47	8.30	15.62	15.4
V	3(α)7(α)12(α)Triacetoxycholane	$C_{29}H_{45}O_6Br$	amorph.						

Lithocholic acid. Lithocholic acid was prepared from desoxycholic acid according to Bergström and Haslewood [8] in 20 g batches in a steel bomb.

3(α)-acetoxycholanic acid. Lithocholic acid (m.p. 181—83°) was dissolved in 2 parts acetic anhydride and 1 part of pyridine and left on the steam bath for 3 hours. After cooling, water was slowly added with stirring when the acetylated acid crystallized. It was filtered off and washed thoroughly with dilute acetic acid, dried and recrystallized from aqueous acetone. Yield: 90 per cent of 3(α)-acetoxycholanic acid, m.p. 166—68°. (*Cf.* Reindel and Niederländer [9]).

23-Bromo-3(α)-acetoxy-norcholane. 6.4 G of the acetylated acid was dissolved in 100 ml ethanol and neutralized with sodium hydroxide (phenolphthalein). This solution was poured into a solution of 4 g silver nitrate and the silver salt was filtered off and washed with aqueous ethanol. The precipitate was dried overnight in a vacuum oven at 60°, pulverized and dried over phosphorus pentoxide *in vacuo*.

To 7.7 g of dry silver lithocholate was added 5.6 g of dry silver acetate and 200 ml of ethyl bromide (purified and freshly distilled over P_2O_5). To the boiling mixture (b.p. 38°) 2.6 ml of dry bromine was added, during 11 minutes, and boiling was continued for another 4 minutes, whereafter the mixture was allowed to cool.

The precipitated silver bromide was filtered off and washed with chloroform. The combined filtrates were washed successively with dilute aqueous solutions of potassium iodide and sodium thiosulphate and evaporated to dryness. The brownish crystalline product (7.3 g) was dissolved in 50 ml benzene and 25 ml light petroleum and filtered through a column of 50 g neutral alumina (activated 10 minutes at 180°).

The main fraction of colourless crystals weighed 4.0 g; m.p. ~180°. Recrystallizations from benzene-light petroleum gave an analytical sample, m.p. 185—88°. Analysis: see Table 1.

23-Bromo-3(α)-hydroxynorcholane was prepared by refluxing 500 mg of the acetate for 2 hours in 20 ml absolute ethanol containing 2.4 millimoles of hydrogen chloride. On

Table 2.

	Acetylated norbromide		Corresponding acetylated acid		M_D (acetyl.norbromide) − M_D (acetyl. acid)
	$[\alpha]_D$*	M_D	$[\alpha]_D$	M_D	
I	+35	+144	+18	+ 64	+80
II	+55	+250	+40	+168	+82
III	+26	+133	+15	+ 72	+61
IV	+97	+498	+92	+438	+60

* All rotations have been determined in chloroform ($c = 1$) at 24−25° in 1 dm micro tube.

careful addition of water the product crystallizes in long needles melting at 130−31°, unchanged on further recrystallization. (Found: Br 19.8. Calc. for $C_{23}H_{39}OBr$: Br 19.4.)

3(α)-hydroxycholanonitrile. 226 mg of the hydroxy bromide was refluxed with 223 mg potassium cyanide in 35 ml of absolute ethanol and 3 ml of water for 48 hours. The solution was then concentrated and the crystalline nitrile (194 mg) isolated. M.p. after recrystallization from aqueous ethanol 193−95°. (Found: C 80.7; H 10.9; N 3.9. Calc. for $C_{24}H_{39}ON$: C 80.61; H 10.99; N 3.91.)

Lithocholic acid. 130 mg of the nitrile was hydrolyzed in a mixture of 25 ml ethanol and 3 ml 30 per cent aqueous potassium hydroxide for 48 hours. The acid fraction weighed 108 mg. Crystallization from aqueous acetone yielded lithocholic acid, m.p. 180−81°.

In the synthesis with labelled potassium cyanide the same conditions were used except that about two moles of bromide was used per mole of cyanide. After refluxing for 48 hours the potassium hydroxide was added directly and the refluxing was continued for a further 48 hours. The overall yield of acid calculated on the labelled cyanide was 80−90 per cent.

Desoxycholic, chenodesoxycholic and cholic acid. These acids were degraded by essentially the same methods. The following simple procedure was found to yield crystalline acetylated acids from all three acids.

The acids were acetylated in an excess of acetic anhydride and pyridine by heating on the steam bath for 3 hours. After cooling, the reaction mixture was very slowly poured into ice cold dilute acetic acid with vigorous stirring when a crystalline precipitate of the acetoxy acids generally formed directly. The acid was filtered off, washed with water and dried *in vacuo* at 50°.

The silver salt was prepared and degraded as mentioned above. The acetylated norbromide was purified by recrystallization after filtering through aluminium oxide. In the isotopic nitrile syntheses from these bromides the acetylated bromide was used directly, as acid catalyzed deacetylation under the conditions used on the acetylated bromide from lithocholic acid apparently caused some dehydration.

In these syntheses with labelled cyanide the usual procedure was otherwise followed except that potassium hydroxide was added in an amount corresponding to the acetoxy groups. The same yields were obtained as reported above.

SUMMARY

Cholanic, lithocholic, desoxycholic, chenodesoxycholic and cholic acid labelled in the carboxyl groups have been prepared by degradation to corresponding norbromides followed by a nitrile synthesis with carbon labelled cyanide.

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On the Complex Chemistry of the Uranyl Ion

VII *. The Complexity of Uranyl Glycolate

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The aim of the present investigation has been to study the formation of complexes of the uranyl ion with a ligand possessing a pronounced tendency to give chelate complexes. If indeed chelates are formed, then the ratios between the stability constants b ** of the consecutive complexes should be greater than in a system where chelates are not formed [1, 2 p. 131]. This is due to purely statistical factors, for a chelating ligand leaves vacant fewer sites for coordination, on which the next ligand can be taken up than does a simply bound one. Thus chelate formation reduces the probability of the formation of the next complex.

For such comparisons to be significant, it is however necessary that the complex systems compared shall have very similar ligands. Only then is it reasonably certain that the other factors governing complex formation — electrostatic as well as those specific for the individual ligands — are approximately of the same magnitude, and the differences between the ratios of the constants thus really due to the statistical factors alone.

A suitable ligand for this investigation is the glycolate ion. Apart from the chelating hydroxy-group, it is completely analogous to the acetate ion, which has no ability to form chelates. The acetate system has been investigated previously [3]. Of other systems studied, the chloroacetate [4] may possibly also be utilized for comparison.

It is of course necessary to investigate the glycolate under the same conditions as those used previously for the selected comparable systems, *i. e.* at the temperature 20° C and the ionic strength I = 1 C, $NaClO_4$ being used as the supplementary neutral salt.

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** defined according to $b_n = \beta_n/\beta_{n-1}$, if $n \geqq 2$; $b_1 = \beta_1$.

The experimental procedure is the same as in the case of acetate and chloroacetate. The complexity constants β are obtained from the complex formation function, *i. e.* from connected values of [A] and $\bar{n}$. These are calculated from $[H^+]'$ of pure glycolate buffers and from $[H^+]$ of the same buffers, containing the total uranyl concentration C_M, according to:

$$[A] = \frac{[H^+]'}{[H^+]} \left(C'_A + \frac{(1+\delta)\,[H^+]' - [H^+]}{\delta} \right) \quad (1)$$

$$\bar{n} = \frac{C'_A + [H^+] - [A]}{C_M} \quad (2)$$

As in [4 p. 791] the approximation $C^0_H = C_s$ is introduced in deducing the equations for $\bar{n}$ and [A]. This implies firstly that the initial concentration of acid in the uranyl perchlorate solution is regarded as arising from the spontaneous hydrolysis of the uranyl ion and, secondly, that this hydrolysis is forced back completely by the complex formation even at the lowest [A] measured. The first condition is well fulfilled, as has been shown by a potentiometric base titration, *cf.* [5 p. 384], which gave $l = 0$, *i. e.* no excess or deficit of acid in the preparation used here. That the second condition is fulfilled, which is of fundamental importance for the success of the investigation [3 p. 203], has been proved by the use of buffers of different ratios $C'_{HA}/C'_A = \delta$. If no perceptible hydrolysis occurs, the complex formation function must be independent of $[H^+]$, *i. e.* δ.

As a rule, two or three different C'_M are used for every buffer, in order to indicate any polynuclear complex formation [5 p. 379] which might occur. In the present system, where the ligand tends to give chelate bonds, polynuclear complexes are in fact very likely to exist.

EXPERIMENTAL

Chemicals used. Two different preparations of *glycolic acid* have been used: Kebo *puriss* and Schering-Kahlbaum *puriss*. Both gave, after drying *in vacuo*, the equivalent weight 76.4 (calc. 76.1). Stock solutions of buffer were prepared by partial neutralization of weighed amounts of dry acid by NaOH and subsequent dilution to the correct volume. They had $C'_A = 1\,000$ mC, and $\delta = 0.4$, 1 and 4, *i. e.* approximately $I = 1$ C. In the following measurements, the buffers of both the acid preparations used gave the same result. The *other chemicals* used are the same as before.

Experimental data. In the buffers of $\delta = 0.4$ and $\delta = 1$, $[H^+]$ and $[H^+]'$ are easily and exactly measured with a quinhydrone electrode in precisely the same manner as in [3] and [4 p. 788]. As before, a quinhydrone electrode RE [5 p. 383] is used as reference electrode, in the present case with $[H^+]_0 = 10.02$ mC. The emf of the cell so formed is denoted E'_q when $[H^+]'$ of pure buffer is measured, and E_q when the buffer contains uranyl salt.

At $\delta = 4$, E'_q is still steady and reproducible, but as soon as uranyl salt is present, the measured E_q moves for some hours towards higher and higher values *. Such an increase indicates that oxidation of the glycolic acid by the quinone is taking place, the uranyl salt evidently being the necessary catalyst. It is not a question of that oxidation of glycolic acid which is brought about by uranyl salt alone and catalysed by light. In the existing conditions, this reaction does not occur with perceptible velocity, as the glass electrode gives fairly constant emfs and thus makes the determination of $[H^+]_0$ possible even at $\delta = 4$.

The experimental setup for the measurements with the glass electrode is the same as described in [5 p. 388]. The glass electrode is immersed in the buffer solution to be measured, and this halfcell is combined with the same quinhydrone RE as above. As in all previous measurements, all buffers of a certain δ and C'_M are prepared in the electrode vessel by a single titration series. The emf is denoted E'_g when a buffer has $C_M = 0$, otherwise E_g. Before and after each such series, the glass electrode is inserted in the reference solution of $[H^+]_0 = 10.02$ mC, $I = 1$, and the emf $E_g^{(10)}$ corresponding to $[H^+]_0$ is measured. If $E_g^{(10)}$ has the same value before and after a buffer series is measured, then the asymmetry potential of the glass electrode has certainly remained constant and hence we get for an arbitrary buffer of the series (*cf.*[5 p. 392]):

$$E_g - E_g^{(10)} = S \cdot \log \frac{[H^+]_0}{[H^+]} \tag{3}$$

When the corresponding uranyl free buffer series is then measured, we get for the same buffer:

$$E'_g - E_g^{(10)'} = S \cdot \log \frac{[H^+]_0}{[H^+]'} \tag{4}$$

where, as a rule $E_g^{(10)'} \neq E_g^{(10)}$ owing to the change of the asymmetry potential which has generally occurred in the time interval between the two series. Hence

$$E'_g - E_g^{(10)'} - (E_g - E_g^{(10)}) = E_A = S \log \frac{[H^+]}{[H^+]'} \tag{5}$$

where E_A is the difference of potential due to complex formation.

From these equations $[H^+]/[H^+]'$, $[H^+]$ and $[H^+]'$ of (1) and (2) may be calculated and hence [A] and $\bar{n}$ for every buffer measured, if only the value of the slope S is known. For the buffer of $\delta = 1$ however, the same E_A:s are obtained with the glass as well as with the quinhydrone electrode. This implies that S must have its theoretical value of 58.2 mV.

The measured values of E'_q and $E'_g - E_g^{(10)'}$ are given in Table 1. As seen from the calculated K_c, glycolate ions as well as undissociated glycolic acid have a marked influence on the ionic medium. If C'_A is kept constant, K_c increases with increasing C'_{HA}, while K decreases with increasing C'_A at constant C'_{HA}. These effects are best observed in the measurements with the glass electrode which are not affected by any salt error. The measurements with the quinhydrone electrode on the other hand contain a salt error, *i. e.* the activity factors of the quinhydrone components in the solution are affected by changing perchlorate for glycolate buffer, as seen from the deviation of E'_q from $E'_g - E_g^{(10)}$. The salt error

* In a less pronounced degree, this behaviour is observed even when a buffer of $\delta = 2$ is used.

Table 1. Determination of E'_q and $E'_g - E_g^{(10)'}$ as a function of C'_A for the different buffers used ($[H^+]_0 = 10.02$ mC).

	quinhydrone electrode						glass electrode			
$\delta \rightarrow$	4		1		0.4		4		1	
C'_A mC	E'_q mV	$K_c \cdot 10^4$ C	E'_q mV	$K_c \cdot 10^4$ C	E'_q mV	$K_c \cdot 10^4$ C	$E'_g - E_g^{(10)'}$ mV	$K_c \cdot 10^4$ C	$E'_g - E_g^{(10)'}$ mV	$K_c \cdot 10^4$ C
13.16	59.6	2.59	94.1	2.51	117.5	2.46	59.6	2.59	95.2	2.39
25.98	58.4	2.60	93.5	2.53	116.9	2.50	58.2	2.62	94.5	2.42
38.5	57.9	2.61	93.3	2.53	116.7	2.50	57.7	2.63	94.4	2.42
50.7	57.5	2.64	93.1	2.54	116.5	2.51	57.4	2.65	94.3	2.42
62.5	57.2	2.66	93.0	2.54	116.4	2.52	57.2	2.66	94.3	2.42
90.9	56.7	2.69	92.9	2.54	116.3	2.52	56.9	2.67	94.3	2.41
117.7	56.3	2.72	92.9	2.54	116.3	2.52	56.7	2.69	94.4	2.40
166.7	55.7		92.8	2.55	116.3	2.52	56.5	2.70	94.7	2.37
210.5	55.2		92.8	2.55	116.3	2.52	56.2	2.73	95.0	2.34
250.0	54.8		92.7	2.56	116.4	2.51	56.1		95.2	2.31
285.9	54.4		92.6	2.57	116.4	2.51	56.0		95.4	2.29
348	53.9		92.5		116.4	2.51	55.9		95.8	
400	53.4		92.4		116.5	2.50	55.8		96.2	
444			92.3		116.5	2.50	55.7		96.5	
500			92.3		116.6		55.6		97.0	
572			92.3		116.7					
$[H^+]'$ mC $\rightarrow$			0.24—0.26		0.10		0.95—1.11		0.21—0.24	

is however without influence on the final result, as E_A (*cf.* (5)), which is of decisive importance for the calculation of [A] and $\bar{n}$, is independent of the electrode used as proved by measurements with $\delta = 1$ using both electrodes, Tab. 3 B. This is a natural consequence of the fact that E_A expresses the emf of a cell with the same buffer concentration and thus the same salt error in both half-cells.

On the other hand, the change in the medium caused by the exchange of perchlorate for glycolate buffer must have some influence on the values of β, as well as on K_c, especially in the case of the higher complexes. The β values of the different complexes thus are not related to the same medium, which renders them less well fitted for comparison with the β values of the acetate system, which to judge from [3, Table 1] does not greatly influence the perchlorate medium.

The values of [A] and $\bar{n}$ obtained for the different electrodes and buffers are given in Tables 2—4. The complex formation functions are given in Fig. 1. As will be seen, the functions do not coincide.

The deviations must be due partly to the influence of hydrolysis. The fact that the difference between $\delta = 0.4$ and $\delta = 1$ is greater than that between $\delta = 1$ and $\delta = 4$ is

Table 2. Determination of corresponding values of $\bar{n}$ and [A] *at the buffer of $\delta = 0.4$. $C_M = 50$ mC. Quinhydrone electrode.*

C_M mC	C'_A mC	E_A mV	$[H^+]$ mC	[A] mC	$\bar{n}$
49.3	13.16	75.9	1.93	0.43	0.298
48.1	38.5	68.2	1.47	2.36	0.782
46.9	62.5	60.5	1.10	5.48	1.24
45.4	90.9	50.0	0.73	12.36	1.74
42.8	142.9	33.1	0.37	38.5	2.45
40.6	189.3	23.3	0.25	75.2	2.82
37.5	250.0	16.0	0.19	132.7	3.13
34.1	318.3	10.7	0.15	208.4	3.23
30.0	400	7.2	0.1	301	3.32
25.0	500	4.5	0.1	419	3.25

in support of this, as is the cessation of the influence of C_M with increasing δ. A variation of C_M produces a rather great effect at $\delta = 1$, but hardly any at $\delta = 4$, *cf.* [3 p. 211]. Now, the pH of the buffer of $\delta = 4$ does not permit any perceptible hydrolysis, as seen from the measurements of [5], if the quite improbable assumption is not made that the degree of hydrolysis is greater for the glycolate complexes than for the uranyl ion itself. The reverse is certainly true and thus the complex formation curve with $\delta = 4$ is in-

Table 3. Determination of corresponding values of $\bar{n}$ and [A] *at the buffer of $\delta = 1$.*

Table 3 A. $C'_M = 10$ mC. Quinhydrone electrode.

C_M mC	C'_A mC	E_A mV	$[H^+]$ mC	[A] mC	$\bar{n}$
9.87	13.16	26.6	0.69	4.52	0.945
9.74	25.98	19.5	0.54	11.99	1.49
9.62	38.5	15.1	0.45	21.19	1.84
9.50	50.7	11.9	0.40	31.7	2.04
9.38	62.5	9.9	0.37	42.4	2.19
9.09	90.9	7.1	0.3	68.9	2.45
8.82	117.7	5.6	0.3	94.6	2.66
8.57	142.9	4.5	0.3	119.9	2.72
8.33	166.7	3.9	0.3	143.2	2.86
7.90	210.5	3.1	0.3	186.6	3.1
7.50	250.0	2.5	0.3	226.5	3.2
7.14	285.9	2.0	0.3	264.9	2.9

Table 3 B. C'_M *= 25 mC and 50 mC. Quinhydrone and glass electrode.*

	C'_M = 25 mC						C'_M = 50 mC					
		quinhydrone				glass		quinhydrone				glass
C'_A mC	C_M mC	E_A mV	[H$^+$] mC	[A] mC	$\bar{n}$	E_A mV	C_M mC	E_A mV	[H$^+$] mC	[A] mC	$\bar{n}$	E_A mV
13.16	24.66	46.5	1.52	1.92	0.518	46.8	49.3	62.2	2.86	0.910	0.306	61.9
25.98	24.35	41.2	1.26	4.93	0.917	41.4	48.7	59.6	2.64	2.25	0.541	59.1
38.5	24.05	35.9	1.03	9.18	1.26	36.1	48.1	56.3	2.33	3.96	0.766	55.9
50.7	23.75	30.8	0.85	14.88	1.545	31.0	47.5	52.5	2.01	6.16	0.979	52.5
62.5	23.45	26.7	0.72	21.7	1.77	26.9	46.9	48.9	1.75	8.84	1.185	48.8
90.9	22.72	19.2	0.5	42.6	2.15	19.1	45.4	40.0	1.2	18.49	1.62	40.0
117.7	22.04	15.1	0.5	64.7	2.43	14.8	44.1	32.5	0.9	32.4	1.96	32.2
142.9	21.42	12.1	0.4	88.5	2.56	12.0	42.8	26.7	0.7	49.6	2.20	26.5
166.7	20.82	10.2	0.4	111.4	2.68	10.3	41.7	22.4	0.6	68.8	2.36	22.4
210.5	19.75	7.8	0.3	154.9	2.83	7.8	39.5	16.7	0.5	108.6	2.60	16.7
250.0	18.75	6.2	0.3	195.5	2.93	6.3	37.5	13.4	0.4	147.2	2.76	13.1
285.9	17.85	5.2	0.3	233.3	2.96	5.3	35.7	10.9	0.4	185.9	2.82	10.6
348.0	16.30	3.9	0.3	298.5	3.05	4.0	32.6	8.0	0.4	253.5	2.91	7.8
400	15.00	3.0	0.3	355	3.0	3.2	30.0	6.2	0.3	312.5	2.92	6.1
444	13.88	2.4	0.3	403	2.9	2.7	27.8	5.2	0.3	361.5	2.99	5.0
500	12.50	1.9	0.3	463	2.9		25.0	3.9	0.3	428	2.9	3.9
572							21.4	2.9	0.3	509	2.9	

dubitably the curve of the uranyl glycolate system, undisturbed by hydrolysis. Its independence of C_M proves that all the complexes are mononuclear.

It is possible that the movement of the curves with δ might be due at least partly to complex formation by the undissociated glycolic acid, by means of its hydroxy-group. Such a competing process would reduce the glycolate complexing, *cf.* Fronaeus [6]. It should however also be possible to prove this spectrophotometrically. The whole extinction curve between 2 250 Å and 4 500 Å has therefore been determined (with a Beckman DU Spectrophotometer) for a solution having $C_M = 15$ mC, [H$^+$] = 500 mC, C_{HA} = 200 mC and I = 1 C ($NaClO_4$), where the dissociation of glycolic acid is almost completely repressed. The curve obtained is however almost identical with that of UO_2^{2+}, the deviations being at most a few per cent. This may be explained by the low [A] ≈ 0.1 mC, which nevertheless exists in the solution. No effect of the glycolic acid itself can thus be proved, certainly it does not form any complexes.

The total deviation between the different complex formation curves can however hardly be ascribed to the hydrolysis, as was the case in the acetate system. In contrast with that case, the curves here do not converge very rapidly but run almost parallel in their upper parts. This behaviour is certainly a consequence of that medium change

Table 4. Determination of corresponding values of $\bar{n}$ and [A] at the buffer of $\delta = 4$. $C'_M = 25$ mC and 50 mC. Glass electrode.

	C'_M = 25 mC					C'_M = 50 mC				
C'_A mC	C_M mC	E_A mV	$[H^+]$ mC	[A] mC	$\bar{n}$	C_M mC	E_A mV	$[H^+]$ mC	[A] mC	$\bar{n}$
13.16	24.66	35.6	3.88	3.24	0.559	49.3	49.1	6.61	1.80	0.365
25.98	24.35	32.4	3.61	7.30	0.92	48.7	48.4	6.79	3.74	0.596
38.5	24.05	28.8	3.2	12.47	1.215	48.1	46.2	6.4	6.12	0.807
50.7	23.75	25.4	2.8	18.75	1.47	47.5	43.7	5.8	8.95	1.00
62.5	23.45	22.2	2.5	26.2	1.66	46.9	40.6	5.2	12.53	1.18
90.9	22.72	16.9	2.1	46.9	2.03	45.4	33.9	4.0	23.8	1.57
117.7	22.04	13.5	1.8	69.5	2.27	44.1	28.5	3.3	38.3	1.88
142.9	21.42	11.2	1.7	92.5	2.43	42.8	24.1	2.8	55.3	2.11
166.7	20.82	9.5	1.6	115.1	2.56	41.7	20.7	2.4	73.8	2.29
210.5	19.75	7.2	1.4	158.9	2.69	39.5	16.0	2.0	112.2	2.54
250.0	18.75	5.8	1.4	199.5	2.77	37.5	12.9	1.8	150.7	2.70
285.9	17.85	4.8	1.3	237	2.8	35.7	10.8	1.7	187.1	2.82
348.0	16.30	3.5	1.3	304	2.8	32.6	8.1	1.5	253.5	2.95
400	15.00	2.7	1.2	361	2.7	30.0	6.5	1.4	309.7	3.06
444	13.88	2.2	1.2	407	2.7	27.8	5.3	1.4	361	3.0
500						25.0	4.2	1.3	424	3.1

which is caused by the glycolic acid, as was observed above. That the curves are parallel indicates, however, that it is the absolute values of the constants which are altered rather than the ratios between them. As the effect of change in medium cannot be separated from that of hydrolysis, it is very difficult to assess the effect of this factor, with any degree of certainty, and it cannot be denied that an error may thereby enter the final result.

THE CALCULATION OF THE CONSTANTS AND THEIR COMPARISON WITH THOSE OF OTHER SYSTEMS WITH SIMILAR LIGANDS

The β values are calculated according to[4 p. 785] using the complex formation function of $\delta = 4$, which at least is free from hydrolytic influence. As the $\bar{n}$/[A]-function has a very steep course in the present system it is necessary to use (5b) of[4 p. 786] with $[A]_0 = 2$ mC, Table 5. In Table 6, the values of β are given, with the maximal random errors assigned, together with values of $\bar{n}$ and α, calculated with the aid of β. The $\bar{n}$ found give the fulldrawn curve of

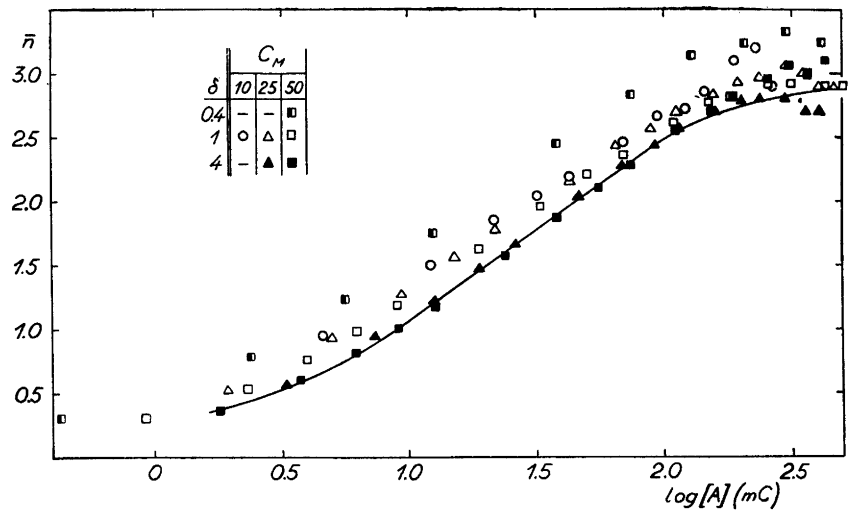

Fig. 1. The complex formation functions. — The signs refer to the series of different δ and C'_M according to the scheme given in the fig. — The fulldrawn curve is obtained from the values of β finally calculated for δ = 4.

Table 5. The X-functions obtained by graphical integration of the $\bar{n}$/[A]-function.

[A] mC	$\ln \frac{X([A])}{X(0.002)}$	$\frac{X([A])}{X(0.002)}$	$X([A])$	X_1 ([A]) C^{-1}	X_2 ([A])·10^{-3} C^{-2}	X_3 ([A])·10^{-5} C^{-3}
0		*0.64*		*263*	*9.1*	*1.62*
2	0	1.00	1.56	280		
4	0.340	1.41	2.19	298		
6	0.624	1.87	2.91	318		
8	0.875	2.40	3.74	342	9.9	
10	1.099	3.00	4.68	368	10.5	
15	1.576	4.83	7.53	436	11.55	1.63
20	1.978	7.21	11.25	513	12.5	1.70
30	2.630	13.87	21.6	687	14.1	1.67
40	3.154	23.4	36.4	885	15.5	1.60
50	3.601	36.6	57.0	1 120	17.1	1.60
75	4.486	88.3	137.8	1 824	20.8	1.56
100	5.173	175.4	273	2 720	24.6	1.55
150	6.210	497	775	5 160	32.7	1.57
200	6.980	1 072	1 672	8 360	40.5	1.57
300	8.145	3 440	5 370	17 900	58.7	1.65
400	8.970	7 850	12 250	30 600	75.8	1.67

Table 6. The ligand number and the composition of the system as calculated for some values of [A] *with the complexity constants found.*

$\beta_1 = 265 \pm 15$ C^{-1} $\beta_2 = (9.1 \pm 0.6) \cdot 10^3$ C^{-2} $\beta_3 = (1.6 \pm 0.2) \cdot 10^5$ C^{-3}

[A] mC	$\bar{n}$	a_0	a_1	a_2	a_3
2	0.385	64	33.5	2.5	0
10	1.05	21	56	19.5	3.5
30	1.74	4.5	37	38.5	20
100	2.47	0.5	9.5	32.5	57.5
200	2.72	0	3	21.5	75.5
400	2.86	0	1	12	87

Fig. 1, which is seen to fit very well the experimental points of the curve of $\delta = 4$.

In Table 7, the constants for the various systems are compared. Besides β, b and the ratios b_n/b_{n-1}, the quantity $1/K_c$ has also been introduced, *i. e.* β_1 $(= b_1)$ of the corresponding acids in the same ionic medium. As a rule, the values of $1/K_c$, β and b decreases in the same sequence: acetate → glycolate → monochloroacetate.

As expected, the affinity of related ligands to H^+ and UO_2^{2+} thus decreases in the same order. β_1 of the glycolate system alone forms an exception to this rule, having an abnormally high value. As a consequence, the ratio b_1/b_2 comes out three times as large here as in the acetate system. The difference between the values of b_2/b_3 are not so marked, but as pointed out above, these values are not very useful owing to the effects of change in medium. For that reason, to which is added a large experimental error of β_3, b_2/b_3 of the mono-

Table 7. The constants of the uranyl acetate, glycolate and chloroacetate systems.

A	$1/K_c$ C^{-1}	$\beta_1 (= b_1)$ C^{-1}	β_2 C^{-2}	β_3 C^{-3}	b_2 C^{-1}	b_3 C^{-1}	b_1/b_2	b_2/b_3
$^{\Gamma}Ac^-$	39 000	240	23 000	$2.2 \cdot 10^6$	96	96	2.5 ± 0.4	1.0 ± 0.4
$HOAc^-$	3 800	265	9 100	$1.6 \cdot 10^5$	34.5	17.5	7.7 ± 1.5	2.0 ± 0.6
$ClAc^-$	450	27	180	(500)	6.7		4.0 ± 0.7	

chloroacetate system should not be used at all. The magnitude of b_1/b_2 for this system seems to fit that of acetate approximately.

Thus it is very possible that the first ligand of the glycolate system is really bound as a chelate. The values of β_1 and, especially, that of b_1/b_2 are both in favour of this hypothesis, when compared with the corresponding values of the other systems. The chelate bond possibly formed is not very strong, however, as it is certainly broken by the addition of the third ligand, UO_2^{2+} being able to coordinate at most three ligands of the acetate type, even if they are coordinated only by their carboxyl radicals.

SUMMARY

The complexity of uranyl glycolate has been quantitatively determined by emf measurements, similar to those used previously in the investigations of the acetate and chloroacetate systems. The presence of three complexes has been proved, *viz.* MA, MA_2 and MA_3, the lattermost being the saturated complex of the system. The first complex MA is remarkably strong as compared with those which follow and might thus possibly be a chelate. If such a complex is formed, it must however be broken when the complex formation proceeds, as the maximum coordination number of the uranyl ion seems to be three for ligands of the acetate type, even if they are bound only by their carboxyls.

Atomkommittén (The Swedish Atomic Committee) has given this work a generous support which I wish gratefully to acknowledge.

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Determination of Pentose Nucleic Acid in Trichloroacetic Acid Extracts of Human Placental Tissue

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The sugar component of pentose nucleic acid (P.N.A.) is the basis of a variety of procedures for determination of this substance [1,2,3]. The specificity of these reactions, however, is poor, and when applying them to the quantitative assay of P.N.A. in biological material great care has to be taken.

A method for quantitative extraction of nucleic acids by hot trichloroacetic acid (T.C.A.) has been devised by Schneider [4]. By applying colorimetric methods to the estimation of pentose and desoxypentose sugars, the nucleic acids can be determined without having to separate the two acids.

In an investigation of the nucleic acids of human placenta the T. C. A. procedure has been used [5,6]. However, a large variety of sugars are present in this tissue. Corey [7] has demonstrated the presence of large amounts of glycogen in the placenta. On T. C. A. hydrolysis this substance may yield glucose. Jones, Gey and Gey [8] have presented ample evidence of the production of chorionic gonadotrophin by placental tissue. This hormone is a mucoprotein containing galactose [9], and the sugar may be split off during the acid hydrolysis. Embryonic tissue has been found to possess considerable fructolytic power [10], and it may be presumed that fructose plays an important role in embryonic tissue [11]. Huggett, Warren and Warren [12] have presented conclusive evidence of the production of fructose by sheep placenta. It is thus necessary to take into consideration the possible presence of all these sugars, the corresponding amino sugars and uronic acids, in the T. C. A. extract of human placenta.

For determination of P. N. A. in the hot T. C. A. extract Schneider [4] uses the orcinol reaction. Some criticism may, however, be raised against this method. As has been shown by Mejbaum [1], the reaction obeys Beer's law only for concentrations between 1 μg and 18 μg pentose per ml. When the

concentration is increased from 18 μg to 24 μg pentose per ml, the extinction coefficient decreases by about 8 %. Frequent dilution of the sample is thus necessary, which introduces some error in the determination. Moreover, the influence of several carbohydrates on the pentose-orcinol reaction has been observed. Schneider[4] emphasizes the necessity of correcting for any desoxypentose nucleic acid (D. N. A.) present, which involves determination of this substance in the sample. Mejbaum[1] points out the influence of glucose. In most determinations of P. N. A. by the orcinol reaction, including that done by Schneider[4,13], no attention seems to have been paid to this source of error. Brown[14] has studied the pentose-orcinol reaction in the presence of glucose, and he has also pointed out the influence of fructose on the reaction.

These considerations and the supposed presence in human placental tissue of a great variety of sugars, which are known to interfere with the pentose-orcinol reaction, made it desirable to find another method for determination of P. N. A. This paper is concerned with the application of Dische's[3] basic cysteine reaction (BCyR) to the determination of P. N. A. in hot T. C. A. extracts of human placental tissue.

The present investigation was carried out along two lines. The influence on the pentose-cysteine reaction of sugars that might be present in the placenta was first studied. This part is a confirmation and extension of some of the studies by Dische[3]. The possible presence of disturbing substances in the hot T. C. A. extract was then investigated.

EXPERIMENTAL

After extraction of cold T.C.A.- and lipid-soluble substances the nucleic acids were extracted by Schneider's[4] method. The P.N.A. content was estimated by a slight modification of Dische's[3] BCyR. One volume, containing the P.N.A. to be estimated, is chilled in an ice-water bath. Four volumes of concentrated chilled (+ 5° C) H_2SO_4 are added. After vigorous shaking for half a minute the tube is placed in a water bath at 60° C for 20 minutes. It is then immersed in a water bath at 25° C, where it is stood for ten minutes. 0.1 ml of 3 % cysteine-HCl is added and the contents are mixed. The tube is again placed in the water bath at 25° C and the sample is read in a Beckman quartz spectrophotometer after exactly ten minutes, using a 1 cm cell. A reagent blank is run. When performing the reaction on T.C.A. extracts from biologic material, corrections are made for the absorption caused by the reaction products with H_2SO_4.

As a standard of reference for the P.N.A. determinations on the T.C.A. extracts, yeast nucleic acid from Hoffmann-La Roche was used. It was purified according to Hammarsten's[15] method and then hydrolyzed in 5 % T.C.A. at 90° C for 20 minutes.

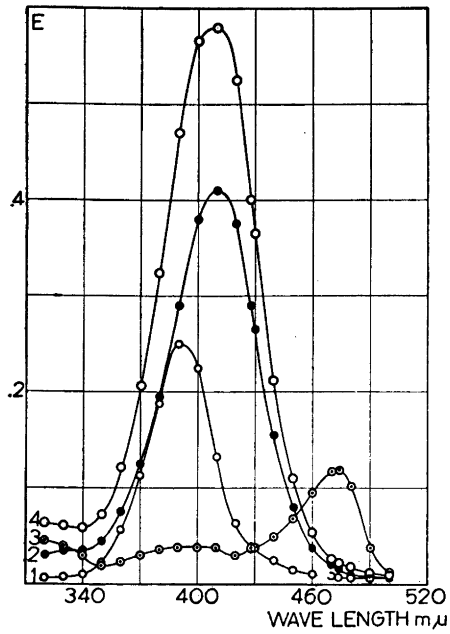

Fig. 1. Basic cysteine reaction. 1. Yeast nucleic acid (3.39 μg P). 2. 32.4 μg dextrose. 3. Desoxypentose nucleic acid (12.05 μg P). 4. 22 μg fructose. All curves read 10 to 20 minutes after adding cysteine HCl.

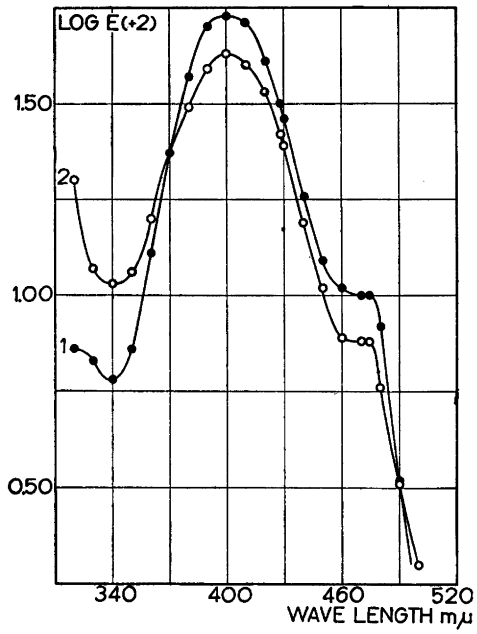

Fig. 2. Basic cysteine reaction. 1. A mixture of yeast nucleic acid (3.39 μg P), 32.4 μg dextrose and desoxypentose nucleic acid (9.6 μg P). 2. T. C. A. extract from human placenta. Curves read 10 to 20 minutes after adding cysteine HCl. Log extinction plotted against wave length.

RESULTS

As pointed out by Dische [3], every class of sugar yields spectrophotometrically measurable reaction products, when the reaction is done as described above. Fig. 1 shows the absorption curves for yeast nucleic acid, D. N. A., dextrose and fructose. Fig. 2 shows the absorption curve for a hot T. C. A. extract from human placenta. On comparing this curve with that obtained from a mixture of yeast nucleic acid, D. N. A. and dextrose, it is clear that one or more hexoses are present.

It will be seen in Fig. 1 that dextrose and D. N. A. show the same extinction at 390 mμ and 428 mμ. The difference in extinctions of P. N. A. at these wave lengths follows Beer's law. Thus, sugars behaving similarly to dextrose and desoxypentose do not interfere with the quantitative determination of P. N. A. Table 1 gives the figures for differences between extinctions at these

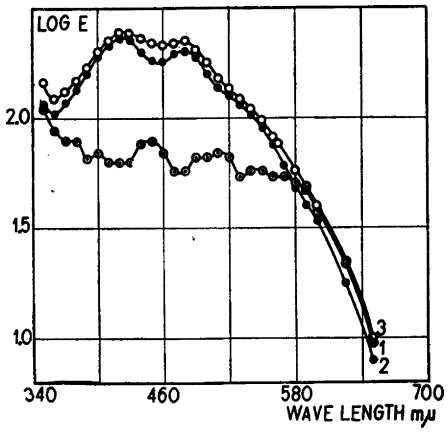

Fig. 3. Resorcin reaction. 1. T. C. A. extract. 2. 441 μg fructose. 3. 441 μg fructose + T. C. A. extract. Log extinction plotted against wave length.

wave lengths for various sugars. It will be seen that quantitative estimation of P. N. A. is possible in the presence of desoxypentose, dextrose, galactose, mannose, glucosamine, and galactosamine, but not in the presence of large quantities of fructose, fucose or uronic acids, especially not galacturonic acid.

The resorcin reaction was carried out to investigate whether fructose is present in the hot T. C. A. extract. Concentrated hydrochloric acid to give a final concentration of 12 %, and 0.1 ml of a 50 % resorcin solution were used. The mixture was placed in a water bath at 100° C for five minutes and the

Table 1. Differences of densities at 390 mμ and 428 mμ for various sugars in Dische's BCyR. Readings 10 minutes after addition of cysteine HCl.

Substance Amount: 10 μg	△ 390.428 × 1 000
Nucleic acid bound pentose *	107
Nucleic acid bound desoxypentose *	zero
Glucose	zero
Galactose	zero
Mannose	zero
Fructose	34
Fucose	16
Glucosamine	zero
Galactosamine	zero
Glucuronic acid	3
Galacturonic acid	32

* Calculated by the percentage of pentose of a structural polytetranucleotide.

Table 2. Recovery tests. Yeast nucleic acid added to T.C.A. extracts of human placenta.

μg P.N.A. phosphorus		Difference in per cent
Added	Found	
3.78	3.85	1.19
3.78	3.88	2.58
3.78	3.90	3.08
9.44	9.70	2.69
9.44	9.56	1.26
9.44	9.56	1.26
12.88	13.38	3.74
12.88	13.43	4.10
12.88	13.33	3.38
	Average	2.59

coloured product was extracted with amyl alcohol. Fig. 3 shows the absorption curves for different solutions. On comparing these curves it will be found that the presence of fructose in the T. C. A. extract is not probable. The fructose test was also carried out according to Roe's [16] method. The hot T. C. A. extract gave no measurable absorption at 490 mμ.

Dische's [3] test for fucose was done to find out if this substance could be detected in the hot T. C. A. extract. The difference between extinctions at 400 mμ and 380 mμ decreased slightly after two hours, as compared with the difference ten minutes after addition of 0.6 ml of water to the mixture. The result obtained speaks in favour of the absence of fucose in the extract. The possibility that the T. C. A. would depress the reaction was ruled out.

Galacturonic acid has never been demonstrated with certainty in animal tissues. Glucuronic acid, on the other hand, may be presumed to be present in placental tissue. It will be seen in Table 1 that the error associated with the estimation of nucleic acid-bound pentose in the presence of an amount of glucuronic acid equal to that of the nucleic acid sugar is fairly small, being about 2.8 %. It was considered desirable, however, to rule out the presence of glucuronic acid in the T.C.A. extract by direct evidence. To this end the glucuronic acid reaction devised by Dische [17] was used. The result of the test was such as to rule out the presence of any significant amount of glucuronic acid in the hot T.C.A. extract. The T.C.A. had no effect on this reaction.

These tests seem to show that the P.N.A. content in T.C.A. extracts of human placenta can be determined by this method.

The relation between concentration and absorption was investigated by the method of least squares [18,19]. When using a 1 cm cell, the method can be employed for amounts of P.N.A. phosphorus between 0.75 μg and 16 μg. The standard deviation ($n = 20$) expressed as a percentage of the average is 2.28.

The extinction per μg phosphorus shows some variation for different batches of yeast nucleic acid. Three batches were investigated. All the preparations were purified by the method of Hammarsten. The extinction values were a maximum of 7.9 % apart, the average extinction being 0.062 per μg phosphorus.

Table 2 summarizes the data from a series of recovery tests. Known amounts of purified yeast nucleic acid were added to T.C.A. extracts of human placenta and estimated by the method outlined above.

SUMMARY

The applicability of a modification of the pentose-cysteine reaction to the estimation of P.N.A. in hot T.C.A. extracts of human placental tissue has been investigated. The influence on the reaction of fructose, fucose and glucuronic acid, carbohydrates which are probably present in this kind of tissue, has been demonstrated. The presence of these substances in the hot T.C.A. extracts has been ruled out. The accuracy of the method has been evaluated by statistical analysis, the standard deviation being 2.28 % of the average.

I wish to express my gratitude to Prof. E. Hammarsten and Prof. E. Jorpes for invaluable advice and discussions, and also to the Reserve Funds of Karolinska Institutet for partially defraying the expenses of this work.

The author wishes to thank Mrs. M. Thorell for the drawings.

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A Spectrophotometric Study on the Desoxypentose Nucleic Acid — Cysteine Reaction

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In 1944 Dische [1] described the reaction between desoxypentose nucleic acid (D.N.A.) and cysteine in sulphuric acid. Stumpf [2] presented a paper dealing with the quantitative colorimetric application of Dische's reaction to the specific estimation of D.N.A. This reaction has been used by Hedén [3], Kurnick [4] and others.

In an investigation [5,6] of the amount of nucleic acids in various organs the present writer used, among other methods, the extraction procedure described by Schneider.[7] D.N.A. was estimated according to Stumpf's method, but the values showed a great scatter. In my experience the error inherent in the method is fairly great. Stumpf gives no information about the standard error, nor do others. According to Stumpf the extinction curve follows Beer's law for amounts between 25 and 550 μg D.N.A. Amounts below 100 μg, however, give an extinction less than 0.1, when read in a Beckman spectrophotometer using a 1 cm cell.

In most of my preparations the total amount of D.N.A. was less than 100 μg. In an attempt to use the D.N.A.-cysteine reaction under these conditions and in order to investigate the various factors affecting the reaction, a systematic investigation of the reaction has been made.

EXPERIMENTAL

Concentration of sulphuric acid. It was observed that the sensitivity of the method was considerably increased when concentrated acid was used. The colour produced is yellow and there is a very sharp absorption peak at 474 mμ (Fig. 1). The optimal final concentration of the acid is 86.5 per cent, which means 5 ml of concentrated acid in a total volume of 5.55 ml (Fig. 2).

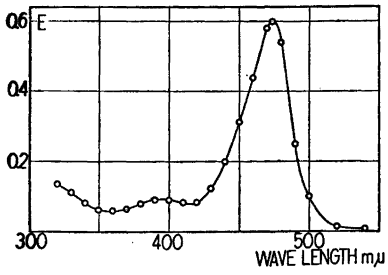

Fig. 1. Absorption spectrum for the reaction product of D.N.A. and cysteine in concentrated sulphuric acid.

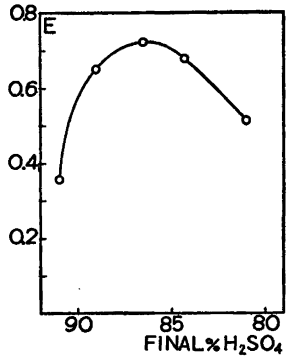

Fig. 2. Influence of concentration of sulphuric acid on the intensity of the D.N.A.-cysteine reaction.

Concentration of cysteine hydrochloride. Maximal absorption, other factors being unchanged, was obtained with 0.05 ml of a 1 % cysteine hydrochloride solution on a total volume of 5.55 ml (Fig. 3). A preparation from Merck (U.S.A.) gave the most consistent results.

Influence of temperature. It was observed that the temperature had a pronounced effect on the reaction. To get reproducible results it is necessary to avoid overheating at the moment of mixing the D.N.A.-solution and the sulphuric acid. The test tubes are therefore thoroughly chilled and the sulphuric acid is cooled to at least $-10°$ C before mixing is done. The cold acid is then pipetted into the test tubes along their sides, so that two layers are formed. This is done with the test tubes immersed in an ice-water bath. The contents are then mixed.

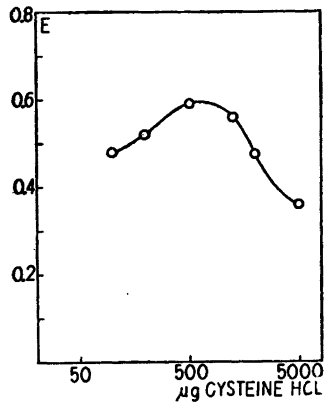

Fig. 3. Influence of concentration of cysteine HCl on the intensity of the D.N.A.-cysteine reaction.

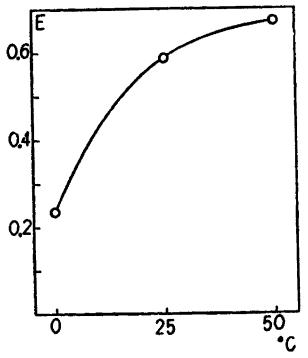

Fig. 4. Influence of environmental temperature during colour development.

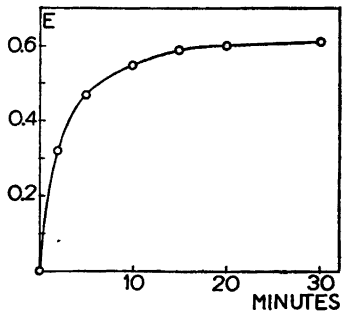

Fig. 5. Rate of colour development.

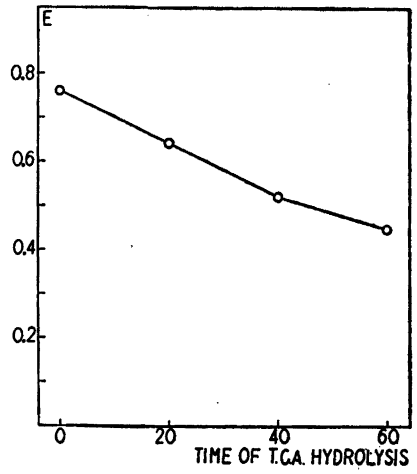

Fig. 6. Influence of trichloroacetic acid hydrolysis of D.N.A. on the intensity of the D.N.A.-cysteine reaction.

When the tubes are allowed to stand at room temperature until reading in the spectrophotometer, there is a considerable scatter in the results. To study the influence of the environmental temperature during colour development the tubes were immersed in a water bath at 0°, 25° and 50° C immediately after mixing. The results are shown in Fig. 4.

Colour development. The increase in colour intensity is very marked during the first few minutes after mixing. After fifteen minutes the curve flattens out (Fig. 5).

Influence of trichloroacetic acid (T.C.A.) hydrolysis of D.N.A. This part of the investigation was carried out with a view to apply this method of determination to nucleic acid extracts according to the technique devised by Schneider [7]. The D.N.A. preparation used

Table 1. Recovery tests.

Mixture	μg D.N.A.P.		Difference in per cent
	Added	Found	
D.N.A. + P.N.A.	1.30	1.34	3.0
—»—	8.90	8.78	1.4
—»—	5.08	4.97	2.2
D.N.A. + Schneider-extract	3.62	3.67	1.3
—»—	2.42	2.47	2.3
—»—	4.83	4.75	1.7
—»—	4.20	4.24	0.9

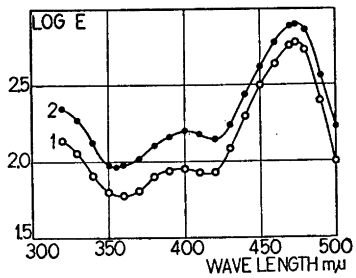

Fig. 7. D.N.A.-cysteine reaction. 1. D.N.A. solution. 2. T.C.A. extract of human placenta (time of T.C.A. hydrolysis 20 minutes). Log extinction plotted against wave length.

as a standard was prepared by the method of Hammarsten [8]. When an aqueous solution of D.N.A. was diluted with the same volume of 10 % T.C.A., a cloudy precipitate was obtained. This precipitate was dissolved by warming the solution in the water bath at 90° C for a few minutes. The influence of T.C.A. hydrolysis for varying length of time at this temperature on the intensity of the D.N.A.-cysteine reaction is shown in Fig. 6. As 0 minutes hydrolysis is taken the value obtained by determination of an aqueous solution of D.N.A.

Specificity of the reaction. 1 mg of yeast nucleic acid and 0.2 mg of either dextrose, galactose, mannose, galactosamine, glucosamine or glucuronic acid gave no extinction at 474 mμ.

Proposed method of determination of D.N.A. The above mentioned observations illustrate the sensitivity of the method. The marked influence of a variety of factors stresses the necessity of working under strictly defined conditions.

The D.N.A. solution to be determined should amount to 0.5 ml. To this is added with an 'Agla' micrometer syringe 0.05 ml of a 1 % l(+)cysteine hydrochloride solution. The test tubes are then placed in an ice-water bath for about ten minutes. 5 ml of properly chilled concentrated sulphuric acid is then added as described above. After mixing the contents of the tubes, these are placed in a water bath at 25° C. The optical densities

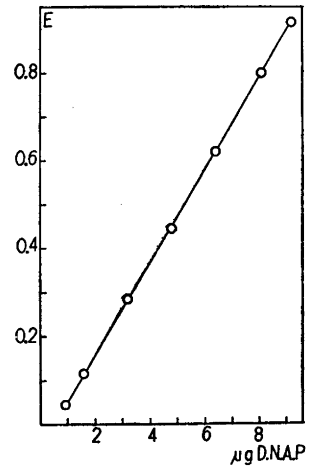

Fig. 8. Standard absorption curve for D.N.A. hydrolysed in 5 % T.C.A. for 20 minutes.
Regression line $y = -0.056 + 0.1054\,x$
$\bar{x} = 60.54 \pm 6.548.$
$\bar{y} = 0.454 \pm 0.0553.$
Regression coefficient 0.1054 ± 0.000625
Correlation coefficient 0.9996 ± 0.000179
$\sigma(y-Y) = [0.006395] = 1.54$ *per cent.*
y = *extinction (E)*
x = *μg D.N.A. phosphorus*

are read after exactly (stop watch) 20 minutes in a Beckman quartz spectrophotometer at 474 mμ wave length using a 1 cm cuvette. A reagent blank is run.

Recovery tests. A known amount of D.N.A. was added to pentose nucleic acid solutions and to hot T.C.A. extracts of human placenta. The D.N.A. was determined according to the method described here. The results are summarized in Table 1.

To demonstrate further the applicability of this reaction to the estimation of desoxypentose nucleic acid in a hot T.C.A. extract the absorption curves for a pure D.N.A. solution and a hot T.C.A. extract according to the reaction described here were determined. (Fig. 7.)

Statistical analysis. The relation between concentration and absorption has been investigated. The data fit a straight line for amounts of D.N.A. phosphorus between 0.96 μg and 9.2 μg (Fig. 8). The rectilinear line has been calculated by the method of least squares [9,10]. The standard deviation is calculated as the square root of the mean square. As this figure is a logarithm, the standard deviation as a percentage of the average has been calculated from the antilog of the standard deviation [11]. It amounts to 1.54 per cent.

SUMMARY

The colorimetric determination of D.N.A. by the cysteine reaction has been investigated and a standard procedure is proposed. The concentration range falls between 0.96 μg and 9.2 μg D.N.A. phosphorus. The error of the method is 1.54 per cent. The influence of T.C.A. hydrolysis of D.N.A. on the cysteine reaction has been studied with a view to apply the method to T.C.A. extracts of nucleic acids.

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Quantitative Studies on the Nucleic Acids in Human Placenta

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Human placental tissue offers a favourable material for the study of quantitative changes of the nucleic acids during development.[1]

During an investigation concerning these problems it was found necessary to make some comparative studies on different procedures for the determination of the nucleic acids. This was considered important from two points of view. Up to the present, the human placenta has not been investigated for nucleic acids and might present some peculiarities. Some earlier comparisons of the procedures of Schneider [2] and Schmidt and Thannhauser [3] have revealed discrepancies between the methods. As these procedures have never been run together with a method that yields pure nucleic acid fractions, it was thought that by comparing them with the method of Hammarsten [4], more precise information concerning the source of error in the respective method would be obtained.

EXPERIMENTAL

Placentas from different stages of pregnancy were obtained at abortions, provoked on social-medical indications according to Swedish law, and at parturitions *. All the patients were in good health. When possible, the age of the placentas was estimated from the over-all (crown-heel) length of the foetus, applying Haase's (quoted from Wylie and Amidon [5]) rule. In some instances, however, the foetus was destroyed at operation, and in these cases the age of the placenta was calculated from the time of the last menstrual period.

Immediately after removal the placentas were freed from the foetal membranes and the decidua. The remaining foetal placenta was cut into small sections, wiped free of

* The material was kindly placed at my disposal through the generosity of Docent S. Karlson, M.D., Head of the General Maternity Hospital, Stockholm, and Dr. E. Westberg, M.D., Head of the Department for Gynecology and Obstetrics at St. Erik's Hospital, Stockholm.

blood and placed in a large volume of absolute alcohol. The dissection of the foetal portion of the placenta from the maternal elements was in some cases rather difficult in the fresh state, especially as it had to be done very quickly. It was much easier carried out after the specimens had been fixed in alcohol for some time.

The placentas were homogenized in alcohol in a Turmix blender. The homogenates were filtered with the aid of a Buechner funnel, repeatedly washed with ethyl ether and allowed to dry at room temperature.

Extraction of trichloroacetic acid- (T.C.A.) soluble phosphorus. About 100 mg of the dry powder is suspended in 15 ml of ice-cold 10 % T.C.A. The suspension is allowed to stand at 0° C for 60 minutes. It is then spun in a refrigerated centrifuge. The tissue is resuspended three times in the same amount of T.C.A., the centrifugate always being discarded.

Extraction of lipid-soluble phosphorus. To remove the T.C.A. the tissue is suspended twice in 80 % alcohol and then washed three times in absolute alcohol. It is then again suspended in a large volume (50 to 80 ml) of an ethyl ether-alcohol mixture (1 : 3). The suspension is boiled for two hours under reflux and then washed twice in alcohol.

Vibration. The tissue residue is then thoroughly homogenized in a Turmix blender and placed in a thick-walled glass tube with a capacity of about 100 ml. This is filled with glass beads, and alcohol is poured into the interstices. The specimen is vibrated for 96—120 hours in the manner prescribed by Hammarsten [4]. The tissue is then washed twice with ethyl ether and allowed to dry at room temperature.

Extraction of nucleic acids according to the method of Hammarsten. The extraction procedure outlined by Hammarsten was followed. Some details, however, may be pointed out. The tissue is extracted thirteen times. The last extraction is done over-night. This comprehensive extraction procedure was found to be necessary particularly to obtain maximum yield of pentose nucleic acid (P.N.A.). After extraction and centrifugation the polynucleotides are precipitated as copper salts. The collected supernates are allowed to stand for 30 to 32 hours.

After alkaline hydrolysis the desoxypentose nucleic acid (D.N.A.) is precipitated with 0.1 *M* lanthanum nitrate in 0.01 *N* H_2SO_4 [6]. The precipitate is finally dissolved in a small volume of 1 *M* K_2CO_3.

The tissue residue after the extractions with the salt solutions, and the undissolved precipitate after the potassium-urea extractions, are repeatedly washed with distilled water and combined.

Nucleic acid extraction according to the method of Schneider. Between 30 and 60 mg of dry tissue is suspended in 2.5 ml of 5 % T.C.A. at room temperature. After centrifuging the tissue is resuspended in 4.5 ml of T.C.A. and allowed to stand at 90° C for 20 minutes. The residue is washed with 2.5 ml of T.C.A. at room temperature. The supernatant solutions are collected and diluted with distilled water to a given volume.

Separation of nucleic acids by the Schmidt and Thannhauser procedure. 75 to 150 mg of dry tissue is suspended in a given volume of 1 *N* KOH, and hydrolysed in the water bath at 37° C for 24 hours. To 2 ml of this solution is added 2.5 ml of an ice-cold mixture of 5 % T.C.A. and 6 *N* HCl (5 : 1). The mixture is spun in the refrigerated centrifuge. The precipitate is washed twice with 5 % T.C.A. The supernatant solutions, containing also P.N.A., are diluted with distilled water to a given volume. The precipitate, containing among other substances, D.N.A. and protein, is dissolved in a given volume of 1 *N* KOH.

Quantitative determination of nucleic acids. Nucleic acids, extracted and separated according to Hammarsten's technique and separated by the Schmidt and Thannhauser method, are estimated as phosphorus. The phosphorus is determined according to Teorell's [7] modification of Fiske and SubbaRow's [8] method.

The nucleic acids in the T.C.A. extract are estimated colorimetrically as sugars. For the determination of D.N.A. the present writer has worked out a modification of Dische's [9] cysteine reaction. A detailed account of this method is given elsewhere [10]. The regression line for the relation between concentration and absorption is satisfied by the equation $y = -0.056 + 0.1054\,x$, y indicating the extinction. The amount of D.N.A. phosphorus present is obtained by using this formula. The assay of P.N.A. is made according to Dische's [11] basic cysteine reaction. Its adoption to T.C.A. extracts has been described elsewhere [12]. The extinction of an amount of P.N.A., equivalent to 1 μg phosphorus, is 0.062, under the experimental conditions described.

All colorimetric determinations were carried out in a Beckman quartz spectrophotometer, using a 1 cm cell.

RESULTS

The analytical results are summarized in Table 1. Every figure is the mean of duplicates. It will be seen that there is good agreement between the values obtained by the method of Hammarsten and by the application of the cysteine reactions to the hot T. C. A. extract. The Schmidt and Thannhauser values, on the other hand, show considerable divergencies.

The amount of nucleic acids per unit dry tissue powder decreases with the aging of the placenta. This decrease is brought about by the reduced P.N.A.

Table 1. Comparison between results obtained with different methods.

Method		Hammarsten						T. C. A. extraction							Schmidt-Thannhauser			
Weeks of pregn.	P_{tot}	NAP	PNAP	DNAP	P_{res}	$P_{tot}-P_{est}$	PNAP/DNAP	NAP	PNAP*	DNAP*	P_{extr}	$P_{extr}-P_{NAP}$	P_{res}	PNAP/DNAP	NAP	PNAP	DNAP	PNAP/DNAP
9	6 235	4 480	2 665	1 815	530	1 225	1.47	4 250	2 500	1 750	5 700	1 450	535	1.43	6 235	4 000	2 230	1.79
12	6 580	4 105	2 315	1 790	495	1 980	1.30	4 260	2 495	1 765	6 125	1 865	455	1.41	6 580	4 000	2 580	1.55
24	5 248	4 075	1 805	2 270	327	846	0.80	4 005	1 760	2 245	4 750	745	498	0.78	5 248	2 878	2 280	1.20
40	4 533	3 250	1 210	2 040	374	909	0.59	3 295	1 235	2 060	4 165	870	368	0.60	4 533	1 943	2 590	0.75

P_{tot}: phosphorus left after lipid and cold T.C.A. extraction.
NAP: nucleic acid phosphorus.
PNAP: pentose nucleic acid phosphorus.
DNAP: desoxypentose nucleic acid phosphorus.
P_{res}: residual phosphorus in tissue after extraction.
P_{est}: NAP + P_{res} according to Hammarsten.
P_{extr}: phosphorus extracted by Schneider's procedure.
The figures are given as μg phosphorus/gram dry tissue powder.

* Calculated from sugar determination.

content. The D.N.A. increases slightly. The P.N.A./D.N.A. ratio will thus show a marked change during the course of pregnancy.

The hot T.C.A. extract contains a considerable amount of phosphorus, which is probably not nucleic acid phosphorus as calculated from the sugar determinations. An equal amount of phosphorus is extracted by the method of Hammarsten, but is not precipitable with copper.

There is an appreciable quantity of phosphorus left in the tissue residue after both extraction procedures.

DISCUSSION

The question of the ability of T.C.A. to remove acid-soluble phosphorus has been discussed by several authors. Barrenscheen and Peham [13] consider that extraction with 10 % T.C.A. at 0° C removes quantitatively the nucleosides and nucleotides. There are, however, also other fractions which have to be removed, and Davidson, Frazer and Hutchison [14] have made a comprehensive investigation of this question. They compared the results following three, six and twenty extractions with 10 % T.C.A. There was little difference after three and six times. Twenty extractions gave a larger yield, but the authors conclude from turnover rate determinations that this phosphorus is not only acid-soluble phosphorus, which has not been removed by earlier washings, but also phosphorus split off from other fractions by T.C.A. hydrolysis. On the other hand, these authors hold that, however numerous the washings, the contamination of protein-bound phosphorus with traces of inorganic phosphate cannot be ruled out. This is of course of utmost importance in tracer work but has no bearing on the results in the present investigation. Four extractions with 10 % T.C.A. at 0° C were supposed to be sufficient.

To find out whether the lipid extraction was complete, two of the placentas were further twice extracted with a $CHCl_3$-alcohol (1 : 3) mixture, each time for 90 minutes, in the water bath at 60° C. The amount of phosphorus in the tissue after this treatment was exactly the same as before.

The values obtained by the extraction procedure of Schneider, together with the colorimetric methods used here for the determination of the sugar components of nucleic acids, are in good agreement with the results obtained by Hammarsten's method. The possible effect of several substances on the P.N.A. determinations in the hot T.C.A. extract has been discussed earlier.[12] Direct evidence of the possibility of determining the nucleic acids by the colorimetric sugar reactions has been produced [10,12]. The agreement between the results obtained by Hammarsten's method and the T.C.A. extraction procedure affords strong indirect evidence that the nucleic acid determinations, based

upon estimation of nucleic acid-bound sugar as performed in this investigation, may be considered reliable. When separation of the nucleic acids is unnecessary, the T.C.A. extraction procedure offers an accurate and convenient means for quantitative determination of nucleic acids.

Contrary to the findings of Schneider [2,15] all the phosphorus in the hot T. C. A. extract of placental tissue could not be accounted for by nucleic acids as calculated from sugar determinations.

The method of Schmidt and Thannhauser has been repeatedly criticized and Schneider [15] made a comparison of his method and that of Schmidt and Thannhauser. He investigated the different Schmidt and Thannhauser fractions for phosphorus, pentose and desoxypentose. The values obtained by the sugar determinations showed good agreement with those obtained by his own method. On the other hand, he states that "results based upon phosphorus determinations were less consistent and reliable". From his tabulated values it will be seen that the amount of phosphorus in the tissue residue after extraction with hot T. C. A. is fairly high, especially in liver, kidney and brain tissue, in the brain tissue amounting to about 63 per cent of the nucleic acid phosphorus. Schneider [15] does not make any comment on this fact. Davidson, Frazer and Hutchison [14] made a comprehensive investigation on the protein-bound phosphorus fractions in rat and rabbit liver. They too found considerable quantities of phosphorus in the residue after hot T. C. A. extraction. They demonstrated that the bulk of this phosphorus cannot be regarded as phosphoprotein and assumed that the hot T. C. A. extraction is incomplete. These authors made no determinations of the amount of the different nucleic acids in the hot T. C. A. extract. From their results, however, it is clear that the amount of phosphorus in this extract is less than the combined amounts of phosphorus in the different Schmidt and Thannhauser nucleic acid fractions. They also state, "It would appear probable, therefore, that fraction A_2S (corresponding to the P. N. A. P. fraction in this investigation) contains phosphorus compounds other than the acid-soluble ribonucleotides and the inorganic phosphate derived from phospho-protein".

With both the Hammarsten and the T. C. A. extraction procedure I obtained a large phosphorus fraction in the present material, which probably is not nucleic acid phosphorus. In the Schmidt and Thannhauser procedure, however, this fraction will be determined as nucleic acid phosphorus. In an early placenta the fraction amounts to 44 per cent and in a late case to 26 per cent of the nucleic acid phosphorus.

It is probable that the residues after the salt as well as the T. C. A. extraction procedure contain small amounts of nucleic acid. In two cases the residues after Schneider extraction were further extracted with T. C. A.

Table 2. Prolonged T.C.A. extraction of Schneider residue.

Weeks of pregnancy	Tissue residue	T.C.A. extract		
	P	P	PNAP	DNAP
40	121	170	83.0	17.5
24	182	332	86.0	19.3

P: μg phosphorus/gram dry tissue powder.
PNAP: μg pentose nucleic acid phosphorus, calculated from sugar determination.
DNAP: μg desoxypentose nucleic acid phosphorus, calculated from sugar determination. For further explanations, see text.

at 90° C for 60 minutes. Phosphorus in residue and extract and nucleic acid-bound sugars in extract were determined. The results are summarized in Table 2. These nucleic acids amount to 3.3 per cent of the total nucleic acids in one case, and to 2.6 per cent in the other. Assuming that the same amount is still left in the residue, though this is not probable, the nucleic acids non-extracted by the Schneider procedure would amount to between 5 and 6 per cent.

SUMMARY

The nucleic acid content of the human placenta during development has been determined using different methods.

1. There was good agreement between the results obtained by Hammarsten's method and by the application of the cysteine reactions to the hot T. C. A. extract. The Schmidt and Thannhauser values showed considerable divergencies.

2. A large, probably non-nucleic acid fraction, in a young placenta amounting to 44 per cent of the nucleic acid phosphorus and in an old placenta to about 26 per cent, is extracted by the methods of Hammarsten and Schneider. This fraction will not be determined as nucleic acids when using the Hammarsten procedure or the cysteine reactions on the hot T. C. A. extract, but introduces a large error when the nucleic acids are determined according to the Schmidt and Thannhauser procedure.

3. After the extraction procedures of Hammarsten and Schneider the tissue residue contains a considerable quantity of phosphorus, constituting about 12 per cent of the nucleic acid phosphorus. It has been demonstrated that the Schneider residue probably contains 5 to 6 per cent of the total amount of nucleic acids.

4. A decrease of P. N. A. per unit of dry tissue powder was obtained with all the methods, and when applying salt or T. C. A. extraction a small increase of D. N. A. was obtained. The P. N. A./D. N. A. ratios decrease considerably with the aging of the placenta.

For valuable discussion and advice I desire to acknowledge my great indebtedness to Professor E. Hammarsten and to Doctor Bo Thorell.

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ACTA CHEMICA SCANDINAVICA 7 (1953) 514–517

Studies on Quinones and Hydroquinones

III. * Bromotrimethoxyhydroquinone

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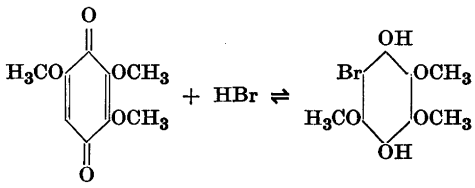
It is well known that hydrogen chloride and bromide react with most quinones to form halogenohydroquinones, although some derivatives, such as 2,5- and 2,6-dimethoxyquinone, do not exhibit any reactivity towards these reagents. In Parts I [1] and II of this series the occurrence of the reverse type of reaction to this addition process was demonstrated with 2-chloro- and 2-bromo-3,5-dimethoxyhydroquinone, these compounds decomposing readily into 2,6-dimethoxyquinone and the corresponding hydrogen halides. The isomeric 2-chloro- and 2-bromo-3,6-dimethoxyhydroquinone were, however, quite stable substances.

The investigation has now been extended to trimethoxyquinone derivatives. Trimethoxyquinone in chloroform was found to add on hydrogen bromide with the formation of a labile substance, which easily decomposed into the starting materials. The decomposition is catalyzed by acids, but in alkali the substance seems to be rather stable. A diacetate was obtained by acetylation with acetic anhydride either in alkaline solution or in pyridine. The diacetate was identical with bromotrimethoxyhydroquinone diacetate, prepared by bromination of the trimethoxyquinone and subsequent reductive acetylation. The labile substance could be obtained by reduction of the bromoquinone by zinc dust in acetic acid. All these facts indicate that it should be bromotrimethoxyhydroquinone, and the reaction



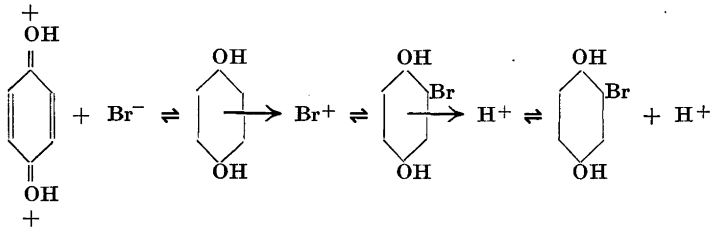
* Part II. *Acta Chem. Scand.* 6 (1952) 1048.

would appear to be the first known example of the reversible addition of a hydrogen halide to a quinone with the formation of a hydroquinone. A similar reaction, the reversible addition of hydrogen chloride to 4,4'-dimethoxydiquinone, was observed by Erdtman [2], but in this case the reaction product was decomposed by alkali, and an investigation by the present author [3] has proved that the substance is not a hydroquinone.

The action of sodium borohydride on the quinones concerned in this investigation and some other quinones was examined. It proved to be very effective, methanolic solutions of the quinones being decolorized instantaneously. However, the method does not seem to offer any advantages over the classical methods for the reduction of simple quinones. Humic acids, both natural and artificial, are also reduced by sodium borohydride, and in this field the reagent might be useful, especially if the reduction can be developed into an analytical method, as has been done for carbohydrates [4].

DISCUSSION

According to Dewar [5], the addition reactions of quinones proceed through π-complexes:



In the example given by Dewar the neutral quinone molecule reacts with the anion, but as these reactions occur in an extremely acid medium, the present author prefers to write the reaction as above.

This scheme also gives a reasonable mechanism for the reverse reaction, which should be catalyzed by hydrogen ions. Electron repelling substituents decrease the redox potential of quinones, so it is natural that quinones with such substituents should show decreased additive reactivity and also that labile substances might be found among the corresponding halogenohydroquinones. It is, however, difficult to interpret the great differences in reactivity between rather similar substances, *e.g.* why 2-bromo-3,6-dimethoxyhydroquinone should be stable but not the isomeric 3,5-dimethoxyderivative. It is possible that an investigation of the redox potentials and other physical-chemical properties of these substances would contribute to a better understanding of these reactions.

EXPERIMENTAL

All melting points uncorrected. Trimethoxyquinone was prepared by the method of Huisman [6].

Trimethoxyhydroquinone diacetate. Reductive acetylation of trimethoxyquinone with zinc dust and acetic anhydride gave an almost quantitative yield of trimethoxyhydroquinone diacetate. Two recrystallizations from methanol yielded the pure substance, m.p. 76.5–77°. (Found: OCH_3 32.6; Calc. for $C_{13}H_{16}O_7$ (784.3): OCH_3 32.8.)

Bromotrimethoxyquinone. A solution of trimethoxyquinone (3 g) and bromine (3 g) in absolute chloroform was refluxed for 15 minutes and then evaporated to dryness under reduced pressure. The crystalline residue was recrystallized from benzene, when unchanged starting material (0.3 g) was obtained. The mother liquors were concentrated and the residue recrystallized from methanol. Repeated recrystallizations yielded almost pure bromotrimethoxyquinone (1.5 g) as red needles, m.p. 89.5–91°. (Found: OCH_3 34.3; Br. 28.0; Calc. for $C_9H_9O_5Br$ (277.1): OCH_3 33.6; Br. 28.8.)

Bromotrimethoxyhydroquinone diacetate. a) Reductive acetylation of bromotrimethoxyquinone (0.5 g) with zinc dust in acetic anhydride yielded the bromotrimethoxyhydroquinone diacetate. The substance was recrystallized twice from methanol. Yield 0.6 g, m.p. 97–98° (Found: OCH_3 25.7; Br. 21.3; Calc. for $C_{13}H_{15}O_7Br$ (363.2): OCH_3 25.6; Br. 22.0.)

b) Hydrogen bromide was passed into a solution of trimethoxyquinone (0.5 g) in chloroform (15 ml). The red colour of the solution rapidly changed to light yellow. Dry air was then bubbled through the solution to remove excess of hydrogen bromide, a small amount of calcium carbonate was added and the solution evaporated to dryness under reduced pressure at room temperature. The yellow residue was acetylated with acetic anhydride (5 ml) and pyridine (1 ml) and poured into water. An oil separated, which soon crystallized. Recrystallization from methanol yielded colourless crystals (0.65 g, 70 %), m.p. 97–98° alone or in admixture with the bromotrimethoxyhydroquinone diacetate described under a). The same acetate was obtained in a yield of 30 % when the yellow residue was dissolved in alkali and acetylated with acetic anhydride.

Decomposition of bromotrimethoxyhydroquinone. Bromotrimethoxyhydroquinone was obtained, as a yellow oil which showed tendency to crystallize either by addition of hydrogen bromide to trimethoxyquinone as described above or by reduction of bromotrimethoxyquinone with zinc dust in acetic acid. The identity of the substance was demonstrated by converting it into the diacetate. When the oil was dissolved in benzene and the solution heated, the yellow colour changed to red. On addition of a drop of conc. hydrochloric acid, the change of colour became very rapid and trimethoxyquinone, identical with authentic material, separated.

SUMMARY

Trimethoxyquinone reacts with hydrogen bromide to form bromotrimethoxyhydroquinone. In contradistinction to other reactions of the same type, this reaction is reversible.

The author wishes to thank *Statens Naturvetenskapliga Forskningsråd* for financial support and Mr. J. Paju for skilful assistance.

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Unsaturated Five-Carbon *Iso*thiocyanates

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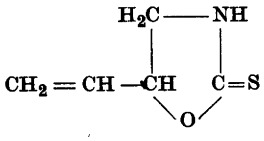
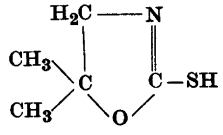
Recently Astwood *et al.*[1] isolated from turnip root and the seeds of various *Brassicae* an antithyroid compound which was demonstrated to possess the thioöxazolidone structure (I), later corroborated by the synthesis of Ettlinger [2]. In 1938 Hopkins [3] obtained a structurally similar compound (II) from the seeds of the crucifer *Conringia orientalis*.

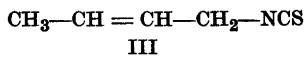


I

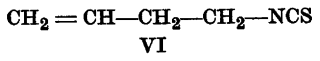


II

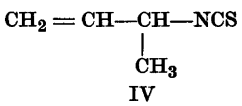
The widely recognised occurrence of mustard oils in seeds of numerous *Cruciferae* led us to consider the unsaturated 5-carbon *iso*thiocyanates as possible biogenetic or chemical precursors of (I) and (II) as has been suggested previously by Hopkins [3] and Astwood *et al.*[1]. Consequently, it was deemed desirable to synthesise the isomeric, unsaturated mustard oils (III)–(VI) and also to test their antithyroid activity. The present paper gives an account of the chemical results. The biological findings will be reported elsewhere.



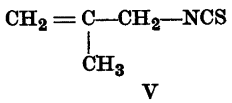
III



VI



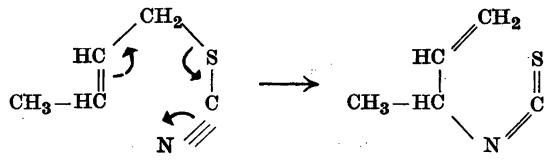
IV



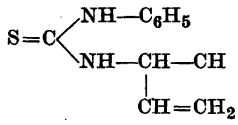
V

Disregarding stereochemical isomerides and structures containing a double bond adjacent to the NCS-grouping the compounds (III)–(VI) represent the theoretically possible unsaturated 5-carbon *iso*thiocyanates.

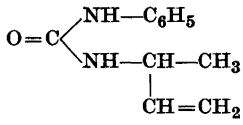
A search of the literature discloses that crotyl *iso*thiocyanate (III) has never been unequivocally prepared and identified. In 1899 Charon [4] obtained a mustard oil from the reaction between crotyl bromide and ammonium thiocyanate, which was characterised as a thiourea derivative with m.p. 105°. The alleged crotyl *iso*thiocyanate was shown by Mumm and Richter [5] in 1940 to be in fact α-methallyl *iso*thiocyanate (IV) formed through intramolecular rearrangement of crotyl thiocyanate according to the following scheme.



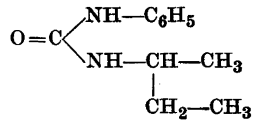
This method of synthesis was employed in the present investigation, and the α-methallyl *iso*thiocyanate (IV) was characterised as thioureas, formed upon reaction with ammonia, aniline, *p*-toluidine and α-naphthylamine. Desulphurisation of N-phenyl-N′-(α-methallyl)-thiourea (VII) with silver nitrate yielded the corresponding urea-derivative (VIII) which, upon catalytic hydrogenation, was transformed into N-phenyl-N′-*sec*-butyl-urea (IX), melting in accord with the literature value [6]. No attempts were made to prepare the optically active isomerides of (IV).



VII



VIII

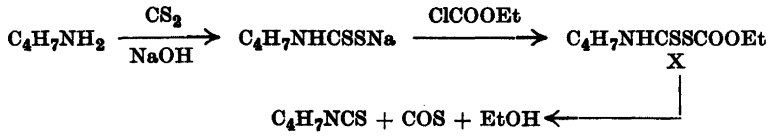


IX

In 1910 a communication from Schimmel & Co.[7] appeared in which a synthesis of crotyl *iso*thiocyanate (III) was described. Crotonaldehyde was transformed into its oxime which, in turn, was reduced with sodium amalgam to crotylamine. The classical Hofmann synthesis finally afforded the desired *iso*thiocyanate which gave a thiourea melting at 65—66°. Numerous attempts in this laboratory to repeat the above sequence of reactions under varying conditions either failed completely or led to minute amounts of reaction products of questionable purity. Therefore, alternative routes of synthesis were explored.

Experiments employing silver thiocyanate instead of the ammonium salt met with no better success, the rearranged α-methallyl *iso*thiocyanate again being the sole reaction product. Attention was next turned to the preparation

of *trans*-crotylamine by a modified Gabriel-synthesis. In dimethylformamide solution, a valuable improvement introduced recently by Sheehan and Bolhofer [8], the reaction between potassium phthalimide and crotyl bromide proceeded exothermically and gave an 83 per cent yield of N-crotylphthalimide. Hydrazinolysis, according to Ing and Manske [9], afforded crotylamine hydrochloride in 92 per cent yield. This was further processed to *trans*-crotyl *iso*thiocyanate by the Andreasch-Kaluza procedure [10,11], utilising the spontaneous decomposition of the ester (X) according to the following scheme.



In one instance, when a crude preparation of crotylamine hydrochloride was employed, a considerable amount of a crystalline by-product of unidentified structure resulted. The analytical data did not suggest any definite composition and no further attempts were made to reveal its chemical nature. The isomeric *cis*-crotyl *iso*thiocyanate will be described in a forthcoming paper.

Crotyl *iso*thiocyanate gave, on reaction with aqueous ammonia, a thiourea melting at 58—60°, somewhat lower but hardly incompatible with the value reported previously by Schimmel & Co.[7]. The mustard oil was further characterized as thioureas formed upon reaction with aniline, *p*-toluidine and *α*-naphthylamine. The presence of an unbranched side-chain was proved by desulphurisation of the phenylthiourea, followed by catalytic hydrogenation to give N-phenyl-N′-*n*-butylurea, whose m.p. was in accord with the literature values and was not depressed when mixed with an authentic sample.

To prove rigorously that no isomerisation of the double bond had taken place, N-crotyl-N′-phenylthiourea was submitted to various degradative treatments. Attempts to oxidise the thiourea with alkaline permanganate at 0° proved abortive, while oxidations at higher temperature yielded an unidentified volatile acid, isolated as its S-benzyl thiuronium salt and apparently different from formic and acetic acid. Performic acid at 0° oxidised the thiourea, largely to free sulphur. The method of Criegee [12] for oxidative cleavage of unsaturated compounds to aldehydes by means of ethereal hydrogen peroxide and osmium tetroxide gave interesting results in our hands. As expected from the structure of the thiourea studied, acetaldehyde was consistently obtained in these experiments. It was demonstrated in blank experiments, however, that the ether used as a solvent was partly oxidised to acetaldehyde under the usual reaction conditions. Consequently, informations gained by

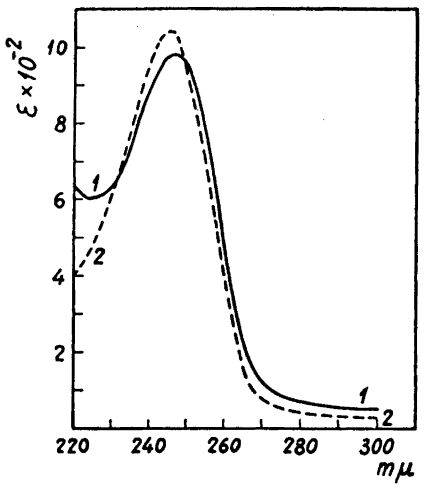


Fig. 1. Ultra-violet spectra of 1: α-methallyl isothiocyanate and 2: β-methallyl isothiocyanate, both in methanolic solution.

application of the Criegee-method to unsaturated compounds must be interpreted with caution. Reaction of the thiourea with hydrogen peroxide and osmium tetroxide in tertiary butanol, followed by treatment with periodic acid, yielded a carbonyl-fraction, again originating mainly from oxidative attack of periodic acid on the solvent.

Eventually, confirmation of the structure (III) was provided by synthesis of the sole alternative isomeride, *viz.* 3-butenyl *iso*thiocyanate (VI). The readily accessible allyl cyanide was reduced with lithium aluminium hydride to 3-butenylamine which was transformed into 3-butenyl *iso*thiocyanate by the procedure described above. Again, reaction with ammonia gave the corresponding thiourea, m.p. 65—66°, and aniline, *p*-toluidine and α-naphthylamine yielded the analogous substituted derivatives.

The only remaining isomeride, β-methallyl *iso*thiocyanate (V) was obtained upon reaction between β-methallyl chloride and ammonium thiocyanate, following the directions given by Bruson and Eastes [13]. The thiourea, m.p. 93—94°, as well as the usual substituted thioureas were prepared for comparison purposes.

A "crotonylsenföl", yielding a thiourea with m.p. 85°, was reported in 1874 by Hofmann [14]. From its mode of synthesis in conjunction with the melting point reported for the thiourea, it appears safe to conclude that Hofmann's mustard oil represents an impure preparation of β-methallyl *iso*thiocyanate. Less readily interpreted is a recent patent claim by Searle [15] accord-

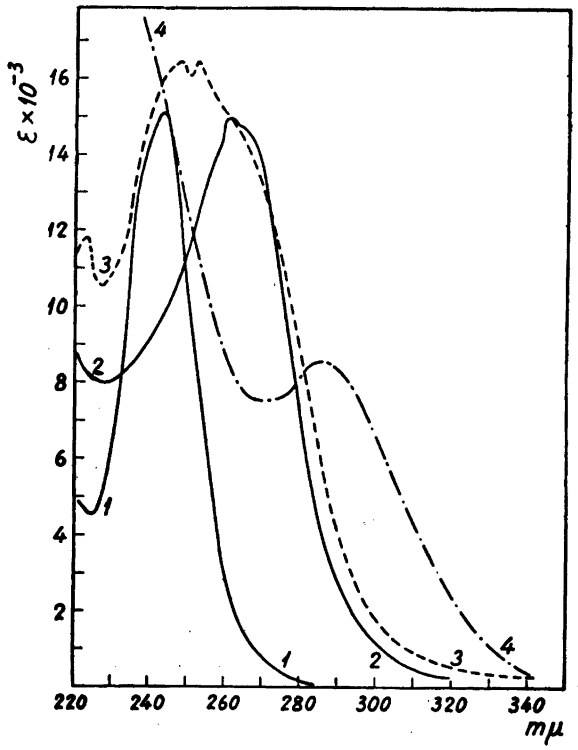


Fig. 2. Ultra-violet spectra of 1: allylthiourea, 2: phenylthiourea, 3: N,N'-crotylphenylthiourea and 4: N,N'-(α-methallyl)-α-naphthylthiourea, all in methanolic solution.

ing to which the reaction between crotyl chloride, sodium cyanide and sulphur gives rise to crotyl *iso*thiocyanate, characterised as its thiourea with m.p. 91.8—92.5°. From the results in the present paper it can be definitely excluded that Searle's compound represents the authentic *trans*-crotyl *iso*thiocyanate (III). The melting point of the corresponding thiourea points to its structure being that of (V) although the formation of α-methallyl *iso*thiocyanate (IV) under the conditions used would have been far less surprising in view of the well-established intramolecular rearrangement mentioned above.

The ultraviolet absorption spectra of the different types of compounds reported above were determined. In Fig. 1 the curves for α- and β-methallyl *iso*thiocyanate are given as representatives of the isomerides (III)—(VI). The curves are in accord with previously reported absorption data for allyl *iso*thiocyanate [16] and *n*-butyl *iso*thiocyanate [17]. These findings serve to confirm the conclusions of Pestemer and Litschauer [18] that the characteristic absorption

is due solely to the NCS-grouping and not interfered with by unconjugated double bonds in the remaining part of the molecules.

In Fig. 2 the absorption curve for allylthiourea is presented, again as typical for all of the isomeric N-alkenylthioureas prepared in the present study. The spectroscopically non-interfering character of the unsaturated substituents appears from the accord in absorption data with the ones reported earlier for unsubstituted thiourea [19]. Somewhat different results are found for the aromatically substituted thioureas. In Fig. 2 the spectrum of N-phenylthiourea is presented. In this case a noticeable and practically constant hypsochromic and hyperchromic effect is the result of introducing the crotyl-, α- or β-methallyl residue in the molecule. A marked difference in absorption is noticed on introducing the 1-naphthyl-residue in the thioureas (Fig. 2). The intensive end-absorption in conjunction with the maximum at 280—290 mμ is reminiscent of the absorption of naphthalene itself and points to less interference with the remaining part of the molecule than in the corresponding phenyl-derivatives. Once again the highly complex nature of the influence of substituents on absorption in the thiourea-series has been recognised.

EXPERIMENTAL *

trans-*Crotyl bromide.* To 93 g of phosphorus tribromide was cautiously added drop by drop a mixture of 72 g of *trans*-crotyl alcohol [20] and 8 g of pyridine. After the initial reaction had ceased the mixture was heated for 15 minutes at 70°. Distillation at 100 mm and a bath temperature of 50—60° gave 125 g (92 %) of colourless bromide, which before being further used, was fractionated in an all-glass column. 116 g, b.p. 100—105°, of the equilibrium mixture described by Young and Winstein [21] was obtained.

This procedure gave better yields and a higher grade of purity than the one hitherto recommended [4,5] employing aqueous hydrobromic acid.

α-Methallyl iso*thiocyanate* (IV). Prepared from ammonium thiocyanate and crotyl bromide as previously described [4,5] with the single deviation, that the reaction mixture was heated under reflux for 3 hours. An 87 % yield of colourless, analytically pure substance was obtained, b.p. 70—72° at 34 mm. The preparation slowly took on a slightly yellow colour, even when kept in the dark at 0° in sealed vessels.

Reaction with concentrated aqueous ammonia at room temperature overnight yielded N-(α-methallyl)-thiourea which, after recrystallisation from water, melted at 109—110°. (Lit. 105° [4] and 107—108° [5]).

General procedure for the preparation of aromatically substituted thioureas. A solution of 0.5 g of the appropriate *iso*thiocyanate and slightly more than one molecular equivalent of aniline, *p*-toluidine or α-naphthylamine in 1 ml of ethanol was heated on the steam-bath for 2 hours. After cooling and scratching, crystalline but somewhat oily products usually formed. These were freed of excess of amine by treatment with a few drops of

* All melting points are uncorrected and determined in capillary tubes, those above 80° in an electrically heated block, the remaining in a water bath.

Table 1. N-Alkenyl-N'-phenylthioureas, R-NH-CS-NH-C_6H_5.

No.	R	M.p., °C	Analyses * C %	H %	N %	S %
1.	$CH_2=CH-CH(CH_3)-$	109–110[a]	64.28	6.89	13.67	15.47
2.	$CH_2=C(CH_3)-CH_2-$	80–81	64.13	6.83	13.59	15.71
3.	$CH_3-CH=CH-CH_2-$	105.5[b]	64.28	7.02	13.75	15.75
4.	$CH_2=CH-CH_2-CH_2-$	123–127[c]	64.17	6.98	13.40	15.42

* Calc. for the isomeric substances: C 64.03 %; H 6.84 %; N 13.58 %; S 15.54%. [a] Ref.[22] 110°. [b]Mixed with No. 1, m.p. *ca.* 80°. Ref.[25] 106°. [c]A non-analysed preparation with this alleged structure is claimed to melt at 91° [25].

Table 2. N-Alkenyl-N'-p-tolylthioureas, R-NH-CS-NH-C_6H_4-CH_3.

No.	R	M.p., °C	Analyses * C %	H %	N %	S %
1.	$CH_2=CH-CH(CH_3)-$	125	65.54	7.35	12.85	14.50
2.	$CH_2=C(CH_3)-CH_2-$	106–107	65.63	7.57	12.93	14.70
3.	$CH_3-CH=CH-CH_2-$	81–82	65.42	7.18	12.94	14.77
4.	$CH_2=CH-CH_2-CH_2-$	66–67	65.39	7.38	12.74	14.60

* Calc. for the isomeric substances: C 65.41 %; H 7.32 %; N 12.72 %; S 14.55 %.

Table 3. N-Alkenyl-N'-(α-naphthyl)-thioureas, R-NH-CS-NH-$C_{10}H_7$.

No.	R	M.p., °C	Analyses * C %	H %	N %	S %
1.	$CH_2=CH-CH(CH_3)-$	129–130	70.30	6.47	11.17	12.60
2.	$CH_2=C(CH_3)-CH_2-$	100–101	70.26	6.29	11.10	12.41
3.	$CH_3-CH=CH-CH_2-$	129–130[a]	70.33	6.10	11.09	12.79
4.	$CH_2=CH-CH_2-CH_2-$	109–110	70.31	6.33	11.04	12.69

* Calc. for the isomeric substances: C 70.26 %; H 6.30 %; N 10.93 %; S 12.50 %. [a]Mixed with No. 1, m.p. *ca.* 110°.

aqueous hydrochloric acid. The colourless products were recrystallised to constant melting points from aqueous ethanol. Melting points and analytical data are presented in the Tables 1, 2 and 3.

N-(α-Methallyl)-N'-phenyl-urea (VIII). To 1 g of α-methallyl-phenylthiourea dissolved in 5 ml of hot ethanol was added a solution of 1.8 g of silver nitrate in 10 ml of 50 % (v/v) ethanol. The mixture was refluxed for 15 minutes and cooled. On addition of water and chilling, crystals separated and were filtered off. They were recrystallised from dilute ethanol with addition of decolourising charcoal. A final crystallisation from aqueous ethanol yielded thin colourless needles, m.p. 154.0–154.5°.

$C_{11}H_{14}ON_2$ (190.2) Calc. C 69.45 H 7.42 N 14.73
Found » 69.65 » 7.69 » 14.77

When a solution of 0.11 g of the unsaturated urea (VIII) in 10 ml of ethanol, containing 5 mg of Adam's platinum-catalyst, was shaken in a hydrogen atmosphere at ordinary

pressure and room temperature, 15.7 ml of hydrogen (theoretical: 15.6 ml) were absorbed within 30 minutes. After filtering off the catalyst and concentrating the filtrate, a colourless crystalline product remained, which separated in clusters of thin needles from aqueous ethanol, m.p. 155.0—155.5°. This value is in accord with the literature value [6] for the m.p. of DL-N-*sec*-butyl-N'-phenylurea (IX) but because no depression was noticed on admixture with the starting material, an analysis served to ascertain its identity. No depression was observed on mixing with an authentic sample of the urea.

$C_{11}H_{16}ON_2$ (192.3)	Calc.	C 68.69	H 8.39	N 14.57
	Found	» 68.89	» 8.46	» 14.60

Reaction of crotyl bromide with silver thiocyanate. When these reagents were brought together in ethanol and left overnight at room temperature, a 90 % reaction took place as estimated from the amount of silver bromide formed. Upon reaction with the usual amines, the ethanolic solution gave thioureas identical with those just described, indicating that allylic rearrangement had occured under these conditions also.

N-Crotylphthalimide. To the well-stirred, thin suspension of 9.8 g of potassium phthalimide in 40 ml of pure dimethylformamide was added 6.7 g of crotyl bromide. The temperature rose spontaneously to 95° and after the first reaction had ceased the mixture was kept for one hour at about 75° by means of a water-bath. 60 ml of chloroform and 200 ml of water were added, the chloroform layer separated and the aqueous phase extracted twice with 20 ml-portions of chloroform. The extracts were washed with 0.2 *N* sodium hydroxide and water, dried over sodium sulphate and the chloroform removed *in vacuo*. The remaining oil solidified in a freezing-mixture and was recrystallised from aqueous ethanol, yielding 8.4 g (84 %) of crotyl phthalimide. An analytical sample was prepared by two additional recrystallisations from ethanol. M. p. 75—76°.

$C_{12}H_{11}O_2N$ (201.2)	Calc.	C 71.63	H 5.51	N 6.96
	Found	» 71.52	» 5.70	» 7.11

Crotyl iso*thiocyanate* (III). A solution of 20 g of crotylphthalimide and 6 ml of hydrazine hydrate in ethanol was boiled under reflux for one hour. After addition of 250 ml of concentrated hydrochloric acid and 250 ml of water, heating was continued for another hour. After cooling, the phthalylhydrazide which crystallised from the mixture was removed. Most of the alcohol was distilled off, additional phthalylhydrazide removed by filtration and the filtrate evaporated *in vacuo* to dryness. By repeated evaporations with fresh portions of water the excess hydrochloric acid was removed. The remaining crotylamine hydrochloride weighed 9.3 g (84 %) after drying. Titration of an aliquote amount of amine, liberated by distillation with strong alkali, indicated a content of *ca.* 95 %. The phthalylhydrazide isolated represented 92 % of the calculated amount.

The hydrochloride was used directly in the preparation of the *iso*thiocyanate which was perfor medby following the procedure given in Organic Syntheses [23] for methyl *iso*thiocyanate. A 61 % yield of colourless crotyl *iso*thiocyanate, b.p. 67—68° at 11 mm, was obtained after distillation. The analytical data were not very satisfactory, in accord with our generally experienced difficulty in obtaining analytically pure samples of *iso*thiocyanates prepared according to the present method. The originators of the procedure [10,11] also noticed these difficulties and they suggested that traces of symmetrically substituted ureas were responsible for the consistently low nitrogen- and sulphur-values found. In spite of the poor analytical data, we consider our product as being essentially pure *trans*-crotyl *iso*thiocyanate, as evidenced from its reaction products with amines described below.

N-Crotyl-thioureas. When the *iso*thiocyanate was shaken with concentrated aqueous ammonia overnight and then taken to dryness *in vacuo* at room temperature, a crystalline product remained. Two recrystallisations from chloroform-petroleum ether yielded a voluminous mass of colourless needles. M.p. 58—60°.

$C_5H_{10}N_2S$ (130.2)	Calc.	C 46.13	H 7.75	N 21.53	S 24.64
	Found	» 46.18	» 7.73	» 21.59	» 24.33

The aromatically substituted thioureas were prepared as described above and are listed in Tables 1, 2 and 3.

N-Crotyl-N'-phenylurea. When desulphurised according to the directions given above, N-crotyl-N'-phenylthiourea yielded the corresponding N-crotyl-N'-phenylurea as thin colourless needles. M.p. 130.5°.

$C_{11}H_{14}ON_2$ (190.2)	Calc.	C 69.45	H 7.42	N 14.73
	Found	» 69.45	» 7.50	» 14.53

Submitted to catalytic hydrogenation as described above, the unsaturated urea was transformed into N-*n*-butyl-N'-phenylurea, m.p. 131—132°, alone or in mixture with an authentic specimen, prepared from *n*-butylamine and phenyl *iso*cyanate. A large depression was observed on mixing with crotyl-phenylurea.

3-Butenyl iso*thiocyanate* (VI). In a three-necked flask, provided with a dropping funnel and a mercury-sealed stirrer was placed a suspension of 13 g of finely pulverised lithium aluminium hydride in 275 ml of Grignard-ether. The flask was kept under a slight nitrogen pressure, and a solution of 20 g of allyl cyanide [24] was slowly added under vigorous stirring at room temperature. The mixture changed from yellow, through green, to pink. After the reaction ended, the excess lithium aluminium hydride was destroyed by cautiously adding wet ether and water. 6 *N* hydrochloric acid was introduced to dissolve the aluminium salts and the red ether phase was separated from the clear and colourless aqueous layer, which was extracted with two additional portions of fresh ether. The aqueous solution was made alkaline with strong sodium hydroxide and distilled. The volatile amine was collected in strong hydrochloric acid and 3-butenylamine hydrochloride isolated by evaporation to dryness *in vacuo*. Yield 9.3 g (30 %). The hydrochloride was further processed to the corresponding *iso*thiocyanate in the usual way. 3-Butenyl *iso*thiocyanate distilled at 60° at 12 mm.

C_5H_7NS (113.2)	Calc.	C 53.04	H 6.23	N 12.38	S 28.32
	Found	» 52.99	» 6.32	» 12.50	» 28.45

N-(3-Butenyl)-thioureas. When a suspension of the *iso*thiocyanate in concentrated aqueous ammonia was left for two days at room temperature, a homogenous solution resulted which, on evaporation *in vacuo* at room temperature, yielded a colourless crystalline product. This was recrystallised from water for analysis. M.p. 65—66°.

$C_5H_{10}N_2S$ (130.2)	Calc.	C 46.13	H 7.75	N 21.53	S 24.64
	Found	» 45.95	» 7.99	» 21.50	» 24.47

Reactions with the usual aromatic amines yielded products which crystallised less readily than in the above cases. The phenylthiourea was induced to crystallise only after sublimation in high-vacuum. The derivatives are listed in Tables 1, 2 and 3.

β-Methallyl iso*thiocyanate* (V). This isomeride was prepared from β-methallyl chloride and ammonium thiocyanate following the directions given by Bruson and Eastes [13]. A possible allylic rearrangement obviously leads to no ambiguity in structure in this case.

N-(β-Methallyl)-thioureas. The reaction product with ammonia was prepared as described by the same authors [13]. M.p. 93—94°. The derivatives with the usual aromatic amines are listed in Tables 1, 2 and 3.

Ultraviolet absorption spectra. All the spectra reported in this paper were determined in methanolic solution with a Beckman model DU quartz spectrophotometer.

SUMMARY

Four unsaturated 5-carbon *iso*thiocyanates (III)—(VI) have been unequivocally synthetised. No stereoisomerides of these have been considered.

The mustard oils have been transformed into characteristic thiourea-derivatives upon reaction with ammonia, aniline, *p*-toluidine and α-naphthylamine.

Structure proofs of the isomeric *iso*thiocyanates have been forwarded by transformation into known derivatives.

The microanalyses have been performed in this laboratory by Mr. A. Grossmann.

We are much indebted to the *Danish Shell Company* for a generous supply of β-methallylic chloride.

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Paper Chromatography of Thioureas

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In connection with a current investigation of some natural products it became necessary to separate and identify minute amounts of N-substituted thioureas. The success which has attended the use of paper chromatography for the identification of a great variety of widely differing substances suggested the application of this technique to the present problem. No previous examples of paper chromatography of thioureas seem to be on record. A satisfactory method for separating a large number of thioureas has been worked out and a brief account of the method given in a preliminary paper [1]. The present communication deals with a more detailed description of the technique and results.

Because, for other reasons, we were mainly interested in N-substituted thioureas, most of the substances studied were of this type. In addition, however, we investigated some N,N'- and N,N-disubstituted, N-trisubstituted and N-tetrasubstituted thioureas while *iso*thioureas have not been considered. The scope of the method was further explored by studying some thiosemicarbazides, a few thio-amides and -hydrazides and a series of heterocyclic thiourea-derivatives (*e. g.* thiobarbituric acids). Various substituents were considered, including alkyls, alkenyls, aralkyls and aryls as well as combinations of these. From the outset of the present investigation the highest accuracy attainable was aimed at, because highly reproducible results were a prerequisite for the application of the method to the currently studied problems.

EXPERIMENTAL

Spraying Reagents. For location of the spots on the paper two different and about equally satisfactory reagents were found to be applicable. Grote [2] described a colour reaction for thioureas and similar compounds with a specially prepared reagent, produced from sodium nitroprusside, bromine and hydroxylamine. We found the intensive blue or turquoise colours produced to be sufficiently stable for application in paper chromato-

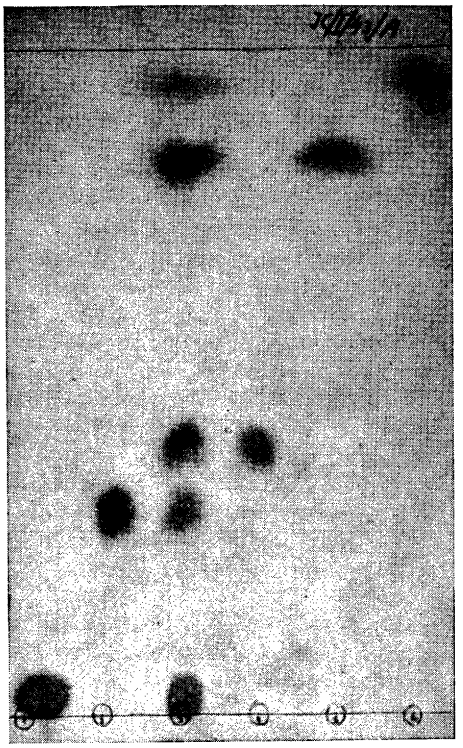

Fig. 1. Paper-chromatogram on No. 1 Whatman paper of N-methylated thioureas. Solvent system chloroform-water. Temperature 23.5°. Sprayed with Grote's reagent. 1. Mono-methyl-; 2. N,N'-Dimethyl-; 3. Mixture of mono-methyl-, N,N'-dimethyl-, N,N-dimethyl-, trimethyl- and tetramethyl-; 4. N,N-Dimethyl-; 5. Trimethyl-; 6. Tetramethyl-.

graphy. Dilution tests demonstrated that a solution containing 0.1 mg of thiosinamine per ml still gave an easily detectable blue spot on filter paper.

Likewise, good results were obtained on consecutive spraying of the paper with a starch-solution and the iodine-azide reagent of Feigl [3]. Owing to the ability of thioureas to catalyse the reaction

$$I_2 + 2\ N_3^- \rightarrow 2\ I^- + 3\ N_2$$

white spots on a uniformly blue background appeared where the individual thioureas were located. This catalytic action of sulphur compounds on the reaction above is of a rather general nature as far as thio-compounds containing divalent sulphur are concerned (*cf.* Ref.[3]). Therefore, we believe that the iodine-azide-reagent will prove useful for the detection of a great many sulphur compounds in paper chromatography. When this work was about complete, we incidentally noticed one previous application of the reagent to paper chromatography. Chargaff, Levine and Green [4] utilised the Feigl reagent for the

detection of cysteine, cystine and methionine, although without the preceding treatment with a starch-solution, which latter certainly increases the sensitivity considerably.

Because of its convenience, we generally preferred the Grote reagent in our paper-chromatographic work. Heating at 100° for about one minute accelerated the colour formation, whereas prolonged heating or the application of higher temperatures should be avoided due to the thermolabile character of the colours produced.

Solvents. After many experiments with various solvents and solvent mixtures, chloroform-water was eventually found to be the most suitable system for the separation of mixtures of mono-substituted thioureas. Water-saturated chloroform was used throughout as the mobile solvent with water as the stationary phase. The application of chloroform, apparently a little used solvent in paper chromatography, at first presented certain difficulties in this investigation. Due to the high density of chloroform it proved difficult to secure the necessary equilibrium in the chromatographic chamber until special precautions were introduced as described below. Also, mixtures of butanol-dibutyl ether proved to be of value in many cases but suffered from the inherent disadvantage of rapidly developing peroxides, deleterious to the thioureas under investigation.

Paper. Throughout this work, No. 1 Whatman paper was used, the strips always being cut parallel to the long side of the commercial sheets. Several experiments, however, were conducted on No. 2 Whatman paper with about equally satisfactory results. The very slow No. 50 Whatman paper proved useful in some separations, especially for substituted thioureas with R_{Ph}-values (see below) over 1.20. The texture of this paper, however, makes it less suitable for general application.

Temperature. In order to obtain highly reproducible results it appeared necessary to keep the temperature of the chromatographic system rather constant. Therefore, all determinations reported in the present paper were performed in a room kept at 23.5° ± 0.2°.

Working procedure. For several reasons the ascending technique was preferred during the present investigation. It combines speed and high reproducibility with simplicity in apparatus and performance. Especially when chloroform is used as the mobile solvent, the descending technique results in an inconveniently high flow-rate, mainly due to the high density of the solvent.

The chromatograms were run in a rectangular all-glass case (23 × 11 cm, 32 cm high), provided with a plane, ground upper edge and covered with a glass-plate, tightened by means of silicone grease. On the bottom of the chamber was placed a rectangular glass dish (20 × 10 cm, 5 cm high), containing water-saturated chloroform and surrounded by a thin layer of chloroform-saturated water. The back-wall of the chamber was completely lined with filter paper dipping into the chloroform in the dish. The two narrow end-walls were similarly lined with paper, soaked in the aqueous phase on the bottom of the chamber. Only with this arrangement was it possible to create the necessary liquid-vapour equilibrium throughout the entire system. A rack of pyrex glass rods, standing on the bottom and extending along the end-walls right to the top of the chamber, was here provided with two bendings at slightly different levels. These supported a horizontal glass rod to which the chromatogram was attached by means of small stainless steel springs.

The Whatman paper was cut into broad strips, 28 cm long and 17.5 cm wide. Two cm from the lower edge of the paper the starting line was marked, and another horizontal pencil-line drawn in a distance of 23 cm from the starting line, indicating the end-position of the rising liquid-front. The strip was fixed to the glass rod along its upper edge, and at

the opposite end it was burdened with another rod in order to keep the strip in a vertical position. The solutions to be investigated, usually containing 10 mg of solute per ml in ethanol or chloroform, were applied with micropipettes as circular spots of 6—8 mm diameter along the starting line at previously marked points. This required *ca.* 1—2 μl of solution, *i. e.* each spot contained 10—20 μg of the individual compound. It may be noticed, however, that mixtures containing as little as 1 mg/ml of each compound still gave detectable spots after development and spraying. Six individual samples were run simultaneously on the same strip. Spots No. 1 and 6 were applied at a distance of 2 cm from the vertical edges of the paper while the remaining samples were arranged equidistantly in between, all along the starting line.

The air-dried paper was now placed at the upper support of the rack in the chromatographic chamber with its lower edge a few mm above the chloroform-surface in the dish on the bottom. It was kept here for 16—18 hours to acquire complete equilibrium. This stage proved very important for the obtainment of reproducible results. Next day the glass cover was removed, the strip lowered to the second bending in the rack and the lid rapidly replaced. The chloroform was now allowed to rise to the upper line (23 cm). By proper operation the chloroform-front rose as an even, horizontal line, easily observed in transmitted light. The development should require 110—120 minutes. Periods outside this range were indicative of imperfect equilibrium and led to less satisfactory results. It should be pointed out, however, that no difficulties in this respect were encountered when the directions given above were carefully followed. The strip was now removed from the chamber, air-dried and sprayed with Grote's reagent. On brief heating at 100°, sharp, deep-blue spots revealed the location of the individual thioureas. The boundaries of the patches were marked with a pencil because the colour gradually faded.

Even in experiments carefully conducted as described, some variations in the R_F-values were noticed from one run to another, probably caused by factors outside the control of the experimenter (unevenness in paper texture, traces of impurities *etc.*). These fluctuations were circumvented, however, by relating the distances travelled by the individual compounds not as usually to the solvent front, but rather to some arbitrary reference compound. For this purpose we chose phenylthiourea which was placed as a standard sample on each chromatogram. For convenience, we introduced the term R_{Ph}, defined as the ratio between the distances travelled by the individual compounds and phenylthiourea.

Quantitative studies. No extensive study of the application of the present technique to quantitative determinations of thioureas was made. Exploratory experiments, however, indicated an accuracy of *ca.* ±5 %. In these experiments the solutions to be investigated were applied to two identical paper sheets as 6 mm broad streaks along the starting lines. After chromatography in the usual way, one of the sheets was sprayed with Grote's reagent and the other cut in horizontal strips according to the locations determined on spraying. The strips were separately extracted in micro-Soxhlet extractors with methanol and the concentrations determined spectrophotometrically.

Model substances

Only a few of the substances examined and listed in the accompanying tables were commercially available. Most of them were synthesised according to descriptions in the literature and found to possess physical data in agreement with those previously reported. In some cases, however, we found physical properties which differed considerably from

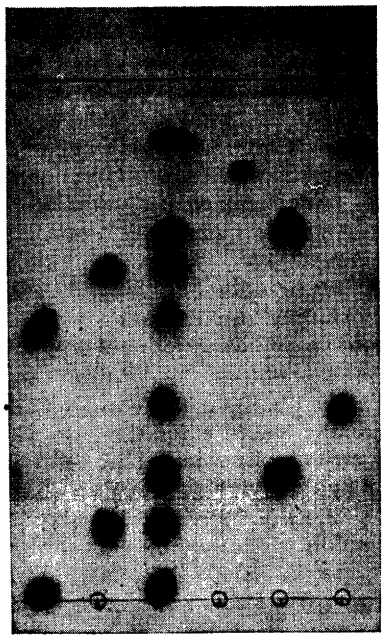

Fig. 2. Paper-chromatogram on No. 1 Whatman paper of different mono-substituted thioureas. Solvent system chloroform-water. Temperature 23.5°. Sprayed with Grote's reagent. 1. Methyl- and β-methallyl-; 2. Ethyl- and α-methallyl-; 3. Mixture of methyl-, ethyl-, allyl-, iso-propyl-, β-methallyl-, α-methallyl-, n-butyl- and β-phenylethyl-; 4. Phenyl-; 5. Allyl- and n-butyl- ; 6. iso-Propyl- and β-phenylethyl-.

the literature values. The revised data together with compounds not previously reported will be briefly discussed below.

N-Ethylthiourea. Two widely differing melting points are found in the literature. Hofmann [5] reports the m.p. 113°, while Dyson and Hunter [6] list the value 144° *. Our preparation, carefully recrystallised from ethyl acetate, melted at 110—111° **.

$C_3H_8N_2S$ (104.2) Calc. N 26.89
Found » 26.93

N-iso-*Propylthiourea.* Jahn [7] reports the m.p. 157° while a non-analysed specimen is claimed by Mathes *et al.*[8] to melt at 168—169°. Our product, recrystallised from water as flat prisms, melted at 169—170°.

$C_4H_{10}N_2S$ (118.2) Calc. N 23.70
Found » 23.79

N-iso-*Butylthiourea.* This compound was previously reported by Hofmann [9] with m.p. 93.5°. Our preparation, synthesised from *iso*-butylamine and thiocarbonyl chloride, appeared as colourless, nacreous plates after recrystallisation from water. M. p. 100—101°.

$C_5H_{12}N_2S$ (132.2) Calc. C 45.42 H 9.15 N 21.20
Found » 45.63 » 8.96 » 21.13

Compounds No. 14—17 in Table 1. These substances have been reported in a previous paper [10].

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* misprint for 114°?

** all our melting points are uncorrected.

N-n-*Butyl-N'-methylthiourea.* This was prepared from *n*-butyl *iso*thiocyanate and methylamine in the usual way. The thiourea was recrystallised from ethyl acetate-petroleum ether. M. p. 40—41°.

$C_6H_{14}N_2S$ (146.3)	Calc.	C	49.26	H	9.65	S	21.92
	Found	»	49.35	»	9.42	»	21.93

4-Morpholinethiocarboxamide (Table 2, No. 11). A compound with this alleged structure was recently described by Henry and Dehn [11], m.p. 111.5—112.5°. It was prepared by evaporating an aqueous solution of morpholine hydrochloride and potassium thiocyanate on the water-bath. The generally experienced difficulties in rearranging thiocyanates of secondary amines in conjunction with the agreement in m. p. with the long known morpholinium thiocyanate, rendered it probable that the compound in question was in fact the salt. On repeating the experiment we found this to be the case.

An authentic sample of 4-morpholinethiocarboxamide was synthetised by the Wallach-procedure [12]. An ethereal solution of 7.3 g of cyanogen bromide was added dropwise to an ice-cooled solution of 12.0 g of morpholine in ether. The morpholine hydrobromide was removed by filtration, the ether evaporated at ordinary pressure and the residue distilled twice *in vacuo* to give 5.5 g of pure *4-cyanomorpholine* as a colourless liquid. B. p. 74.5° at 0.9 mm.

$C_5H_8N_2O$ (112.1)	Calc.	C	53.58	H	7.19	N	25.00
	Found	»	53.39	»	7.15	»	24.95

When a solution of 4-cyanomorpholine in ethanol was saturated with ammonia and hydrogen sulphide the separation of crystals started spontaneously. After standing overnight, the reaction mixture was concentrated on the water-bath and filtered hot. On dilution with ether crystalline 4-morpholinethiocarboxamide separated. It was recrystallised from ethanol-ether for analysis. M. p. 171°.

$C_5H_{10}N_2OS$ (146.2)	Calc.	C	41.07	H	6.90	N	19.17
	Found	»	41.14	»	6.82	»	19.16

N-Methyl-4-morpholinethiocarboxamide. This compound was prepared from methyl *iso*thiocyanate and morpholine in the usual way. The m. p. was 102.5—103.5° after recrystallisation from ethyl acetate-petroleum ether.

$C_6H_{12}N_2OS$ (160.2)	Calc.	C	44.98	H	7.55	N	17.49	S	20.02
	Found	»	44.75	»	7.59	»	17.56	»	20.03

N-Ethyl-4-morpholinethiocarboxamide. The reaction of ethyl *iso*thiocyanate and morpholine in ethanol yielded the substituted thiourea in high yield. It was recrystallised twice from ethanol-ether. M. p. 81.5—82.5°.

$C_7H_{14}N_2OS$ (174.3)	Calc.	C	48.24	H	8.10	N	16.08	S	18.40
	Found	»	48.32	»	8.19	»	00.00	»	18.51

N-Allyl-4-morpholinethiocarboxamide. Henry and Dehn [11] recently described a compound, m. p. 56—57°, with this alleged structure. On reaction of allyl *iso*thiocyanate with morpholine we obtained, however, a compound which melted at 64.5° after recrystallisation from aqueous ethanol. This we consider to be authentic N-allyl-4-morpholinethiocarboxamide.

$C_8H_{14}N_2OS$ (186.3)	Calc.	C	51.58	H	7.58	N	15.04	S	17.21
	Found	»	51.90	»	7.38	»	14.98	»	17.18

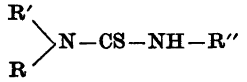
Table 1. R_{Ph}-values of N-mono-substituted thioureas on No. 1 Whatman filter paper with chloroform-water as the solvent system. Temperature 23.5°.

No.	Substituent	R_{Ph}	No.	Substituent	R_{Ph}
1.	Hydrogen	0.00	12.	β-Phenylethyl	1.10
2.	Methyl	0.04	13.	Allyl	0.26
3.	Ethyl	0.15	14.	α-Methallyl	0.74
4.	*n*-Propyl	0.42	15.	β-Methallyl	0.62
5.	*iso*-Propyl	0.41	16.	*trans*-Crotyl	0.78
6.	*n*-Butyl	0.84	17.	3-Butenyl	0.61
7.	*iso*-Butyl	0.76	18.	*p*-Bromophenyl	1.00
8.	DL-*sec.*-Butyl	0.74	19.	*p*-Iodophenyl	1.06
9.	*tert.*-Butyl	1.00	20.	*o*-Tolyl	1.19
10.	*iso*-Pentyl	1.07	21.	*m*-Tolyl	1.25
11.	Benzyl	0.90	22.	*p*-Tolyl	1.22

RESULTS

The R_{Ph}-values for the compounds investigated were determined at least three times and found to be reproducible within ± 0.01. No change in the values was observed even in mixtures containing eight or nine individual compounds. Only a few of the thioureas investigated had R_{Ph}-values which precluded their separation by the present technique. A general tendency to

Table 2. R_{Ph}-values of symmetrically and unsymmetrically N-disubstituted thioureas on No. 1 Whatman filter paper with chloroform-water as the solvent system. Temperature 23.5°.



No.	R	R′	R″	R_{Ph}
1.	Methyl	Hydrogen	Methyl	0.42
2.	Methyl	Hydrogen	Ethyl	0.89
3.	Ethyl	Hydrogen	Ethyl	1.24
4.	Methyl	Hydrogen	*n*-Butyl	1.35
5.	*n*-Butyl	Hydrogen	*n*-Butyl	1.41
6.	Methyl	Hydrogen	Phenyl	1.40
7.	Ethyl	Hydrogen	Phenyl	1.41
8.		Ethylenthiourea		0.26
9.	Methyl	Methyl	Hydrogen	0.60
10.		Pentamethylene	Hydrogen	1.18
11.		3-Oxapentamethylene	Hydrogen	0.55

Table 3. R_{Ph}-values of N-tri- and N-tetrasubstituted thioureas on No. 1 Whatman paper with chloroform-water as the solvent system. Temperature 23.5°.

No.	Substance	R_{Ph}
1.	Trimethylthiourea	1.21
2.	N-Methyl-4-morpholinethiocarboxamide	1.17
3.	N-Ethyl-4-morpholinethiocarboxamide	1.27
4.	N-Allyl-4-morpholinethiocarboxamide	1.31
5.	N-Methyl-1-piperidinethiocarboxamide	1.37
6.	N-Methyl-1-pyrrolidinethiocarboxamide	1.35
7.	N-Ethyl-1-pyrrolidinethiocarboxamide	1.37
8.	Tetramethylthiourea	1.43

form green-blue spots of lower intensity was noticed for the aromatically substituted thioureas. Thiourea itself did not move on the paper, while gradual lengthening of the carbon chain of the substituents resulted in increasing R_{Ph}-values as would be expected. The aromatically substituted, as well as most di-, tri- and tetrasubstituted derivatives, possessed R_{Ph}-values above 1.00. For mixtures containing compounds with R_{Ph}-values far above 1.25 the present solvent system was not very suitable. No efforts were made, however, to extend the technique to such compounds because our main interest centred on the aliphatic mono-substituted derivatives. In Fig. 1 and Fig. 2 two typical chromatograms are shown.

Table 4. R_{Ph}-values of various thio-compounds on No. 1 Whatman filter paper with chloroform-water as the solvent system. Temperature 23.5°.

No.	Substance	R_{Ph}	Colour
1.	Thioacetamide	0.25	weakly violet
2.	Thiobenzamide	0.79	brownish
3.	Thiosemicarbazide	0.00	violet
4.	4-Methylthiosemicarbazide	0.20	violet
5.	1,1,4-Trimethylthiosemicarbazide	1.32	faintly blue
6.	1-Phenylthiosemicarbazide	0.55	turquoise
7.	Thiobarbituric acid	0.00	blue-green
8.	5-Ethylthiobarbituric acid	0.00	yellow
9.	5,5-Diethylthiobarbituric acid	1.30	yellow
10.	5-Methyl-5-allylthiobarbituric acid	1.19	yellow
11.	5,5-Diallylthiobarbituric acid	1.37	yellow

The compounds investigated are listed in the Tables 1, 2 and 3 along with their R_{Ph}-values, determined as described in the experimental section.

A few thioamides and thiohydrazides were investigated. With these, Grote's reagent gave yellow or brownish spots of much lower intensity. Variously substituted thiosemicarbazides were also studied but with less satisfactory results than the thioureas. Due to the formation of only weak and transient colours, varying in an unpredictable manner from blue or violet to red with change in kind and position of substituents, the present method is not considered suitable for this class of compounds. A series of thiobarbituric acids was investigated, all producing yellow spots on spraying with Grote's reagent. The results obtained are listed in Table 4.

SUMMARY

A paper-chromatographic method for the separation and identification of thioureas has been developed. Experimental data for 41 substituted thioureas are presented.

Some related compounds, *viz.* thioamides, thiohydrazides, thiosemicarbazides and thiobarbituric acids have been briefly studied by the same technique.

Some new thioureas are described and revised data for a few previously reported compounds given.

The valuable technical assistance of Mr. Josef Conti in part of this work is greatly acknowledged. The microanalyses were performed in this Laboratory by Mr. A. Grossmann. The photographic assistance of Dr. N. Berg is highly appreciated. Thanks are due also to Dr. A. Friediger and Dr. J. Hald for their valuable gifts of some of the compounds investigated.

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The Solubility of Carbon Dioxide in Perfluoro-*n*-heptane, Normal Heptane, *Cyclo*-hexane, Carbon Tetrachloride, Benzene, Carbon Disulphide and Aqueous Solution of Aerosol

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In three recent papers dealing with the solubility of nitrogen [1], chlorine [2], hydrogen, oxygen and carbon monoxide [3] in non-polar solvents, it was shown that it is possible to account for the solubility by theories based upon a model process of mixing two liquids. The calculation of the solubility was, in the case of chlorine, carried out using heats of vaporization and molal volumes for chlorine and for the solvents. In the case of gases having low solubility (nitrogen, hydrogen, oxygen, carbon monoxide) the solubility parameters for the gases were empirically adjusted.

It is the purpose of this paper to investigate whether it is possible without empirical adjustment to account for the solubility of carbon dioxide which is another gas of relatively high solubility and for which we have all necessary physical data. We would expect the linear carbon dioxide molecule to obey the equations which are valid for mixtures in which association and formation of chemical compounds are absent. Contrary to chlorine, carbon dioxide is at room temperature close to its critical temperature (31° C) and it was difficult in advance to know whether this fact would influence the calculation of the solubility.

APPARATUS, MATERIALS AND PROCEDURE

The solubility determinations were carried out in the same apparatus and using the same procedure as described in a previous paper [3].

Carbon dioxide 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 consisting of two

one liter flasks placed beside each other, and in the bottom connected with glass tubing about 20 cm long and 1 cm in diameter. The carbon dioxide was analysed by absorption in 35 per cent potassium hydroxide in a gas analysis apparatus as devised by Christiansen[4]. When analysis showed a content of less than 0.1 per cent impurities the gas was regarded as pure. It appeared that the carbon dioxide in the gasometer was gradually contaminated with atmospheric air which, presumably, was diffused through the concentrated sulphuric acid in the gasometer. In the course of three weeks the gas contained about two per cent atmospheric air. The gas employed was in most experiments 99.9 per cent CO_2. In experiments carried out with gas containing a greater amount of impurity (until 0.5 per cent) the solubility measurements were converted by a method as described below.

The solvents used were all purified by fractionation in a wire-gauze column. The employed perfluoro-*n*-heptane (C_7F_{16}), *n*-heptane, benzene and carbon disulphide were of the same purity as described in the previous paper[3]. Carbon tetrachloride (analytical reagent, Riedel-E. de Haën) showed boiling point 76.80° C at 759 mm and refractive index $n_D = 1.4573$ at 25° C. *Cyclo*-hexane purified by fractionated freezing, dried by phosphorous pentoxide and distilled, showed boiling point 80.69° C – 80.71° C at 760 mm and freezing point 6.2 – 6.4° C. The solution of aerosol was a 0.15 per cent aqueous solution of aerosol OT 100 % (dioctylester of the sodium salt of sulphotartaric acid, Amer. Cyanamid Co.). In the solution pH was 7.4.

Values for vapour pressures and densities used in the calculation of the solubility experiments are given in Table 1 in the previous paper[3]. Further, the vapour pressure and density of carbon tetrachloride at 25° C equal 114.5 mm[5] and 1.5843[5], respectively, was used. For the vapour pressure of the aerosol solution at 25° C the value valid for pure water (23.8 mm) was used.

Since carbon dioxide in the solvents used is considerably more soluble than oxygen and nitrogen even small amounts of impurity of atmospheric air will cause an essential error in the solubility determinations. The error depends on the amount of impurity as well as on the volume of the gas before and after absorption has taken place. A calculation, originating from an experiment on the solubility of "carbon dioxide" (99.5 per cent CO_2, 0.5 per cent atm. air) in 8.54 ml water at 25° C, is given in the following.

The volume before absorption was 6.647 ml (0°, 760 mm) corresponding to a content of 0.0352 ml atmospheric air. After the absorption had taken place the reduced volume of the gas was 0.970 ml (corresponding to 1.170 ml at 25° C and $p = 687.9$ mm), *i.e.* the pressure of the carbon dioxide has been about $\frac{0.0332}{0.970}\ 100 = 3.4$ per cent less than one atmosphere. Making allowance for the small amount of atmospheric air which has been dissolved in the water we calculate that the pressure of carbon dioxide after equilibrium has taken place is $\frac{0.0284}{0.970}\ 100 = 2.9$ per cent less than one atmosphere. If we calculate the solubility ignoring the impurity (0.5 per cent) we get $\alpha = 0.733$. Adding to this value 2.9 per cent we get $\alpha = 0.754$. An experiment carried out with pure carbon dioxide (99.997 per cent) resulted in the value $\alpha = 0.754$.

EXPERIMENTAL RESULTS

The results of the solubility determinations have been reproduced in Table 1, expressed as Bunsen absorption coefficient (α), *i. e.* the number of ml of the gas reduced to 0° C and 1 atm. which can be dissolved in 1 ml solvent at the

temperature concerned when the partial pressure of the gas is 1 atm. By converting the experiments into a partial pressure of 1 atm. carbon dioxide has been considered to obey Henry's law. That this is correct at pressures approximately 1 atm. appears from Table 1, where the partial pressures of carbon dioxide in the equilibrium are stated. Even at five atmospheres Hähnel [7] found for the solubility of carbon dioxide in water only about eight per cent deviation from Henry's law at 15° C.

According to Horiuti [8] one ml of carbon tetrachloride will expand 0.00215 ml for each ml carbon dioxide dissolved at 25°. At a one atmosphere partial pressure of carbon dioxide the carbon tetrachloride will dissolve about 2.5 ml *i. e.* the total expansion is only 0.0054 ml per ml carbon tetrachloride. This negligible correction has been disregarded in the calculation of the experiments. In the other solvents used the involved error is still smaller.

For the solubility in water the experimental value was $\alpha = 0.754$ at 25° C. In the 0.15 per cent aerosol solution the values at 25° C were $\alpha = 0.750$ ($p_{CO_2} =$ 793.4 mm) and $\alpha = 0.751$ ($p_{CO_2} = 725.2$ mm) *i. e.* 0.4 per cent less than in pure water. The relative surface tension of the aerosol solution was measured to be

Table 1. Solubilities of carbon dioxide expressed in Bunsen absorption coefficent (α). Experimental values at t° C.

n-C_7F_{16}	t			25.1	25.1			
	p[a)]			669.3	686.4			
	α			2.09	2.09			
n-C_7H_{16}	t		20.5	25.1	25.1			34.5
	p		718.6	712.4	710.3			727.7
	α		1.98	1.84	1.85			1.62
cyclo-C_6H_{12}	t			25.0	25.0			
	p			700.3	814.1			
	α			1.57	1.59			
CCl_4	t			25.0	25.1			
	p			613.5	652.1			
	α			2.47	2.46			
C_6H_6	t	20.4	20.5	25.0			29.7	34.2
	p	714.2	556.6	656.1			663.5	609.4
	α	2.65	2.62	2.43			2.33	2.17
CS_2	t			24.9	25.2	25.3		
	p			433.0	394.8	479.2		
	α			1.21	1.21	1.22		

a) p is the partial pressure of carbon dioxide in mm Hg.

Table 2. Solubility (a) of carbon dioxide at 25° C. Comparison with previous measurements.

	Water	Carbon tetrachloride	Benzene	Carbon disulphide
Just 1901 [9]	0.7564	2.102	2.222	0.797
Geffcken 1904 [10]	0.759			
Findlay and Shen 1912 [6]	0.756			
Kunerth 1922 [11]	0.749			
Gatterer 1926 [12]	0.757			
Morgan and Pyne 1930 [13]	0.7572			
Horiuti 1931 [8]		2.444		
Orcutt and Seevers 1936 [14]	0.753			
Kiss, Lajtai and Thury 1937 [15]	0.7504			
Present investigation	0.754	2.47	2.43	1.21

0.431 in a stalagmometer according to Traube, the number of drops of water being 62 and for the aerosol solution 144.

It appears from Table 2, which includes solubilities found by other investigators that the present results for the solubility in carbon tetrachloride, benzene and carbon disulphide are higher than the results stated by Just. In carbon tetrachloride the present value differs only 1.5 per cent from the value found by Horiuti.

CORRELATION WITH THEORY

It was desired to investigate whether the solubility of carbon dioxide in non polar solvents can be represented by the equation

$$\log x_2^i = \log \Phi_2 + 0.4343 \left(1 - \frac{V_2}{V_1}\right) \Phi_1 + \frac{0.4343\, V_2\, \Phi_1^2}{RT} (\delta_1 - \delta_2)^2 \qquad \text{(I)}$$

where x_2^i denotes the 'ideal' solubility expressed in mole fraction, Φ volume fraction, δ solubility parameter, V molal volume, R the gas constant and T absolute temperature. Subscript 1 refers to the solvent and subscript 2 to carbon dioxide. With regard to the derivation of equation (I) reference should be made to Hildebrand and Scott [16]. The introduction of the approximation $\Phi_1 = 1$ just evolves a slight error since Φ_2 in all the solvents used is less than 0.0058. If further $\Phi_2 = \dfrac{V_2 x_2}{V_2 x_2 + V_1 x_1}$ is substituted, equation (I) can be written

$$-\log x_2 = -\log x_2^i + \log \frac{V_2}{V_1} + 0.4343 \left(1 - \frac{V_2}{V_1}\right) + \frac{0.4343\, V_2}{RT} (\delta_1 - \delta_2)^2 \qquad \text{(II)}$$

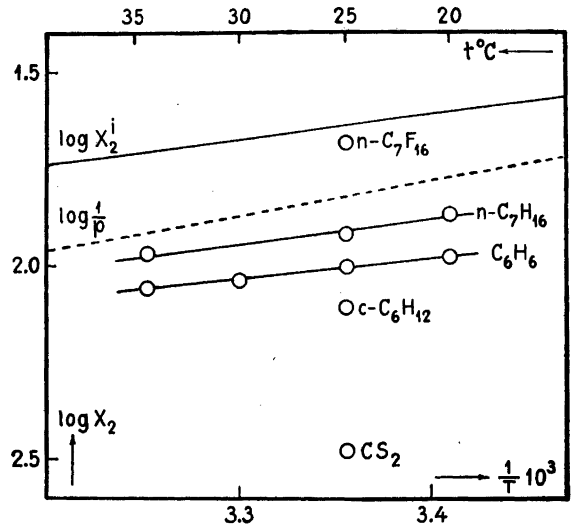

Fig. 1. The solubility of carbon dioxide
——— *log x_2^i ideal solubility of carbon dioxide*
- - - - - - - *ideal solubility disregarding deviations from perfect gas laws*
○ *experimental values of the solubility*

where x_2 is the solubility expressed in mole fraction. If we introduce the approximation $V_1 = V_2$ equation (II) is simplified into

$$-\log x_2 = -\log x_2^i + \frac{0.4343\, V_2}{RT}(\delta_1 - \delta_2)^2 \qquad \text{(III)}$$

The ideal solubility is calculated from

$$x_2^i = f_2/f_2^0 \qquad \text{(IV)}$$

where f_2 denotes the fugacity of carbon dioxide at one atmosphere and f_2^0 the fugacity at the saturation pressure, both calculated at the same temperature. The values of the fugacities are taken from Deming and Deming [17].

At 25° C we calculate $x_2^i = 0.9947/43.3 = 0.0230$. The curve for the ideal solubility is drawn in Fig. 1, using the reciprocal absolute temperature as abscisse and the logarithm of the ideal solubility as ordinate. For comparison the dotted curve shows values of $1/p_2^\circ$, which do not allow for the deviations of carbon dioxide from perfect gas laws. Also there are in Fig. 1 plotted experimental values for the solubilities converted to mole fraction by

$$x_2 = \frac{\alpha/22\,250}{\alpha/22\,250 + d/M}$$

where 22 250 is the molal volume in ml of carbon dioxide at 0° and 1 atm., and d and M is the density and molecular weight, respectively, of the solvent.

Table 3. Calculation of the solubility parameter (δ_2) for carbon dioxide at different temperatures.

$t°$	ΔH cal/g	ΔH cal/mole	p atm.	Specific vol. in ml liq.	Specific vol. in ml gas	Molal volume liq.	Molal volume gas	δ_2
−50	80.6	3 540	6.75	0.867	55.4	38.2	2 440	9.10
−40	76.6	3 370	9.93	0.897	38.2	39.5	1 680	8.68
−30	72.4	3 180	14.1	0.931	27.0	41.0	1 190	8.25
−20	67.8	2 980	19.4	0.971	19.5	42.7	859	7.80
−10	62.5	2 750	26.1	1.019	14.2	44.8	625	7.30
0	56.1	2 470	34.4	1.081	10.4	47.6	457	6.68
10	48.1	2 120	44.4	1.166	7.52	51.3	332	5.95
20	37.1	1 630	56.5	1.298	5.26	57.1	232	4.94
25	28.5	1 255	63.5	1.417	4.17	62.3	184	4.13
30	15.1	665	71.2	1.677	2.99	73.8	132	2.76
31	0	0	73.0	2.18	2.18	96.	96	

The value of V_2 for carbon dioxide (47.7 ml) is calculated as the average value of the partial molal volumes in carbon tetrachloride and benzene on the basis of Horiuti's experiments [8]; these values are only to a slight extent dependent on the solvent.

The solubility parameters (δ_1) of the solvents, defined as the square roots of their energy of vaporization per ml, are taken from Hildebrand and Scott [16]. For carbon dioxide δ_2 has been calculated from

$$\delta_2 = \sqrt{\frac{\Delta H - p\Delta V}{V_2}} \qquad \text{(V)}$$

Table 4. Solubility of carbon dioxide in non polar solvents at 25° C. Comparison of experimental and calculated values. $x_2^i = 0.0230$; $V_2 = 47.7$ ml; $\delta_2 = 5.1$

	V_1	δ_1	$x_2 \cdot 10^4$ exp.	$-\log x_2$ exp.	$-\log x_2$ calc. eq. II	ΔII	$-\log x_2$ calc. eq. III	ΔIII
n-C_7F_{16}	227	5.85	209	1.68	1.32	−0.36	1.66	−0.02
n-C_7H_{16}	147	8.1	121	1.92	1.76	−0.16	1.83	−0.09
cyclo-C_6H_{12}	109	8.2	77.2	2.11	1.86	−0.25	1.97	−0.13
CCl_4	97.1	8.6	107	1.97	1.98	+0.01	2.07	+0.10
C_6H_6	89.3	9.15	96.6	2.02	2.14	+0.12	2.21	+0.19
CS_2	60.6	10.0	32.9	2.48	2.47	−0.01	2.48	+0.00

where ΔH is the molal heat of vaporization and ΔV the difference between the molal volume of carbon dioxide in the gaseous and the liquid state (V_2) at the pressure p. Requisite data (Quinn and Jones [18]) are given in Table 3. It turned out that it was not possible to represent the experimental values using equations (II) or (III) and the value $\delta_2 = 4.1$ at 25° C. The proper value of δ_2 to use for carbon dioxide was then determined from the solubility data themselves. The accuracy with which equations (II) and (III) together with the constant thus determined ($\delta_2 = 5.1$) represent the experimental results, will appear from Table 4. It can be seen that equation (III) expresses the experimental data somewhat better than equation (II).

It is interesting to notice that the experimental value for the solubility of carbon dioxide in benzene is 40—50 per cent higher than calculated. Possibly this higher solubility can be explained by assuming that carbon dioxide and the electron donor benzene form a weak compound. Recently Benesi and Hildebrand [19] proved by spectrophotometric measurements that there is a complex formation between iodine and benzene, and showed how this can explain the abnormal high solubility of iodine in benzene.

Correlation between the solubility and the surface tension of the solvents. The Ostwald's solubility coefficient, λ, can be calculated according to Uhlig [20] from

$$\ln\lambda = \frac{-4\pi r^2\sigma + E}{kT} \qquad \text{(VI)}$$

where r denotes the radius of the dissolved gas molecule, σ the surface tension of the solvent, k Boltzmann's constant and T absolute temperature. E is an energy quantity originating in the attraction between the dissolved gas molecules and the molecules of the solvent. The value of E can be evaluated from the solubility measurements. It should be emphasized that equation (VI) is only valid for the solubility in pure solvents and not for the solubility in mixed solvents or *e. g.* for the solubility in a solution of aerosol in water. By dissolving even minor amounts of aerosol in water we get a considerable change of the surface tension, which for the 0.15 per cent solution used had a value less than half of the value for pure water. In spite of this the solubility of carbon dioxide showed the same value in both solvents. In such a diluted solution E may be assumed to have the same value as in pure water, and it is obvious that in this case equation (VI) will fail. For a pure solvent the surface tension is a function of the forces of attraction between the molecules of the solvent. Thus it is possible to calculate an approximate solubility parameter of a pure solvent from its surface tension, see Hildebrand and Scott [16 p. 431].

SUMMARY

1. Following values for the solubility of carbon dioxide, expressed as Bunsen coefficient, have been determined. In perfluoro-*n*-heptane 2.09 (25°), *n*-heptane 1.98 (20.5°), 1.85 (25°), 1.62 (34.5°), *cyclo*-hexane 1.58 (25°), carbon tetrachloride 2.47 (25°), benzene 2.63 (20.5°), 2.43 (25°), 2.33 (29.7°), 2.17 (34.2°) and in carbon disulphide 1.21 (25°).

2. The solubility equation for regular solutions, with an empirical adjustment of the solubility parameter of carbon dioxide, yields calculated values in reasonably good agreement with the experimental ones.

3. It is shown by experiment that the solubility of carbon dioxide in water and in a solution of 0.15 per cent aerosol in water have the same value to within the accuracy of the experimental determination. This gives an example in which the solubility is independent of the surface tension of the solvent.

The present work has been performed in the Department of Inorganic Chemistry of the Pharmaceutical College of Denmark. I wish to express my gratitute towards the chief of this laboratory, Professor Carl Faurholt, for the unfailing interest he has shown in my work.

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Decomposition of Hydrogen Peroxide by Catalase

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Fifty years ago Senter [1] showed that the decomposition of hydrogen peroxide by catalase from erytrocytes followed a first order reaction, in case the substrate concentration was sufficiently low. At higher substrate concentration the reaction velocity constant decreased during the reaction. In the following years these results were not supported, to the contrary it was demonstrated that catalase was a very fragile enzyme and that it was inactivated by hydrogen peroxide during the reaction.

A few years ago Bonnichsen, Chance and Theorell [2] demonstrated that the reaction did follow the expression for a first order reaction. They used catalase in concentration about fifty times higher than previously and the activity was measured by rapid manipulation of the titration technique or a special polarographic method.

This paper supports the results of Senter and in addition it is shown that under special experimental conditions it is possible to repeat the reaction by further addition of hydrogen peroxide without change in the first order reaction velocity constant.

METHOD

Catalase was prepared from horse kidney after the method of Bonnichsen [3]. Perhydrol "Merck" diluted with redistilled water was used as substrate. Buffer solution $M/150$ phosphate "Sørensen", pH 7.0, temperature 20.0° C. The reaction flask (Pyrex) was treated with chromic-sulfuric acid cleaning mixture and thoroughly washed with redistilled water. After this the flask was inverted over a glass tube through which steam was flowing, so that the inside was rinsed by the condensed water. Between the experiments the flask was only washed with glassdistilled water and treated with steam. The diluted solution of hydrogen peroxide was stable in flasks treated in this way, the spontaneous decomposition being less than 0.5 per cent in twentyfour hours.

The reaction was started by adding the measured amount of catalase solution in a "watchglass" made from the bottom of ordinary test tubes. The reaction was followed by titration of samples at different times. Due to the content of oxygen bubbles in the

reacting mixture it was impossible to take out an accurate volume of the solution. According to Sten Andersen [4] the sample was taken up in a 20 ml pipette with a fairly large delivery orifice. The sample was at once allowed to run into a previously tared Erlenmeyer flask, which contained sulfuric acid in such an amount that the final concentration was 0.5 *M*. The flask was weighed within an accuracy of 0.01 g.

The hydrogen peroxide was titrated with potassium permanganate or ceric sulfate in both cases using Ferroin as indicator. The solution of Ferroin used was 1/400 *M*, which showed to be stable for some weeks. 25 μl of this solution was found to be a sufficient amount to give a distinct endpoint, at the same time requiring only a small correction for the titration of the indicator.

In the titration of the most diluted solutions of hydrogen peroxide with potassium permanganate it was necessary to add manganous salt just before the titration. A total concentration about 10^{-4} *M* of manganous sulfate was used.

EXPERIMENTAL

In some experiments with a catalase concentration of about 2×10^{-10} *M* it was found that high concentrations of hydrogen peroxide inactivated the enzyme. That was not the case at low concentrations of hydrogen peroxide.

The catalase was added to the buffered hydrogen peroxide solution, which had been equilibrated in a water bath at 20.0° C. After six half changes or more the hydrogen

Table 1. The variation of the velocity constant in successive experiments. The reaction at time 0 has been started by adding catalase, the other by adding hydrogen peroxide at the initial concentration designed a. For explanation see text.

Time in hours	k'	a
0	0.311	0.0024 *M*
1.5	0.233	0.0023 »
25	0.198	0.0020 »
25.5	0.198	0.0013 »
0	0.309	0.0024 »
0.5	0.284	0.0024 »
50	0.136	0.0024 »
51	0.136	0.0025 »
0	0.325	0.0015 »
0.5	0.307	0.0015 »
24	0.265	0.0014 »
24.5	0.263	0.0014 »
48	0.216	0.0015 »
48.75	0.215	0.0015 »
49.5	0.215	0.0015 »

Table 2. The velocity constant with varying concentration of catalase.

Initial hydrogen peroxide concentration	Catalase concentration	k
0.0019 M	$3.33 \times 10^{-10} M$	2.64×10^7 l, mole^{-1}sec^{-1}
0.0019 »	$1.67 \times 10^{-10} M$	2.68×10^7
0.0019 »	$8.36 \times 10^{-11} M$	2.63×10^7 *
0.0019 »	$4.20 \times 10^{-11} M$	2.60×10^7 *
0.0005 »	$3.33 \times 10^{-10} M$	2.85×10^7
0.0005 »	$1.67 \times 10^{-10} M$	2.73×10^7
0.0005 »	$8.36 \times 10^{-11} M$	2.53×10^7
0.0005 »	$4.20 \times 10^{-11} M$	2.60×10^7
	Average	2.65×10^7 l, mole^{-1}sec^{-1}

* The velocity constant has been calculated from the first half change.

peroxide was brought to the initial concentration by adding hydrogen peroxide in such a concentration that the dilution effect on the catalase concentration could be ignored.

With an initial hydrogen peroxide concentration of 0.1 M the catalase could decompose only fifty per cent, whereas it decomposed all the hydrogen peroxide when the initial concentration was 0.05 M. When the hydrogen peroxide again was brought to 0.05 M no reaction occurred.

By gradually decreasing the initial substrate concentration the catalase subsisted through more runs before total inactivation occurred. With an initial substrate concentration of 0.01 M the inactivation was complete after the fourth addition of hydrogen peroxide. In experiments with an initial substrate concentration of 0.005 M the activity decreased only about 35 per cent during four runs. In experiments with an initial substrate concentration like this the reaction followed a first order reaction during the first or the first and second half change. With initial substrate concentration of 0.0024 M or lower the reaction followed a first order reaction in its whole course.

By repeating the reaction it was found that the velocity constant after a certain decrease did not change during two or three runs. The results are given in Table 1.

In some experiments the catalase concentrations were determined photometrically just before the measurement of activity by taking the extinction as ε (405 mμ, 10^{-3} M) = 380 (Bonnichsen [5]).

Using 0.0019 M hydrogen peroxide the reaction was first order when the catalase concentration was 1.67 and 3.33×10^{-10} M. With a catalase concentration of 4.2 and 8.3×10^{-11} M the velocity constant decreased after the elapse of the first half change. Calculation of the velocity constant during the first half change gave the same value as that found in experiments with initial substrate concentration of 0.0005 M, in which the reaction followed the expression for a first order reaction from six to more than 95 per cent decomposition of hydrogen peroxide (Fig. 1).

DISCUSSION

Using catalase prepared from horse kidney it is shown that the decomposition of hydrogen peroxide follows a first order reaction at a catalase con-

centration from 4.2×10^{-11} M to 3.33×10^{-10} M if the hydrogen peroxide concentration is sufficiently low. The results confirm Senter's experiments.

The reaction is described by the expression for a first order reaction

$$\ln \frac{a}{x} = k \cdot E \cdot t$$

where t is in seconds, E is the molar enzyme concentration, a is the initial substrate concentration at $t = 0$, and x is the substrate concentration at the time t.

In some experiments where the enzyme concentration was not known with sufficient accuracy the following expression has been used

$$\ln \frac{a}{x} = k' \; t$$

where t is in minutes and

$$k' = 60 \; E \cdot k$$

Table 2 gives the values of the velocity constants which will be seen to agree fairly well. The lower values than that previously found by Bonnichsen [5], 3.5×10^7 1 $mole^{-1}sec^{-1}$, are perhaps due to the fact that the catalase solution at the time of measuring had been kept in refrigerator for several months.

The inactivation of catalase by the substrate is very well known from the literature. It is possible to get rid of it in two ways, either by increasing the enzyme concentration as used by Bonnichsen *et al.*[2] and Beers and Sizer [6] or by decreasing the substrate concentration. It is possible that a catalase concentration lower than 4×10^{-11} M at 20° will require a substrate concentration lower than 0.0005 M.

Many years ago it was assumed that the oxygen set free during the reaction was the agent which caused the inhibition. In experiments at atmospheric pressure and with hydrogen peroxide concentration of about 0.05 M the formation of oxygen is so fast that the solution is saturated with oxygen to such an extent that it is equivalent to a solution saturated with oxygen under pressure of several atmospheres. This is the case before bubbles of oxygen can be seen. Decreasing the total pressure over the solution to about 80 mm of mercury and thorough aeration does not change the course of the reaction. Therefore one must conclude in agreement with Michaelis and Pechstein [7] that the inactivation is not due to oxygen.

Previously it has been observed that catalase solutions, especially if dilute, will show a decreasing activity upon standing. As the decrease is more pronounced when the glass surface is increased by adding glass beads (Bonnichsen *et al.*) it has been ascribed to an adsorption phenomenon.

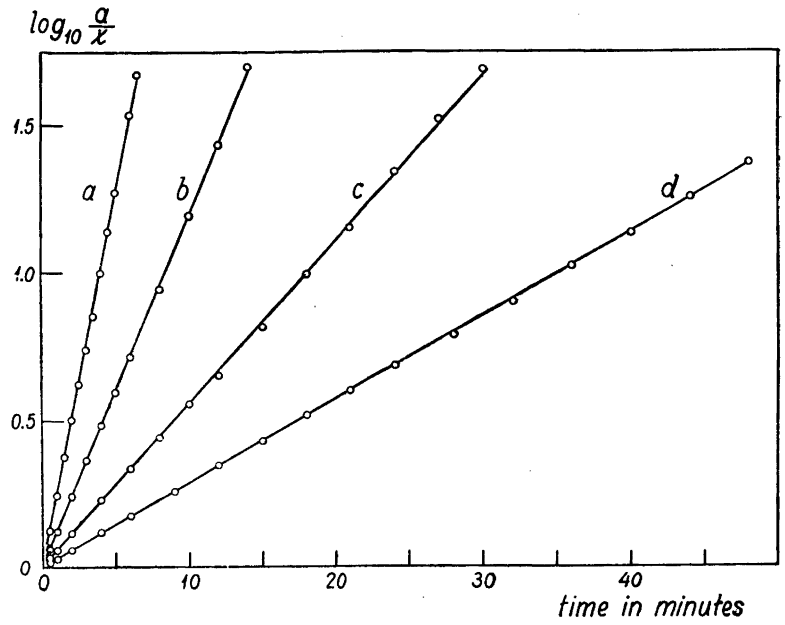

Fig. 1. First order rate curves of decomposition of hydrogen peroxide by varying concentrations of catalase ($a = 3.33 \times 10^{-10}$, $b = 1.67 \times 10^{-10}$, $c = 8.36 \times 10^{-11}$ and $d = 4.20 \times 10^{-11}$ M). Initial concentration of hydrogen peroxide 0.0005 M. Temperature 20.0° C, pH 7.0, M/150 phosphate buffer.

The decrease of k' in Table 1 is probably caused by adsorption of catalase at the glass surface. If the decrease in catalase concentration caused by adsorption is small during the time it will take to run two or three kinetic experiments, one would expect an approximately equal velocity constant in the experiments. Table 1 shows that in 24 hours the adsorption will proceed at a sufficiently low rate to make k' a real constant.

These experiments thus only demonstrate in another way that at low hydrogen peroxide concentration the catalase is not inactivated by the substrate.

As shown the reaction follows the expression for a first order reaction from six to more than 95 per cent decomposition of the substrate, which indicates that the adsorption phenomenon does not take place in solutions in which there is a measurable amount of hydrogen peroxide. As the activity is decreased in the reaction flask from the first run to the second and third, one must assume that it is the hemin group, which is adsorbed on the glass surface.

SUMMARY

Using catalase prepared from horse kidney it is shown that inactivation by the substrate does not take place in case the initial hydrogen peroxide concentration is sufficiently low, and under these circumstances the reaction is a first order reaction.

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Adsorption in Solutions of Catalase

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It is known that dilute solutions of catalase upon standing will show a decrease in activity. The decrease is accentuated when the glass area is increased by addition of glass beads (K. Agner, personal communication to Bonnichsen *et al.*[1]). Previously it was shown by Harkins, Fourt and Fourt [2] that catalase is adsorbed in a monolayer at various interfaces. It has not been possible to find any information on how to minimise the adsorption in dilute solutions.

In kinetic experiments it is of interest to minimise the adsorption or at least to have some information about it, and some experiences will be reported.

METHOD

As previously described the decomposition of hydrogen peroxide by catalase follows a first order reaction in case the substrate concentration is low [3]. As a measure of activity or concentration of catalase the velocity constant is used as taken from the expression

$$\ln \frac{a}{x} = k' \, t,$$

where a is the substrate concentration at $t = 0$, x the substrate concentration at the time t (in minutes).

The substrate, Perhydrol "Merck", was used at a concentration of 0.0019 M or lower. The activity measurement was carried out at 20.0° C and at pH = 7.0 (M/150 phosphate "Sørensen"). The measurement of k' was usually based upon four to eight samples in each activity determination. From the catalase solution to be tested, an amount was taken for activity determination, such that k' was about 0.2 to 0.3. If it was lower more samples were taken.

The catalase was prepared from horse kidney according to Bonnichsen [4]. A solution with a molar concentration of catalase about 4×10^{-6} was diluted to give the desired concentration. The activity determination was started within one minute from the dilution. The amount of catalase used for activity determination was measured with a pipette,

which was blown out into the reacting mixture. In several experiments the initial k' only varied about two per cent. Some experiments, in which the catalase was measured by means of a Krogh syringe, the initial k' showed a great variation, probably caused by adsorption at the ground glass surface of the syringe.

The area of glass surface, which is in contact with the catalase solution, is taken from the internal diameter of the flask and the height of the solution.

EXPERIMENTAL

In the first experiment an attempt was made to elute the adsorbed catalase. After standing for two weeks a solution of catalase in distilled water showed $k' = 0.295$. Potassium chloride was added and chloride titration showed 24 meq./l of chloride *. Two hours later $k' = 0.404$ showing that the activity was increased about 37 per cent.

A 25 ml volumetric flask, in which a catalase solution had been stored for seven days, was washed once with distilled water, and filled to the mark. Fifteen minutes later the content of the flask was poured into a hydrogen peroxide solution for activity determination, which gave a k' of 0.016. The flask was then filled with 0.01 M KCl, and fifteen minutes later the activity was determined as before. This time the $k' = 0.081$ or about five times as high as when washing had been done with pure water.

Table 1. Activity determination in solutions of catalase with molar concentrations at 6.9×10^{-8}.

Time	Catalase dissolved in H_2O k'	0.01 M KCl k'
0	0.301	0.303
1 hour	0.297	0.303
1 day	0.276	0.286
2 days	0.263	
3 »	0.250	0.285
4 »	0.251	0.290
5 »	0.248	0.294
7 »	0.246	0.290
8 »	0.255	0.286
9 »	0.245	
10 »		0.292
12 »	0.247	0.289

After addition of potassium chloride (chloride concentration 1.3 meq./l).

15 minutes	0.296
1 hour	0.307
1 day	0.284
57 days	0.275

* The chloride determinations have kindly been performed by Dr. Barker Jørgensen and Mr. P. Rosenkilde.

Table 2. Activity determination in solutions of catalase with molar concentration at 6.9 × 10⁻⁹.

Time	Catalase dissolved in H_2O without stirring	with stirring
0	0.303	0.312
65 minutes	0.303	
85 »		0.288
150 »	0.307	
1 day	0.291	0.248
3 days		0.231
4 »	0.249	
5 »		0.218
6 »	0.221	0.216
7 »	0.218	
8 »		0.205
10 »	0.198	
14 »	0.173	
35 »	0.127	
After addition of potassium chloride to 0.01 *M*.		
1 hour		0.227
1 day		0.233

Solutions stored in flasks made from ordinary glass sometimes will show a small increase in activity some days after the initial decrease. Because of this it was decided to use only new flasks made from "Pyrex" glass. The flasks were treated as previously described [3].

In the flasks in which the catalase was diluted to a molar concentration at 6.9×10^{-8} with distilled water, the activity was decreased to 82 per cent of the initial. Equilibrium was reached in four days and afterwards no changes occurred for nearly two weeks. By that time a few milligrams of potassium chloride or sodium chloride were added. The final chloride concentration was determined by titration. It varied from 0.6 to 1.3 meq./l. Samples withdrawn within fifteen minutes to two hours after the addition of salt showed that the activity had increased again to between 96 to 102 per cent of the original. During the following twentyfour hours the activity decreased to about 95 per cent.

A similar decrease is found when catalase is dissolved in 0.01 *M* KCl or NaCl to 6.9×10^{-8} *M*. In such solutions equilibrium was reached in a day and remained unchanged for at least 12 days. In Table 1 the results from two experiments are given.

Dilution of the catalase with 0.005 *M* sodium or potassium sulfate, *M*/15 or *M*/150 phosphate (Sørensen) at pH = 7.0 seemed to inhibit the adsorption to a similar degree. Dilution with 0.01 *M* sodium acetate however gave an activity loss of ten per cent.

A solution of catalase diluted with water to 6.9×10^{-8} *M* and stored in a flask made from polyethylene showed a decrease in activity to about 75 per cent in thirteen days. Addition of potassium chloride to a concentration at 1.02 meq. increased the activity to 85 per cent of the initial value.

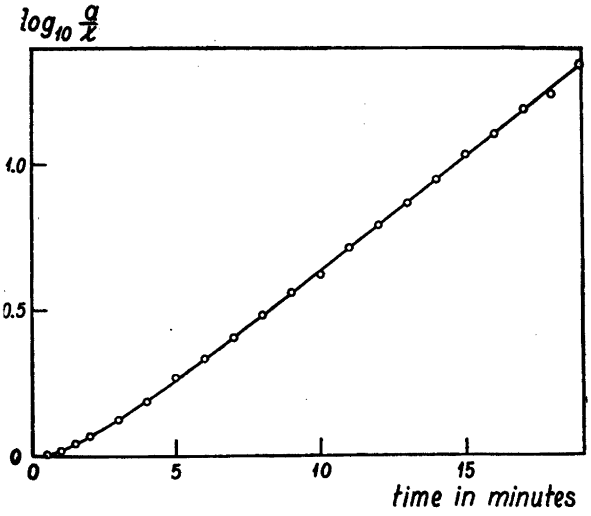

Fig. 1. The rate of reaction in a solution of catalase in which the decrease in activity was allowed to go far, before the reaction was started by adding hydrogen peroxide.

In solutions diluted to a molar concentration at 6.9×10^{-9} it was not possible to reach equilibrium (Table 2) during the period of observation. Difficulties in avoiding bacterial contamination restricted the extension of the experiments in time.

In a catalase solution of molar concentration 1.7×10^{-10}, the loss in activity was about twenty five per cent during the first hour, and forty per cent in the following twenty hours. Addition of potassium chloride (0.01 *M*) or phosphate buffer (pH = 7.0) at a total concentration at *M*/150 did not change these results. In these experiments the adsorption was allowed to take place in the reaction flask.

A catalase solution with a molar concentration at 1.6×10^{-10} was stored for three days at 20.0 °C. The initial activity was determined in an experiment carried out immediately after the dilution ($k' = 0.298$). After the elapse of three days hydrogen peroxide was added to make a concentration of 0.0018 *M*. The reaction was followed by taking several samples. The rate of the reaction is shown in Fig. 1. From the first, second and third sample the k' equalled 0.101, or 34 per cent of the initial. At that time about ten per cent of the hydrogen peroxide had been decomposed. The k' increased from this value to 0.177 and remained constant from about 60 to more than 95 per cent decomposition of the hydrogen peroxide.

In a similar experiment the k' value increased during the reaction from 28 to about 48 per cent of the initial.

DISCUSSION

The experiments show that the activity decreases in dilute solutions of catalase. The decrease depends to a high degree upon the initial concentration of catalase. As it is possible by elution to bring about a return of the activity,

in some cases to the initial level, one may conclude, that the decreased activity is mainly caused by adsorption and not by other types of inactivation.

Assuming the catalase molecules to be adsorbed in a monolayer [2] we can calculate the cross section of the molecule. Three experiments with a molar concentration of catalase of 6.9×10^{-8} showed a 17—18 per cent decrease, corresponding to adsorption of 17.7×10^{13} molecules on a glass surface of about 36 cm^2 indicating a cross section of the catalase molecule about 2 000 square Å. From another experiment with half the catalase concentration (3.4×10^{-8} M) the cross section was calculated to be 2 100 square Å.

A comparison of the adsorbed catalase molecule with molecules of simpler composition will give an impression of the result and its order of magnitude: Palmitic acid with a molecular weight (256) approximately 1/1 000 of the catalase (225 000), has a cross section about 21 square Å [5]. If the molecular weight of the palmitic acid were increased a thousand times the cross section would be increased a hundred times, or to 2 100 square Å, a value which agrees with that found for the catalase molecule.

At equilibrium one finds in saltfree solutions from the measurements that each cm^2 carries about 5×10^{12} molecules of catalase. Upon addition of salt or dilution of the catalase with salt solutions, the equilibrium is displaced, so that only 1.5×10^{12} molecules/cm^2 remains adsorbed.

In saltfree solutions where the molar concentration of catalase was 6.9×10^{-9} it was not possible to obtain equilibrium. A calculation shows that nearly all catalase molecules in solution could be adsorbed on the glass surface, carrying about 5×10^{12} molecules on each cm^2.

The addition of potassium chloride causes a change in the adsorption, and if one is allowed to assume equilibrium this is reached when each cm^2 carries 1.3×10^{12} molecules. This value is to be compared with the value 1.5×10^{12} from the experiments using a catalase concentration ten times higher.

In solutions with catalase concentration at 1.6×10^{-10} M a complete adsorption will put only about 2×10^{11} molecules on each cm^2. This number is so much lower than that found at equilibrium in solutions containing salt, that it may account for the lack of effect of adding potassium chloride or buffer.

During reaction with hydrogen peroxide the activity is increased as mentioned above. The velocity constant does not change from about 60 to more than 95 per cent decomposition of hydrogen peroxide, and during this part of the reaction some sort of equilibrium has therefore probably been reached. In that case addition of hydrogen peroxide elutes catalase molecules much more efficiently than addition of salt as only about 1×10^{11} molecules can be left on each cm^2.

As the number of adsorbed molecules on each cm^2 seems to be independent of the molar concentration of catalase, the activity will be most constant in concentrated solutions, and when the area of glass surface in contact with the solution is small.

Addition of a salt as potassium chloride will shift the equilibrium so that a smaller number of molecules are adsorbed on each cm^2.

SUMMARY

Dilute solutions of catalase, prepared from horse kidney, will show a decrease in activity upon standing. It is found that this decrease is mainly caused by adsorption of catalase on the glass surface.

The number of catalase molecules carried on each cm^2 seems to be independent of the concentration of catalase. It is lower in dilute salt solutions than in pure water.

The cross section of the catalase molecule is calculated to be about 2 000 square Å.

The support of a grant from "*Købmand i Odense Johann og Hanne Weimann, f. Seedorffs legat*" was of great help.

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Short Communications

On the Mechanism of the Hydrolysis of Glycerides by Pancreatic Lipase

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Schönheyder and Volqvartz [1] have found that by the action of pancreatic lipase tripropionyl glycerol most probably is degraded to 1,2-dipropionyl glycerol. Further degradation was not observed in their experiments.

Quite recently Mattson, Benedict, Martin and Beck [2] have provided evidence that during lipolysis of long-chain glycerides in the lumen of the small intestine of the rat, the monoglycerides formed predominantly are of the 2-configuration. As a consequence of this they found it reasonable to assume that the diglyceride formed is the 1,2-isomer. In *in vitro* experiments they were, however, able to identify only 1-monoglycerides.

Studying the course of the *in vitro* hydrolysis of longchain triglycerides by rat pancreatic lipase we have found that the monoglycerides formed *in vitro* under our conditions predominantly are of the 2-configuration. Further we have obtained definite evidence that the diglyceride formed is the 1,2-isomer, by using the chromic acid oxidation method described by Bergström, Theorell and Davide [3].

One experiment is given as an example:

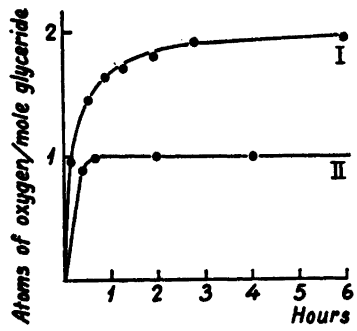

Fig. 1. Oxidation by chromic acid in 97.5 per cent acetic acid at 37° of synthetic 1,3-dipalmitin (II) and of hydrogenated diglyceride obtained from in vitro hydrolyses of olive oil (I).

2 ml olive oil was shaken for 1 hour at 40° with a mixture of 10 ml rat bile-pancreatic juice and 2 ml 0.1 *N* HCl (pH 6.4) [4]. After dilution with water the lipid mixture was extracted with ether, that was washed, dried and evaporated off. From the mixture of glycerides and fatty acids obtained the later were removed by passing the mixture through a column of Amberlite IRA-400. 1.215 g of glycerides was obtained. This was distributed in four separatory funnels between heptane and 80 per cent aqueous ethanol saturated with each other. The ethanol phase, in which the monoglycerides were to be expected contained after evaporation 57.2 mg substance. This was dissolved in 5 ml $CHCl_3$ and 1-monoglycerides assayed on two 1 ml portions by the periodic acid method. The values obtained corresponded to 2.2 mg 1-monoolein. After isomerisation of two other 1 ml portions by ferric chloride a total amount of 10.0 mg 1-monoglyceride was estimated. Thus 50.0 mg total monoglyceride was found in the 80 per cent ethanol phase, 22 per cent of which was 1-monoglycerides and 78 per cent 2-monoglycerides. About the same figures were obtained from several similar experiments.

The glycerides of the heptane phase were separated into tri- and diglycerides on a column of silicic acid [5].

To prove the configuration of the diglyceride a sample of this fraction was hydrogenated (PtO_2, acetic acid) and oxidized with chromic acid in 97.5 per cent acetic acid. Using this method a 1,3-diglyceride consumes one atom of oxygen per mole, while a 1,2-diglyceride takes two. The results of such oxidations on synthetic 1,3-diglyceride and on the hydrogenated diglyceride obtained in the *in vitro* hydrolysis are seen in Figure 1 and proves that the configuration of the diglyceride formed during the pancreatic lipolysis was the 1,2-isomer.

Thus, by the action of pancreatic lipase *in vitro* long-chain triglycerides are degraded *via* the 1,2-diglyceride, mainly to the 2-monoglyceride. The monoglycerides, however, also contain about 20 per cent 1-monoglyceride. It remains to be elucidated whether this 1-monoglyceride is formed directly from the 1,2-diglyceride or is formed by isomerisation of 2-monoglyceride.

A method for distinguishing 1,2-diglycerides from 1,3-diglycerides is given.

With the methods outlined above 1,2-diglycerides can be prepared with lipase. This might prove valuable for preparation of 1,2-diglycerides containing unsaturated fatty acids as hitherto described chemical methods only can be used for the preparation of saturated 1,2-diglycerides.

This work is part of investigations supported by „*Statens Medicinska Forskningsråd*".

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On the Protein Character of a Slime Produced by *Streptococcus cremoris* in Finnish Ropy Sour Milk

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In a previous paper [1] the author proved the earlier statement that the extreme viscosity of Finnish ropy sour milk is due to the intense production of capsular mucus by the milk streptococci in question. Since some other non-haemolytic streptococci, *viz. Str. salivarius* and *Str. bovis*, are known to produce viscous polysaccharides [2] it seemed feasible to assume that the mucus in ropy sour milk should be a polysaccharide too. The capsular material, however, failed to give the Hotchkiss reaction for polysaccharides [3] when studied microscopically in stained smears. In order to settle whether the slime produced by *Str. cremoris* strains is of polysaccharide character the mucous substance of ropy sour milk was investigated in greater detail.

Culture medium. Whey has proved to be the most favourable medium for slime production. Whey was prepared by clotting skimmed milk with rennet at 37° C for 1 hour, followed by filtration through cloth and sterilisation at 110° C for 15 min. No attention was paid to the clearness of the whey.

Preparation of the slime. Portions of sterile whey were inoculated with 2 % of a ropy sour milk and incubated at 25° C for 48 hours. It was necessary to use a natural ropy sour milk as inoculum in order to obtain sufficient amounts of the slime. In pure cultures of the streptococci in question the slime production is less vigorous and the slime formed does not always precipitate with ethanol as well as the slime in a mixed population. Apart from slime-producing and normal milk streptococci, the ropy sour milk contained *Oospora lactis* and some unidentified yeasts. During incubation the turbid whey turned clear and a flocculent sediment was formed. The sediment was centrifuged off and discarded. To the viscous clear supernatant were slowly added 1.25

The glycerides of the heptane phase were separated into tri- and diglycerides on a column of silicic acid [5].

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volumes of ethanol under vigorous stirring. A sticky, filamentous precipitate settled immediately. The thin filaments were often more than 15 mm in length. No precipitate is formed in whey incubated with non-ropy streptococci. The precipitate was washed twice in 60 % ethanol and redissolved in water by dialysis against running tap water for 48 hours. This viscous dialysed solution was centrifuged to remove possibly undissolved particles and used for further analysis.

Analytical methods. After hydrolysis the composition of the slime was investigated by paper partition chromatography. For sugar analyses 1—2 ml of the dialysed slime solution was hydrolysed in N H_2SO_4 for 2 hours on a boiling water bath. The hydrolysate was neutralised to pH 4 with baryta using bromocresol green test paper as an indicator. The $BaSO_4$ precipitate was centrifuged off and the solution evaporated to a small volume (< 100 μl). The solution was chromatographed according to Sundman [4]. n-Butanol saturated with 16.7 % acetic acid and containing 2.5 % phtalic acid was used as solvent. The standards contained galactose, glucose, fructose, mannose, arabinose, xylose and ribose. The amount of analysis and standards applied on the same paper were always equal, mostly 10—15 μl, but the same standard was used in different concentrations. Thus the spots on the developed chromatogram are comparable. The use of different standard concentrations makes it possible to estimate the quantities of the sugars in the analysis from the chromatogram.

The protein component of the slime solution was investigated after hydrolysis at 100° C in 25 % H_2SO_4 for 15 hours. Neutralisation was performed as described above. The clear solution was evaporated to 1 ml, and examined by a modification of Woiwod's one-dimensional technique [5]. Whatman's filter paper No. 1 was used instead of No. 4 and the eluant contained 0.1 % ninhydrin to avoid spray development. A casein hydrolysate prepared in the same way as the sample to be analysed, and known amounts of 14 amino acids were run as standards. The key chromatogram given by Proom and Woiwod [6] for casein hydrolysates was also used to identify the spots in the casein-hydrolysate standard.

The filamentous precipitate formed by 1.25 volumes of ethanol in ropy whey is extremely sticky. When washed and centrifuged off it forms a gummy substance which can be handled without changing shape. Some results of the quantity and composition of the washed undialysed precipitate are listed in Table 1.

From the results it seemed evident that the slime could not be a polysaccharide. The low sugar content was probably due to lactose retained by the precipitate from the whey.

When dialysed the slime lost about one fourth of its dry weight. The sugar content was reduced to less than 1 %. Chromatograms of the dialysed slime showed that galactose is the main carbohydrate. Traces of glucose are always present. The results are given in Table 2.

No other carbohydrates could be demonstrated. Chromatograms where galacturonic acid was run as a standard showed that the galactose spot at R_F 0.16 did not contain galacturonic acid ($R_F = 0.14$).

Table 1. Properties of the slime precipitated by 1.25 vol. ethanol from whey.

Percentage of the precipitate in the whey. Calculated from the weight of washed, centrifuged precipitate		Composition of the dried precipitate		
Before drying	After drying	Ash	Nitrogen [1]	Carbohydrates [2]
1.36 %	0.08 %	15 %	8.9 %	4 %

[1] Micro Kjeldahl determinations.

[2] Estimated as equal parts of galactose and glucose from paper chromatograms of the hydrolysed precipitate.

Table 2. The sugar content of the dialysed slime, estimated from paper partition chromatograms.

Experiment No.	Sugar content, calculated as % of dried dialysed slime		
	Galactose	Glucose	Total
1	0.40	< 0.10	< 0.50
2	0.72	< 0.35	< 1.07
3	0.54	< 0.21	< 0.75
Average	0.55	< 0.22	< 0.77

The presence of amino sugars was investigated according to Morgan and Elson [7], but with a negative result.

The dialysed slime gave a strong biuret reaction and paper chromatographical protein analyses showed that the major part of the slime is a protein. The amino acid composition does not differ remarkably from that of casein. The spot pattern in the analysis run and in the casein hydrolysate run were nearly equal. Of the 11 spots and spot-groups respectively, visible in the one-dimensional casein run, 10 were present in the slime run. The spot at R_F 0.30 (20° C), in the position of prolin, was lacking. Some differences in spot intensities were also evident. No significant difference could be noticed with this technique in the amino acid composition of the slime precipitate before and after dialysis.

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Synthesis and Reactions of α-(3-Methoxy-4-hydroxyphenyl)-glycerol ("Guaiacylglycerol"). I. Preliminary Experiments

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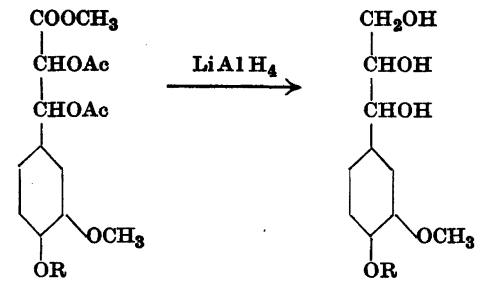
Recently, Adler and Björkqvist [1] reported the synthesis of α-(3,4-dimethoxyphenyl)-glycerol ("veratrylglycerol") (II) by lithium aluminium hydride reduction of the methyl ether of α,β-diacetoxyhydroferulic acid methyl ester (I). The behaviour of veratrylglycerol, especially on heating with sulphite solutions and with ethanolic hydrochloric acid proved to be of considerable interest in connection with the chemistry of lignin [2].

Simultaneously, attempts have been made to prepare the hitherto unknown phenolic compound, α-(3-methoxy-4-hydroxyphenyl)-glycerol ("guaiacylglycerol") (IV). This compound could be expected to be prepared by lithium aluminium hydride reduction of α,β-diacetoxy-acetylhydroferulic acid methyl ester (III). The preparation of this substance and its conversion into guaiacylglycerol are described in the following paper [3].

The present communication deals with some preliminary experiments in this field. They resulted in two different procedures for the preparation of methyl α-bromo-β-acetoxy-acetylhydroferulate (XVIII), which can be further converted, *via* III, into guaiacylglycerol [3]. Although both procedures are — due to the lower yields obtained — of less practical value for the present purpose than the procedure given in the following paper [3], they will be briefly reported as they involve some unexpected observations.

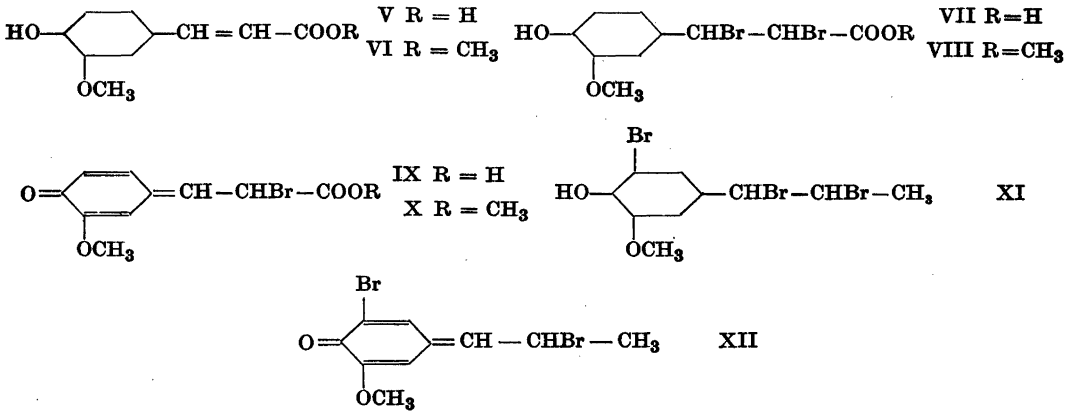
The α,β-dibromo-hydroferulic acid (VII) and its methyl ester (VIII) seemed to be suitable starting materials for the preparation of the triacetate III



I R = CH_3
III R = Ac

II R = CH_3
IV R = H

or the corresponding free carboxylic acid, or of similar products (with OH instead of one, two, or three OAc groups) which with lithium aluminium hydride could be expected to be converted into guaiacylglycerol (IV).



The dibromides VII and VIII were easily obtained from ferulic acid (V) and methyl ferulate (VI), respectively.

When the ether, chloroform, or benzene solutions of the dibromides VII or VIII were shaken with aqueous sodium bicarbonate (or acetate) they turned yellow, and this in analogy with the behaviour of other *p*-(and *o*-) hydroxybenzyl halides must be due to the formation of the "quinone methides" IX and X, respectively. In contrast with simple quinone methides, which rapidly undergo polymerization, "quinone ethides" and "propides" have previously been found to be comparatively stable. For instance, substance XII, which is obtained from XI, has been isolated by Zincke and Hahn [4] in a crystalline state. The yellow-coloured products obtained from VII and

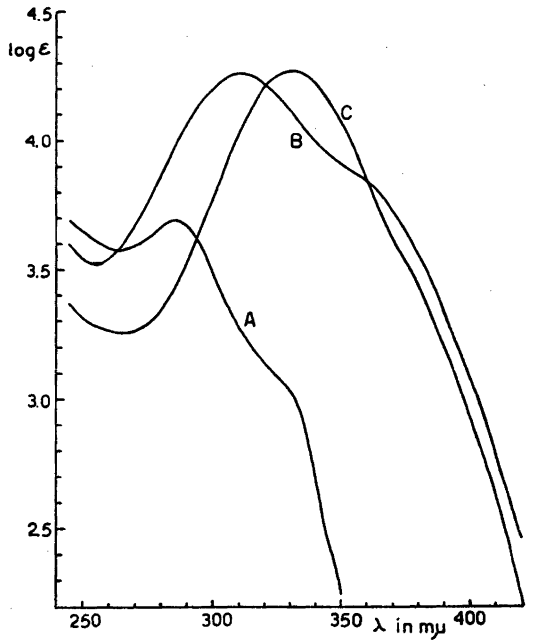


Fig. 1. Light absorption curves of A: α,β-Dibromohydroferulic acid methyl ester (VIII), B: Quinone methide X (based on the concentration of VIII), C: Quinone methide XII, in chloroform solutions.

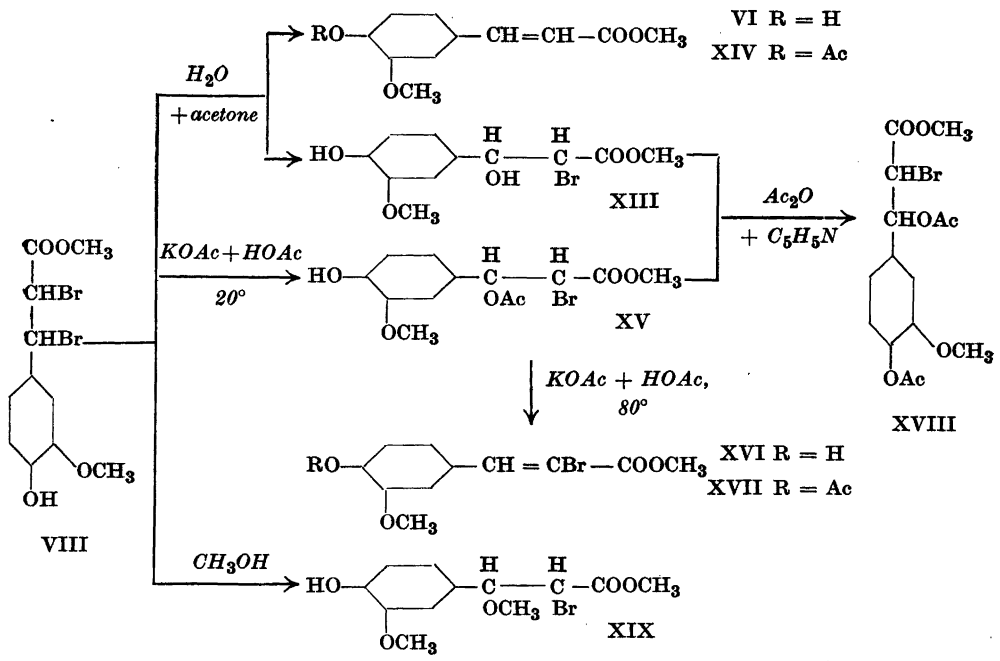
VIII are rather stable in the organic solvents mentioned above, but attempts to isolate the presumed quinone methides (IX, X) led only to amorphous, probably polymerized products.

Fig. 1 shows the absorption spectra of the dibromide VIII and of the yellow chloroform solution, presumably containing X, obtained from it on treatment with aqueous sodium bicarbonate. For comparison, the spectrum of the crystalline quinone methide XII has been included.

Further reactions of the dibromide VIII, characteristic of *p*-hydroxybenzyl halides, are presented in Scheme 1.

Treatment of VIII with aqueous acetone at room temperature produced the carbinol XIII. The yield of this substance, however, did not exceed 15 per cent, the bulk of the reaction product being an oil, from which after acetylation methyl acetylferulate (XIV) was isolated. The main reaction, therefore, consisted in debromination to methyl ferulate (VI), which is in agreement with previous observations made in similar cases (*cf.* [1,5]).

Scheme 1.



Attempts to replace the bromine atom in XIII by acetoxyl were unsuccessful. Treatment with potassium acetate (acetic acid solution, 2 h, 100°) yielded unchanged material, whereas only ill-defined oily products were obtained under more drastic conditions.

As could be expected, the bromine atom in the "benzyl" position in VIII could be replaced by acetoxyl under very mild conditions. The action of potassium acetate in cold acetic acid solution was sufficient to produce the acetate XV, although the yield of this substance was only slightly better than that of the carbinol XIII. Treatment of XV with potassium acetate in acetic acid at 80° did not result in the desired exchange of the second bromine atom, but yielded the unsaturated bromide XVI, which was purified as acetate (XVII). The structure of XVII is also supported by the fact that its ultraviolet absorption spectrum is very similar to that of methyl acetylferulate (XIV) (Fig. 2).

On acetylation (acetic anhydride-pyridine) the carbinol XIII and the mono-acetate XV yielded the same diacetate (XVIII), which can be further converted into the triacetate III [3]. Due to the low yields of XIII and XV, the over-all yield of XVIII, based on the dibromide VIII, was, however,

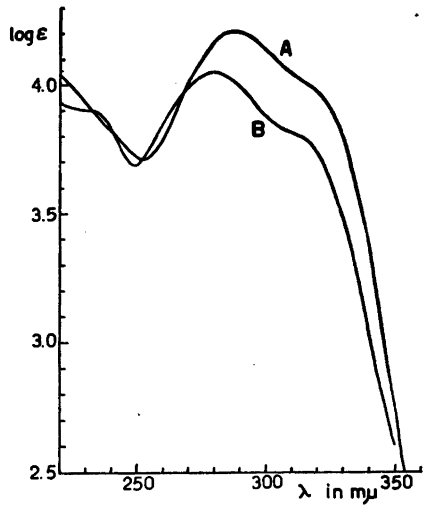


Fig. 2. Ultraviolet absorption curves of A: α-Bromo-acetylferulic acid methyl ester (XVII), B: Acetylferulic acid methyl ester (XIV), in 96 per cent ethanol.

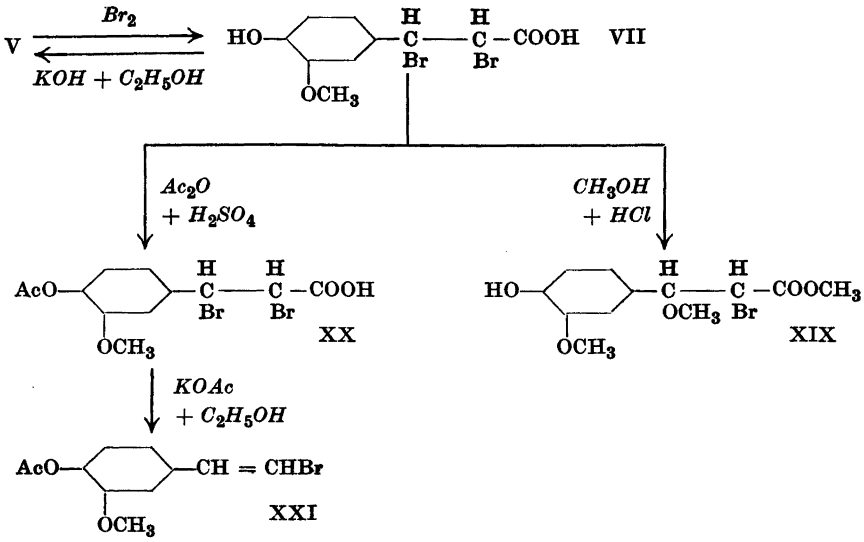
not satisfactory, and a better procedure for the preparation of XVIII (and III) will be given in the following communication [3].

In a "normal" hydroxybenzyl halide reaction, the dibromide VIII, when dissolved in cold methanol, was converted in good yield into the benzyl methyl-ether XIX.

The dibromide of the free ferulic acid (VII) could be expected to be less favourable than VIII as a starting material for the production of suitably hydroxylated or acetoxylated hydroferulic acids. It was already known that similar brominated *p*-hydroxy- or *p*-alkoxy cinnamic acids undergo complete debromination or lose hydrogen bromide and carbon dioxide yielding the corresponding ω-bromo-vinyl compounds when treated with aqueous acetone or with potassium acetate (*cf.*[1]). These reactions were therefore not investigated.

The action of ethanolic potassium hydroxide on the dibromide VII resulted in debromination, ferulic acid (V) being the only reaction product which could be isolated. An attempt was then made to stabilize the substituents of the side-chain by acetylating the phenolic hydroxyl group in VII. Due to the high reactivity of the bromine atom in "benzyl" position, pyridine could not be used as an acetylation catalyst; the acetate XX was, however, readily formed with acetic anhydride and very little concentrated sulphuric acid at room temperature. When treated with potassium acetate (in ethanol) the acetate

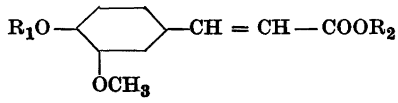
Scheme 2.



XX, however, lost hydrogen bromide and carbon dioxide and yielded the bromostyrene XXI (Scheme 2).

The normal formation of the methyl ether XIX (*cf.* also Scheme 1) by the action of methanol and dry hydrogen chloride on VII may also be noted.

As shown in Scheme 2, ferulic acid (V) was first brominated and the dibromide (VII) obtained was then acetylated. It may be of interest to mention that this sequence of reactions could not be reversed. Surprisingly enough it was found that acetylferulic acid (XXII), in chloroform or acetic acid solutions, does not consume bromine. Similarly, it proved impossible to brominate the methyl ester of acetylferulic acid (XIV), whereas methyl ferulate (VI) is readily brominated.



XXII R_1 = Ac, R_2 = H
XIV R_1 = Ac, R_2 = CH_3

XXIII R_1 = CH_3, R_2 = H
XXIV R_1 = CH_3, R_2 = CH_3

Contrary to acetylation, methylation of the phenolic hydroxyl group in ferulic acid or methyl ferulate, however, does not affect the reactivity of the double bond, both ferulic acid methyl ether (XXIII)[6] and the corresponding

ester (XXIV)[1] being smoothly brominated. Isoeugenol acetate also adds bromine very readily, and hence, in XXII and XIV, the inertness of the double bond towards bromine seems to be the result of the combined influences of the *p*-acetoxyl and the carboxyl (carbomethoxyl) groups.

EXPERIMENTAL

α,β-Dibromo-hydroferulic acid (VII). A solution of 32 g of bromine (0.2 mole) in 250 ml acetic acid was added dropwise to a solution of 38.8 g of ferulic acid (V) (0.2 mole) in 250 ml of the same solvent. The temperature was kept below 10°. The colourless crystalline substance which precipitated in nearly quantitative yield was filtered off and dried in a desiccator over potassium hydroxide. After recrystallization from ethylacetate-hexane it formed needles, m.p. 158—159° (decomposition). (Found: C 34.0; H 2.82; Br 44.6. Calc. for $C_{10}H_{10}O_4Br_2$ (354.0): C 33.9; H 2.85; Br 45.1.)

α,β-Dibromo-hydroferulic acid methyl ester (VIII). Bromine (77 g) was added during five minutes to an ice-cooled, slowly stirred solution of methyl ferulate (VI) (100 g) (prepared from ferulic acid [7] with methanol-hydrogen chloride, m.p. 63—64° [8]) in chloroform (250 ml). After the bromine was added, the dibromide crystallized out, was filtered off and washed with a chloroform-hexane mixture. Recrystallization from ethylacetate-hexane and benzene-hexane yielded needles of m.p. 133—134° (decomposition). Yield 76 %. (Found: C 35.6; H 3.23; Br 43.2; OCH_3 16.8. Calc. for $C_{11}H_{12}O_4Br_2$ (368.1): C 35.9; H 3.29; Br 43.4; OCH_3 16.9.)

α-Bromo-β-hydroxyhydroferulic acid methyl ester (XIII). Water (30 ml) was added to a solution of α,β-dibromohydroferulic acid methyl ester (36.8 g) in acetone or dioxan (200 ml). The solution acquired a yellow colour (quinone methide?), which disappeared within a few minutes. After 3 hours the solution was evaporated under reduced pressure. The oily residue was dissolved in chloroform and the solution was dried (Na_2SO_4) and concentrated under reduced pressure. The crystalline material obtained on addition of hexane was recrystallized from chloroform and from benzene. Prisms, m.p. 118—119°; yield about 15 %. (Found: C 43.3; H 4.38; Br 25.6; OCH_3 20.1. Calc. for $C_{11}H_{13}O_5Br$ (305.1): C 43.3; H 4.29; Br 26.2; OCH_3 20.3.)

Acetylation with acetic anhydride in pyridine at room temperature yielded α-bromo-β-acetoxy-acetylhydroferulic acid methyl ester (XVIII), m.p. 100—101°, no depression with a sample obtained according to the procedure given in the following paper [3].

Methyl acetylferulate (XIV). The oily residue obtained from the mother liquor of XIII, when treated with acetic anhydride and pyridine at room temperature, yielded a considerable quantity of acetylferulic acid methyl ester (XIV), m.p. 122—123°, undepressed on admixture of a sample of the substance prepared by acetylation of methylferulate. (According to Pacsu and Stieber [9], who prepared substance XIV by methylation of acetylferulic acid with diazomethane, the m.p. is 124°.) (Found: C 62.6 H 5.73; OCH_3 25.0. Calc. for $C_{13}H_{14}O_5$ (250.2): C 62.4; H 5.64; OCH_3 24.8.)

α-Bromo-β-acetoxyhydroferulic acid methyl ester (XV). Anhydrous potassium acetate (10 g = 0.1 mole) dissolved in acetic acid (150 ml) was added to a solution of the dibromide VIII (36.8 g = 0.1 mole) in acetic acid (150 ml). After 24 hours at room temperature, potassium bromide was filtered off, and the solution was evaporated under reduced pressure. The oily residue was dissolved in benzene, and the benzene solution was washed with water, dried (Na_2SO_4) and diluted with hexane. The semi-crystalline

material which precipitated was recrystallized from benzene-hexane and from benzene yielding prisms, m.p. 113—114°. Yield 15—20 %. (Found: C 45.0; H 4.29; Br 22.7; OCH_3 17.9. Calc. for $C_{13}H_{15}O_6Br$ (347.2): C 45.0; H 4.36; Br 23.0; OCH_3 17.9.)

Acetylation with acetic anhydride in pyridine solution at room temperature yielded XVIII, m.p. 100°, no depression with samples obtained from XIII or according to the procedure described in the following paper [3].

α-Bromo-acetylferulic acid methyl ester (XVII). A solution of 6.0 g of XV and 1.75 g of potassium acetate in 100 ml of acetic acid was heated at 80° for 10 hours, then concentrated under reduced pressure and diluted with water. The oil which separated was extracted with benzene, and the benzene solution was washed with water, dried over anhydrous sodium sulphate, and concentrated. On the addition of hexane a crystalline substance of m.p. 72—79° (impure substance XVI) was obtained. Acetylation with acetic anhydride and pyridine at room temperature yielded thin plates (recrystallized from 80 per cent methanol and abs. ethanol), m.p. 96—97° (XVII). (Found: C 47.6; H 3.87; Br 24.4; OCH_3 18.9. Calc. for $C_{13}H_{13}O_5Br$ (329.2): C 47.4; H 3.98; Br 24.3; OCH_3 18.9.)

α-Bromo-β-methoxy-hydroferulic acid methyl ester (XIX). 1. A solution of 15 g α,β-dibromohydroferulic acid methyl ester (VIII) in 100 ml of methanol was set aside overnight at room temperature, then neutralized with excess calcium carbonate, filtered, diluted with water and extracted with chloroform. The chloroform solution was dried over sodium sulphate and concentrated to about 25 ml. On addition of hexane the methyl ether separated, and after recrystallization from methanol-water formed needles, m.p. 97—98°. (Found: OCH_3 28.6; Br 25.0. Calc. for $C_{12}H_{15}O_5Br$ (319.2): OCH_3 29.2; Br 25.0.)

2. The same substance was obtained when a methanol solution of α,β-dibromo-hydroferulic acid (VII) was set aside overnight at room temperature and dry hydrogen chloride was subsequently passed into the solution for one hour (*cf.* Scheme 2). The substance decomposes on storage.

α,β-Dibromo-acetylhydroferulic acid (XX). α,β-Dibromo-hydroferulic acid (VII) (50 g) was dissolved by gentle warming in a mixture of 300 ml of acetic acid and 300 ml of acetic anhydride. After cooling 3 ml of conc. sulphuric acid was added dropwise. After two days standing at room temperature the mixture was poured onto ice. The resulting solid was recrystallized from benzene, forming prismatic plates, m.p. 166—167°. (Found: C 36.9; H 3.18; OCH_3 7.88. Calc. for $C_{12}H_{12}O_5Br_2$ (396.1): C 36.4; H 3.06; OCH_3 7.84.)

β-Bromo-3-methoxy-4-acetoxy-styrene (XXI). A solution of 17 g of α,β-dibromo-acetylhydroferulic acid (XX) in 400 ml of ethanol was refluxed with 12 g of potassium acetate for 8 hours. Potassium bromide was filtered off, the solution was evaporated and the residual oil was dissolved in chloroform. The chloroform solution was extracted with sodium bicarbonate solution, washed with water, dried over anhydrous sodium sulphate, and evaporated. After several days the oily residue had partly crystallized. Recrystallization from methanol yielded prismatic plates, m.p. 109—110°. (Found: C 48.7; H 4.07; OCH_3 11.8. Calc. for $C_{11}H_{11}O_3Br$ (271.1): C 48.7; H 4.09; OCH_3 11.5.)

SUMMARY

α,β-Dibromo-hydroferulic acid methyl ester has been prepared and converted into the corresponding β-hydroxy and β-acetoxy derivatives. Attempts

to replace the α-bromine atom in these derivatives by acetoxyl were unsuccessful. On acetylation both derivatives yielded α-bromo-β-acetoxy-acetylhydroferulic acid, in which the bromine is easily replaced by acetoxyl.

Similar attempts to obtain hydroxy and acetoxy derivatives of hydroferulic acid and its acetate are described.

Whereas ferulic acid and methyl ferulate are readily brominated in the side-chain, the corresponding acetyl compounds proved to be inert towards bromine.

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Synthesis and Reactions of α-(3-Methoxy-4-hydroxyphenyl)-glycerol ("Guaiacylglycerol"). II. Synthesis

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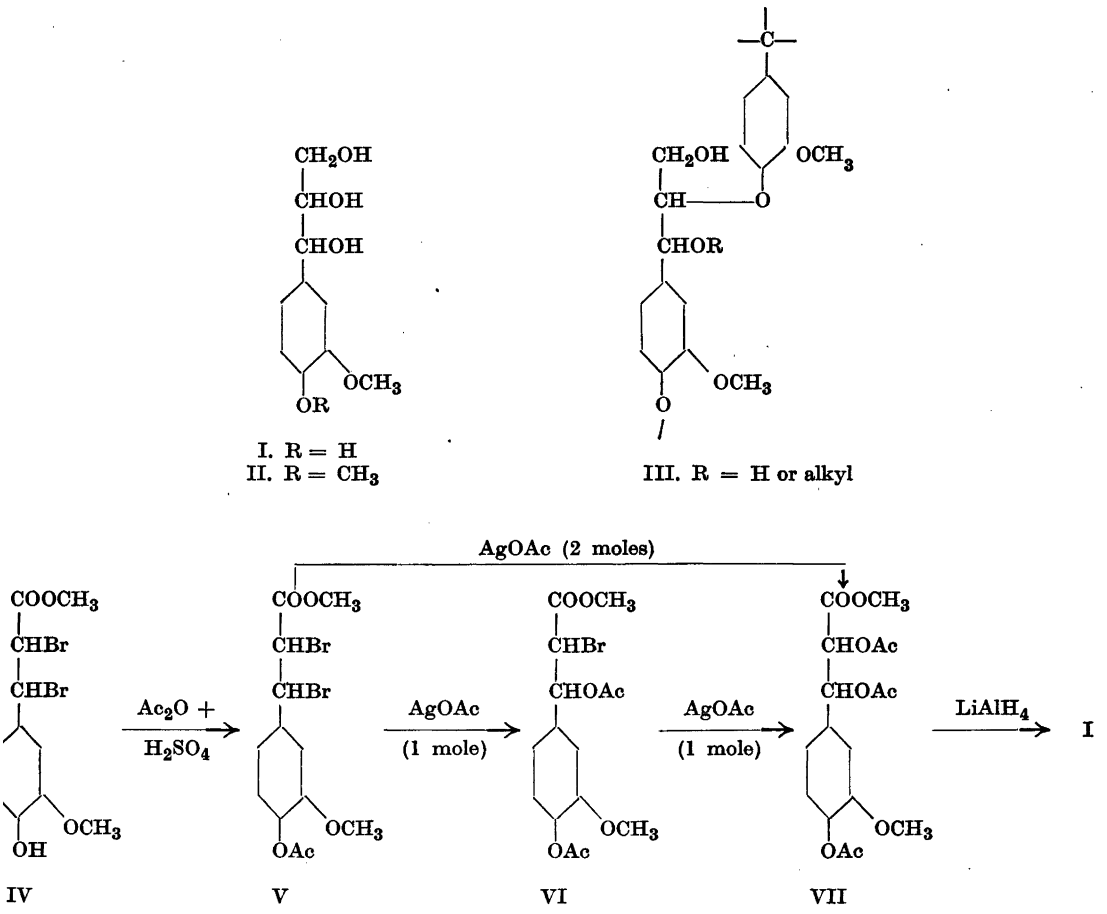
Guaiacylglycerol (I) has been considered to represent the basic structure of at least some of the building stones of lignin [1,2,3]. In lignin, guaiacylglycerol monomers may be assumed to be linked to adjacent monomers by aryl ether linkages in the β-(or γ-)position, and in addition, the hydroxyl group in the α-position may be etherified with a similar benzyl alcohol group or another aliphatic carbinol group belonging to another lignin unit. A possible structure of this kind is presented in the schematic formula III [4,5,6].

The synthesis of "veratrylglycerol" (II) [3] and its behaviour in some reactions related to lignin chemistry [7] have been reported earlier, and in the preceding paper [8], some preliminary experiments concerning the preparation of guaiacylglycerol have been described. The present communication deals with the synthesis of this substance.

This synthesis followed a route (Scheme 1) very similar to that used in the preparation of veratrylglycerol (II) [3].

α,β-Dibromohydroferulic acid methyl ester (IV), prepared according to the directions given in the preceding paper [8], was acetylated, and the bromine atoms in V were replaced by acetoxyl groups, either in a two-stage reaction (*via* VI), or directly, yielding the triacetate VII.

The reduction of the triacetate VII with lithium aluminium hydride yielded a water-soluble, colourless syrup which could not be distilled without decomposition. The identification of this product as guaiacylglycerol (I) was carried out as follows:



Scheme 1.

1. *General properties.* The ultraviolet absorption curve of the product (Fig. 1) is similar to that of phenols with no double bond in conjugation to the aromatic nucleus. The aqueous solution of the product is neutral, and treatment with alkali does not liberate any acidic groups. Hence, all three acetoxyl groups and the carbomethoxyl group in VII must have reacted with the lithium aluminium hydride.

In the absence of solvents, or in neutral solution, the product appears to be rather stable. On treatment with mineral acids, however, carbonyl compounds which yield orange-coloured precipitates with 2,4-dinitrophenylhydrazine, are formed. In this respect, the product behaves like veratrylglycerol

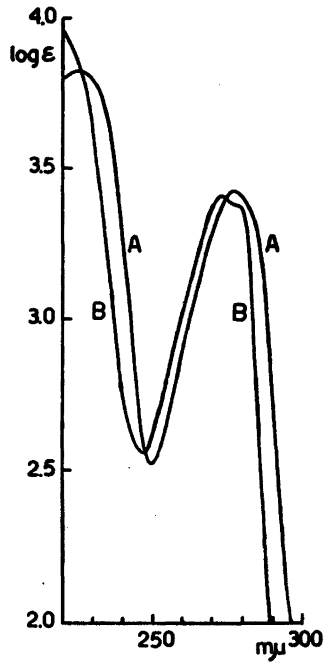


Fig. 1. Ultraviolet absorption of

A: Guaiacylglycerol (I) in water, based on methoxyl concentration,

B: Guaiacylglycerol tetraacetates (VIII, 1 and 2) in 96 per cent ethanol.

(II) [7]. Contrary to veratrylglycerol, however, it is also unstable in alkaline solution. (The mechanism of the alkaline breakdown is being further investigated.)

2. *Tetraacetates.* On treatment with acetic anhydride in pyridine, the syrupy product (I) yielded two crystalline tetraacetates, m.p. 84—85° (VIII, 1) and m.p. 113—114° (VIII, 2) respectively, of which the first-mentioned represented the major product (*cf.* scheme 2). Both tetraacetates distilled without decomposition and the distillates crystallized readily on treatment with suitable solvents such as ether. The ultraviolet absorption spectra of both tetraacetates proved to be identical (Fig. 1).

These results indicate that the syrupy guaiacylglycerol (I) is a mixture of the two possible D,L-forms, one of them being present in excess.

3. *Periodate oxidation.* Treatment of guaiacylglycerol with periodate should result in the following breakdown of the side-chain:

guaiacylglycerol $\xrightarrow{+\ 2\ HIO_4}$ formaldehyde + formic acid + vanillin.

However, this reaction may be complicated by the fact that, according to Pennington and Ritter [9], certain phenolic nuclei are also attacked by periodate. These authors have reported that, during a reaction period of two hours, about 3 moles of periodic acid are consumed by one mole of guaiacol, vanillylalcohol,

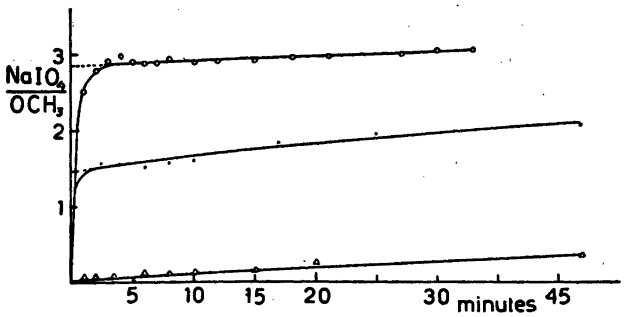


Fig. 2. Consumption of periodate by
O—O—O *guaiacylglycerol (I)*
●—●—● *guaiacol*
△—△—△ *vanillin*

and other guaiacyl derivatives, respectively. Vanillin, however, was practically stable under the same conditions. Therefore, provided that the nuclear oxidation proceeds sufficiently fast compared with the oxidation of the side-chain, it will interfere with the reaction scheme presented above. The periodate consumption will become higher than 2 moles, and the yield of vanillin less than 1 mole per mole of guaiacylglycerol. Due to the fact that vanillin is practically stable towards periodate, the yield of vanillin will essentially depend on the ratio of the rate of the α,β-cleavage in the side-chain to the rate of the nuclear oxidation.

Veratrylglycerol (II, m.p. 109—110°) [3] reacted with periodate in a normal way, consuming 2 moles of the oxidant and yielding 1 mole of formaldehyde, formic acid, and veratric aldehyde, respectively, the veratryl nucleus being stable towards periodate [3]. At a temperature of 12°, the oxidation was complete after two minutes. It may be expected that the glycerol side-chain in guaiacylglycerol (I) is split at a similar rate.

In order to obtain some preliminary information about the rate of the nuclear oxidation, the consumption of sodium periodate by guaiacol at 12° was examined. As shown in Fig. 2, about 1.5 moles of periodate per mole of guaiacol were consumed during the first two minutes, this rapid phase being followed by a comparatively slow further oxidation. During the rapid phase the solution acquired a red-brown colour (*cf.* also [9]).

The rate of the initial phase of this nuclear oxidation appears to be of the same order as the rate of cleavage of the side-chain in veratrylglycerol (II). Hence, in the oxidation of guaiacylglycerol, the breakdown of the phenolic nucleus could be expected to interfere to a considerable extent with the forma-

tion of vanillin. In fact, for each mole of methoxyl contained in the syrupy product (I) approximately 2.9 moles of periodate — instead of 2.0 moles required by the equation above — were rapidly consumed (Fig. 2), the solution turning reddish brown. Vanillin could be isolated as 2,4-dinitrophenyl-hydrazone, but the yield (after an oxidation time of ten minutes) was only 0.54 mole (calc. 1.0 mole) per mole of guaiacylglycerol.

In agreement with the equation above, nearly one mole of formaldehyde and one mole of formic acid per methoxyl were detected in the reaction mixtures obtained after 10 minutes' treatment of the syrupy product (I) with excess periodate.

Neither formaldehyde nor formic acid were obtained, however, on periodate oxidation of guaiacol, thus indicating that the formation of these products from guaiacylglycerol is solely due to the cleavage of the glycerol side-chain.

4. *Diazomethane methylation and subsequent acetylation* (Scheme 2). Provided that the syrupy product has structure I, methylation with diazomethane should yield veratrylglycerol (II). A veratrylglycerol, m.p. 109—110°, had been obtained [3] by the action of lithium aluminium hydride upon the triacetate IX, but diazomethane methylation of I, however, yielded a non-crystallizing syrup, which, in accord with the solid veratrylglycerol (II), could not be distilled without decomposition. When the solid veratrylglycerol (II) was treated with diazomethane, it was recovered unchanged, which indicated that, in the methylation of I, no disturbing reactions in the side-chain were to be expected.

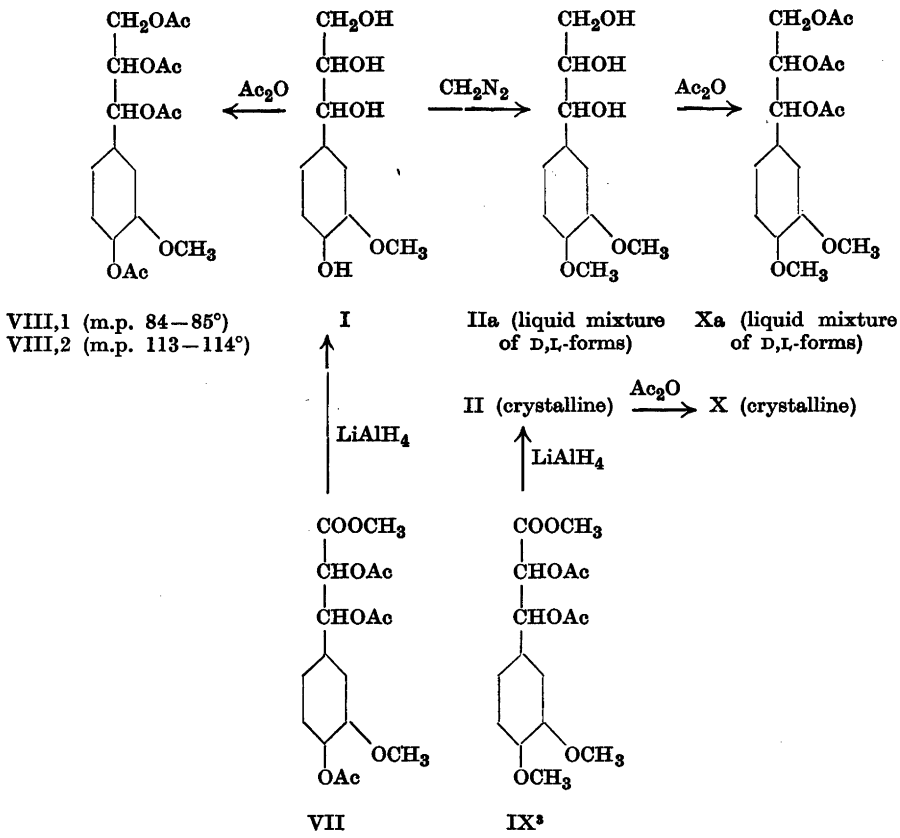
On periodate oxidation, the syrupy methylation product of I consumed 2 moles of periodate and yielded 1 mole each of formaldehyde and veratric aldehyde, based on 2 methoxyl equivalents, which is in harmony with a veratrylglycerol structure (II a).

Treatment of the methylation product (IIa) with acetic anhydride in pyridine yielded a syrup, from which no crystals could be obtained. It could be purified by distillation at 0.05 mm Hg (bath temperature 170°) and then had the analytical composition of the expected veratrylglycerol triacetate (Xa, *cf.* Scheme 2).

When this product was saponified with cold alcoholic potassium hydroxide, the resulting solution, after dilution with water, and reneutralization, consumed 2 moles of periodate per 2 equivalents of methoxyl, which indicates that the glycerol side-chain had been retained during the acetylation and saponification.

The crystalline veratrylglycerol [3], m.p. 109—110° (II), yielded a crystalline triacetate, m.p. 68—69° (X). This substance distilled without decomposition at 0.05 mm Hg (bath temperature 170°), and the oily distillate crystallized

Scheme 2.



readily on treatment with suitable solvents such as ethyl ether. On saponification of the crystalline triacetate X, the crystalline veratrylglycerol II was regenerated.

The similarity of the ultraviolet absorption curves of the oily product Xa and the crystalline veratrylglycerol triacetate (X) (Fig. 3) supports the conception that both products have identical structures.

On the basis of these results and those reported in sections 1, 2, and 3 it can be concluded that the syrupy product (I) is identical with guaiacylglycerol. The non-crystallizability of the methyl ether IIa and its triacetate Xa may be due to the fact that these products are mixtures of the two possible D,L-forms, the presence of which in the guaiacylglycerol product is indicated by the isolation of the two isomeric tetraacetates (VIII, 1 and VIII, 2) (*cf.* p. 572). Which of these belongs to the same steric series as the crystalline veratrylglycerol

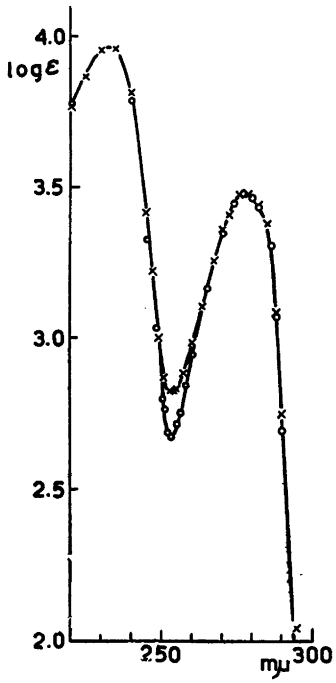


Fig. 3. Ultraviolet absorption of

O—O—O *veratrylglycerol triacetate, crystalline form (X), and*

×—×—× *veratrylglycerol triacetate, liquid mixture of* D,L*-forms (Xa) (in 96 per cent ethanol).*

II[3], previously obtained from IX, and its triacetate X, cannot be decided at the moment.

5. *Sulphonation.* Like veratrylglycerol [7], the syrupy product (I) was converted into a sulphonic acid when heated with a sodium bisulphite solution (pH 3). The barium salt of the sulphonic acid was obtained in a crystalline state, and had the analytical composition expected for the barium salt of a monosulphonic acid. When treated with sodium periodate, it yielded one mole of formaldehyde per mole. (The consumption of periodate exceeded 1 mole IO_4^- per mole of the sulphonic acid, and the reaction mixture turned rapidly red-brown, indicating oxidation of the phenolic nucleus, *cf.* p. 573).

These results establish the structure 1-(3-methoxy-4-hydroxyphenyl)-2,3-dihydroxy-*n*-propane-1-sulphonic acid and constitute additional evidence for the identity of the syrupy starting material with guaiacylglycerol (I).

Experiments on the rate and pH-dependence of the sulphonation as well as on the action of alcoholic hydrochloric acid upon guaiacylglycerol, and some other lignin model reactions of this substance will be reported later.

EXPERIMENTAL

α,β-Dibromo-acetylhydroferulic acid methyl ester (V). Dibromohydroferulic acid methyl ester (IV)[8] (25 g) was dissolved by gentle warming in a mixture of acetic acid (150 ml) and acetic anhydride (150 ml). After cooling 2 ml of conc. sulphuric acid were added. The mixture was set aside at room temperature for 24 hours and then poured into 2 l of ice-water. The resulting oil solidified on standing. Recrystallization from ethylacetate-hexane yielded prismatic plates of m.p. 146—147°. Yield 65 %. (Found: C 38.0, H 3.47, OCH_3 15.1; Calc. for $C_{13}H_{14}O_5Br_2$ (410.1): C 38.1, H 3.44, OCH_3 15.1.)

α-Bromo-β-acetoxy-acetylhydroferulic acid methyl ester (VI). When the dibromide V (1 mole) was heated for five hours at 100° in acetic acid solution with potassium acetate (1 mole) no reaction took place, the unchanged dibromide being recovered on dilution with water. Silver acetate however, reacted readily with the β-bromine atom:

A solution of 5.5 g of dibromide V (13.4 millimoles) in 50 ml of acetic acid was heated on a water-bath for 10 minutes with 2.35 g of silver acetate (13.5 millimoles). The reaction was completed by refluxing the mixture for 3 minutes. The silver bromide (yield almost quantitative) was filtered off and the solvent removed by evaporation in vacuum. The remaining syrup was dissolved in methanol and some colloidal silver bromide removed by filtration through a layer of kieselguhr. The filtrate was concentrated to a volume of 10 ml. On addition of a little water the diacetate VI precipitated. Needles, after recrystallization from 80 per cent methanol and from methanol, m.p. 100—101°; yield 70 %. (Found: C 45.8, H 4.37, Br 19.7, OCH_3 16.1; Calc. for $C_{15}H_{17}O_7Br$ (389.2): C 46.3, H 4.40, Br 20.5, OCH_3 16.0.)

α,β-Diacetoxy-acetylhydroferulic acid methyl ester (VII). — 1. A solution of 69 g α,β-dibromo-acetylhydroferulic acid methyl ester (V) (0.168 mole) in a mixture of 600 ml acetic acid and 500 ml acetic anhydride was refluxed (oil bath) under stirring for 45 minutes with 60 g of silver acetate (0.36 mole). After cooling the silver bromide was filtered off and washed with acetone. The washings were combined with the filtrate, and the solution concentrated in vacuum to about 300 ml. The acetic anhydride was decomposed by the addition of 400 ml of water and the solvent removed under reduced pressure. The remaining oil was dissolved in chloroform, and the chloroform solution was washed with sodium bicarbonate and water, dried (Na_2SO_4), and evaporated in vacuum. A syrup remained which was dissolved in 100 ml of ether. The crystalline material obtained on cooling was recrystallized from ethylacetate-hexane, forming prismatic plates, m.p. 109—110°; yield 70 %. (Found: C 55.7, H 5.60, OCH_3 16.7; Calc. for $C_{17}H_{20}O_9$ (368.3): C 55.4, H 5.47, OCH_3 16.9.)

2. In a similar way the triacetate VII was obtained when the diacetate VI was heated with 1 mole of silver acetate per mole in an acetic acid — acetic anhydride mixture.

α-(3-Methoxy-4-hydroxyphenyl)-glycerol ("Guaiacylglycerol") (I). The centre neck of a three-necked flask was equipped with an extraction tube and a reflux condenser. In the extraction tube was placed a glass-crucible provided with a coarse sintered glass-disk. The crucible contained 4.0 g of α,β-diacetoxy-acetylhydroferulic acid methyl ester (VII). One of the side-necks was provided with a gas-inlet tube, and the other one with an outlet tube which passed to the bottom of the flask and was kept closed during the reduction. The flask contained lithium aluminium hydride (2.2 g) and dry ether (300 ml), and the mixture was heated on a water-bath in a nitrogen atmosphere until the triacetate (VII) had been completely dissolved by the refluxing ether (about 30 minutes).

After a further 30 minutes heating the reaction mixture was cooled and slowly pressed with nitrogen gas through the outlet tube into a flask containing 200 ml of ice-cooled and vigorously stirred 1 *N* sulphuric acid. The aqueous layer was filtered and passed through a column of a cation exchange resin in the H^+-state (Amberlite IR-120). The effluent was immediately neutralized with $BaCO_3$, filtered and concentrated under reduced pressure in an atmosphere of carbon dioxide. Some barium carbonate which precipitated during the evaporation was removed, and the clear solution further concentrated to a volume of about 10 ml. Residual water was removed by repeatedly adding ethanol and benzene and evaporating under reduced pressure. The remaining syrup was purified by dissolving in 100 ml of acetone, filtering off a small amount of insoluble material and evaporating in vacuum.

The product thus obtained was a clear, practically colourless, highly viscous syrup which was free of inorganic material. It was readily soluble in water, alcohols, acetone, acetic acid, ethyl acetate, and dioxan, and sparingly soluble in ethyl ether, chloroform, benzene and petroleum ether.

Guaiacylglycerol tetraacetates (VIII, 1 and 2). Guaiacylglycerol was dissolved in acetic anhydride-pyridine mixture. After 16 hours the mixture was poured into water. The resulting oil was dissolved in ether, and the ether solution was washed with 0.5 *N* sulphuric acid, aqueous bicarbonate, and water, dried (Na_2SO_4), and concentrated. Addition of a little hexane yielded six-edged prismatic plates, recrystallized from ethylacetate-hexane, m.p. 84—85°. (Found: C 56.2, H 5.72, OCH_3 8.20, CH_3CO 46.1; Calc. for $C_{18}H_{22}O_9$ (382.4): C 56.5, H 5.80, OCH_3 8.11, CH_3CO 45.0.)

The ether-hexane mother liquor was concentrated *in vacuo* leaving a yellow oil. This was distilled between 160—170° (bath temperature) (0.01 mm) yielding an almost colourless oil, which crystallized readily on treatment with ether. Regular six-edged plates, recrystallized from ethylacetate-hexane, m.p. 113—114°. (Found: C 56.4, H 5.66, OCH_3 8.03, CH_3CO 42.6; Calc. for $C_{18}H_{22}O_9$ (382.4): C 56.5, H 5.80, OCH_3 8.11, CH_3CO 45.0.)

Oxidation of guaiacylglycerol with periodate. a) *Periodate consumption.* An aqueous solution (100 ml) containing guaiacylglycerol (with 42.2 mg OCH_3 = 1.36 millimoles) and sodium periodate (7.0 millimoles) was kept in a thermostat at + 12°. The consumption of periodate was determined by adding samples of 5 ml of the solution to 10 ml of a solution containing sodium arsenite (0.04 mole per litre), potassium iodide (2.5 per cent), and sodium bicarbonate (1 per cent) and titrating the excess arsenite with 0.05 *N* iodine. The results obtained are presented in Fig. 2.

In a similar way the periodate consumption of veratrylglycerol (II, m.p. 109—110°)[3], guaiacol, and vanillin was determined (*cf.* Fig. 2).

b) *Formation of formaldehyde.* One ml of 0.7 M sodium periodate was added to 10 ml of an aqueous solution of guaiacylglycerol containing 0.136 millimole of methoxyl. After 10 minutes at room temperature excess periodate and the iodate formed were precipitated by the addition of 2 ml of a 20 per cent lead nitrate solution [10]. The precipitate was filtered off and washed with water, and the combined aqueous solutions (about 50 ml) were distilled in an atmosphere of nitrogen, water being added to keep the volume constant.

Dimedone (0.5 g) dissolved in 2 *N* NaOH (2 ml), followed by 2 *N* acetic acid (4 ml), was added to the distillate (200 ml). The mixture was set aside overnight in the refrigerator and the precipitate was collected. Needles, m.p. 183—185°, no depression with an authentic sample of methylene-bis-dimedone. (Calc. for 1 mole HCHO per OCH_3, 39.8 mg; found 34.6 mg = 87 %.)

c) *Formation of formic acid.* Ten ml of an aqueous solution of guaiacylglycerol, containing 1.09 millimole of methoxyl, were mixed with 10 ml of an aqueous solution of 3.27 millimoles of sodium periodate. After 10 minutes at room temperature 0.1 ml of ethylene glycol (to remove the excess periodate) and after further 10 minutes 55 ml of 1 per cent lead chloride were added. The precipitate of lead iodate was filtered off and washed with water. The combined aqueous solutions were evaporated to dryness under reduced pressure (bath temperature 45°). The receiver flask containing 10 ml of 0.1646 *N* potassium hydroxide solution was cooled by running water during the distillation. The residue was then dissolved in 20 ml of distilled water and the solution evaporated under vacuum, this procedure being repeated once. The content of the receiver was titrated with 0.1010 *N* hydrochloric acid. 6.7 millilitres of this solution were required. (Calc. for 1 mole HCOOH per OCH_3, 1.09 millimole formic acid; found 0.97 millimole formic acid = 89 %.) When heated on a water-bath, the neutralized distillate reduced mercuric chloride to calomel.

d) *Formation of vanillin.* One ml of 0.7 *M* $NaIO_4$ was added to 10 ml of an aqueous guaiacylglycerol solution containing 0.136 millimole of methoxyl. After 10 minutes at room temperature 1 ml of 2 *N* H_2SO_4 was added followed by some solid potassium iodide. A concentrated solution of sodium arsenite was then added drop by drop until the iodine colour disappeared. Finally, the solution was extracted six times with benzene, and the combined benzene extracts were shaken with 50 ml of a 0.3 per cent solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl. In order to separate the vanillin-2,4-dinitrophenylhydrazone present in the benzene solution from unreacted dinitrophenylhydrazine and the dinitrophenylhydrazones of benzene-soluble carbonyl compounds which may have been formed by the breakdown of the guaiacyl nucleus, the benzene solution was treated as follows:

After washing with water and drying (Na_2SO_4) the benzene solution was concentrated to a volume of 30 ml and passed through a column (44 × 1.3 cm) of aluminium oxide (Brockmann). From the chromatogram unreacted 2,4-dinitrophenylhydrazine and the unidentified hydrazones mentioned above were eluted with a mixture of benzene and ethanol (99 : 1). The adsorbent was then extracted in a Soxhlet apparatus with ethanol-benzene (1 : 1). The red solid remaining after evaporation of the extract had the m.p. 244—246° (after recrystallization from pyridine-ethanol, m.p. 258—260°), undepressed on admixture with vanillin-2,4-dinitrophenylhydrazone. (Vanillin-2,4-dinitrophenylhydrazone: Calc. for 1 mole of vanillin per mole of methoxyl, 45.1 mg; found 24.6 mg = 54 %.)

Veratrylglycerol [*IIa (liquid mixture of* D,L-*forms)*]. A distilled solution of diazomethane in ether (50 ml) was added to a solution of guaiacylglycerol (0.5 g) in dioxan (10 ml). An amorphous precipitate appeared and was partly redissolved by the addition of methanol (50 ml). After 16 hours the mixture was evaporated in vacuum. Attempts to convert the remaining oil into crystalline material by dissolving in a little chloroform and seeding with veratrylglycerol (II), m.p. 109—110°[3], were unsuccessful. The chloroform was evaporated and residual solvents removed by repeatedly adding water and distilling off under reduced pressure. Finally, the oil was dissolved in water to a total volume of 50 ml (solution A) and the methoxyl content of the solution was determined.

Five ml of solution A, containing 0.37 millimole OCH_3, were treated with excess sodium periodate; IO_4^- *consumption:* Calc. 0.37 millimole; found 0.33 millimole = 90 %.

Twenty ml of solution A, containing 1.47 millimole OCH_3, were oxidized with 0.5 g $NaIO_4$ (2 hrs., room temp.). The *formaldehyde* content of the mixture was determined (*cf.* p. 578). *Methylene-bis-dimedone:* Calc. for 1 mole HCHO per 2 OCH_3, 215 mg;

found 200 mg = 93 %. The methylene-bis-dimedone obtained had m.p. 183—185°, undepressed on admixture with an authentic sample.

Another sample of solution A was oxidized with the calculated amount of periodate, and then precipitated with 2,4-dinitrophenylhydrazine dissolved in 2 *N* HCl. The orange-red precipitate was dissolved in benzene and passed through a column of aluminium oxide (Brockmann). Elution first with benzene and then with 1 % ethanol in benzene yielded the 2,4-dinitrophenyl-hydrazones of formaldehyde, m.p. 162—164°, and of *veratric aldehyde* (84 % of the calculated amount), m.p. 258—260°, no depression with authentic samples of these substances.

Veratrylglycerol triacetate [*Xa (liquid mixture of* D,L*-forms)*]. Diazomethane was distilled from a dioxan solution into the solution of 1 g of guaiacylglycerol in a mixture of 50 ml of dioxan and 25 ml of methanol. After 2 days the solvents were removed under vacuum, and the remaining oil was acetylated with a mixture of 5 ml of pyridine and 5 ml of acetic anhydride. The oily acetate could not be converted into crystalline material. Two subsequent distillations (0.05 mm Hg, bath temp. 170°) yielded an almost colourless syrup. No crystallization occured on treatment with solvents and seeding with solid veratrylglycerol triacetate (X), m.p. 68—69° (see below). (Found: C 57.2, H 6.25, OCH_3 17.7, CH_3CO 35.2; Calc. for $C_{17}H_{22}O_8$ (354.4): C 57.6, H 6.26, OCH_3 17.5, CH_3CO 36.9.)

Veratrylglycerol triacetate [*X (crystalline)*]. Veratrylglycerol (II), m.p. 109—110°[3], was acetylated with acetic anhydride in pyridine solution. The acetate crystallized readily from ether-hexane and when recrystallized from ethylacetate-hexane formed six-edged plates, m.p. 68—69°. (Found: C 57.5, H 6.21, OCH_3 17.6, CH_3CO 36.6; Calc. for $C_{17}H_{22}O_8$ (354.4): C 57.6, H 6.26, OCH_3 17.5, CH_3CO 36.9.)

1-(3-Methoxy-4-hydroxyphenyl)-2,3-dihydroxy-n-propane-1-sulphonic acid, barium salt. A mixture of 30 ml of an aqueous solution of guaiacylglycerol, obtained by lithium aluminium hydride reduction of 4 g of *α,β*-diacetoxy-acetylhydroferulic acid methyl ester (VII) (*cf.* p. 577) and 30 ml of an aqueous $NaHSO_3$-SO_2 solution of pH 3 and a total SO_2 content of 10 %, was heated in a sealed tube for 3 hours at 135°. Na^+ was removed by filtration through a cation exchange resin (Amberlite IR-120), and SO_2 was removed by concentrating the filtrate (300 ml) under reduced pressure. The resulting solution (200 ml) was neutralized with barium carbonate, filtered, and concentrated in vacuum to a volume of about 5 ml and finally filtered through a layer of kieselguhr. Addition of ethanol, followed by ethyl ether, to the clear filtrate, produced an oily precipitate. After several hours the solvent was decanted, and the solid obtained on treating the oil with ethanol was collected and washed with ether. Yield, 2.8 g of crude barium sulphonate.

From a solution of the crude product in 5 ml of water, a mixture of two different types of crystals separated (prismatic plates and fine needles). The prismatic material, which constituted only a small part of the total product was less soluble than the other component and could be partially isolated by fractional crystallization from water. It proved to consist of barium thiosulphate. In order to remove residual thiosulphate from the remaining material, this was dissolved in water and the solution was acidified with sulphuric acid (final concentration about 1 *N* H_2SO_4), which decomposed the thiosulphate yielding $BaSO_4$, SO_2, and S. Sulphur dioxide was distilled off in vacuum, and the remaining solution was neutralized with $BaCO_3$, filtered, and further concentrated in vacuum to a volume of a few millilitres. After a final filtration which removed some residual inorganic material, some acetone was added, and the mixture was set aside in the refrigerator. The uniform, fine needles which separated were collected and washed with acetone

and ether. (Found: OCH_3 9.02, S 9.17, Ba 19.78; Calc. for $(C_{10}H_{13}O_7S)_2Ba$ (691.9): OCH_3 8.97, S 9.27, Ba 19.85.)

Periodate oxidation. An aqueous solution (5 ml) of the barium sulphonate (60 mg) was mixed with 10 ml of 0.1 *M* sodium periodate. After a reaction period of 10 minutes the formaldehyde present in the solution was determined as described above. (Methylene-bis-dimedone: Calc. 50.7 mg; found 43.5 mg = 86 %.) The dimedone compound had m.p. 184—186°, undepressed on admixture of authentic methylene-bis-dimedone.

SUMMARY

α-(3-Methoxy-4-hydroxyphenyl)-glycerol ("guaiacylglycerol") has been prepared by lithium aluminium hydride reduction of α,β-diacetoxy-acetyl-hydroferulic acid methyl ester. It was obtained as a non-crystallizing syrupy mixture of the two possible D,L-forms. Acetylation yielded the two DL-tetraacetyl derivatives in a crystalline state. Methylation with diazomethane gave "veratrylglycerol" which was converted into its triacetate. The latter products were not resolved into the corresponding D,L-forms.

The oxidation of guaiacylglycerol with periodate has been examined.

On heating guaiacylglycerol with aqueous bisulphite solution (pH 3) the hydroxyl group in the position *alpha* to the aromatic nucleus was replaced by SO_3H; a crystalline barium salt of the sulphonic acid was obtained.

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Dispersion Effects in White Light Interferometry

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A micromanometer, which has been used to measure very small osmotic pressures interferometrically, has been devised at this institute [1]. An interferometer of the Rayleigh type [2] has been constructed, designed in the ordinary way with two glassplates, which can be tilted, thus changing the optical path for either light beam. Differences in optical path in this apparatus arise from the light beams passing through liquid layers of different thickness, and these differences are compensated by glass in the original design of the apparatus. Furthermore, it is often convenient to use white light sources (see below). Two disadvantages arise from these facts:

1. The range of measurement is rather limited, as the glass plates for practical reasons cannot be made large enough to compensate for a level difference of more than about 1 mm.
2. The difference in the relative optical dispersions of glass and liquid gives rise to a systematic error which in many cases is considerable.

In view of these drawbacks a new type of compensating arrangement, a liquid compensator, was provided, making it possible to measure osmotic pressures up to 25 mm H_2O and to compensate for the greater part of the differences in optical path with liquid.* In this way the range of measurement has been extended and the dispersion effect almost eliminated at the same time. A certain compensation by means of the glass plates is convenient, however. This paper gives an account of the theoretical background of this dispersion effect, as well as of some experiments carried out in order to obtain the corrections to be made when white light is used.

* Compensation by means of liquid for the determination of refractive index differences between solvent and solution has earlier been described by Borgesius, A. H. *Ann. Physik (Wiedemann)* **54** (1895) 222.

Suppose the two interferring light beams pass through two liquid layers, for which the difference of level is l. The difference in optical path due to l is compensated by tilting the glass plate in one of the beams; let p be the change in optical path due to this movement and N_λ the corresponding number of fringes of wave-length λ. The refractive indices of liquid and glass for wave-length λ are denoted by n^l and n^g respectively. For monochromatic light of wave-length λ the following relation holds:

$$l(n^l-1) = p = N_\lambda\lambda \tag{1}$$

The number of fringes N_λ being known, l can be computed for any liquid with known n^l *. This is the basic idea behind the determination of osmotic pressure by this method. N_λ can be determined using a monochromatic light source. It will be realized, however, that when a monochromatic source is used it is impossible to differentiate between one fringe and another, so a white light source is very often to be preferred since it is then unnecessary to follow the movement of the fringes. But the difference in the relative dispersions of glass and liquid gives rise to a shift $(N'_\lambda-N_\lambda)$ of the achromatic fringe, a systematic error mentioned above. This effect has been theoretically studied by Adams [3], who has also given practical applications of the theory [4]. Assuming the following approximation of the Cauchy dispersion formula

$$n = A + \frac{B}{\lambda^2} \tag{2}$$

and using the condition that at the centre of the achromatic fringe the change of phase with respect to wave-length λ shall be a minimum, he derives the following equation [(11) in Adams's paper [3]] for this shift $(N'_\lambda-N_\lambda)$:

$$N'_\lambda-N_\lambda = \frac{2}{\lambda^3}(B_1-B_2)\,l + \frac{\mathrm{d}p}{\mathrm{d}\lambda} \tag{3}$$

Applied in the present case, where $N'_\lambda = N_s$, $B_1 = B^l$, $B_2 = 0$ and $p = p_s$, the index s referring to white light, (3) gives

$$N_s-N_\lambda = N_t = \frac{2}{\lambda^3}\left[B^l l - B^g\frac{\mathrm{d}p_s}{\mathrm{d}n}\right] \tag{4}$$

This means that the monochromatic fringe is displaced fewer wave-lengths than the achromatic one, *i.e.* (1) becomes

$$l(n^l-1) = (N_s-N_t)\lambda \tag{5}$$

* or n^l for a known l.

The value of l obtained from the uncorrected equation is called l_s, and is defined by

$$l_s(n^l-1) = p_s = N_s\lambda \tag{6}$$

Combining (5) and (6), we get

$$\frac{l}{l_s} = 1-\frac{N_t}{N_s} = 1-\frac{2}{\lambda^3}\left[B^l\frac{l}{N_s}-B^g\frac{1}{N_s}\frac{\mathrm{d}p_s}{\mathrm{d}n}\right] \tag{7}$$

or

$$\frac{l}{l_s} = 1-\frac{2}{\lambda^2}\left[B^l\frac{l}{p_s}-B^g\frac{1}{p_s}\frac{\mathrm{d}p_s}{\mathrm{d}n}\right] \tag{8}$$

Furthermore, (2) gives

$$B = \frac{n_F-n_C}{\frac{1}{\lambda_F^2}-\frac{1}{\lambda_C^2}} \tag{9}$$

Put

$$\frac{1}{p_s}\frac{\mathrm{d}p_s}{\mathrm{d}n} = P \tag{10}$$

Finally, for $\lambda = \lambda_D$,

$$\frac{l_s}{l} = K_1\frac{n_F^l-n_C^l}{n_D^l-1}+K_2 \tag{11}$$

where

$$K_1 = \frac{1}{\frac{\lambda_D^2}{2}\left[\frac{1}{\lambda_F^2}-\frac{1}{\lambda_C^2}\right]+P(n_F^g-n_C^g)} \tag{12}$$

$$K_2 = \frac{\lambda_D^2}{2}\left[\frac{1}{\lambda_F^2}-\frac{1}{\lambda_C^2}\right]K_1 \tag{13}$$

In the case where $\Delta n = n_1-n_2$, the change of th refractive index between a solution 1 and solvent 2, is measured in white light in a cuvette of length l, according to (3) we obtain

$$\frac{\Delta n_s}{\Delta n} = K_1\frac{\nu_1-\nu_2}{\Delta n}+K_2 \tag{14}$$

where ν_1 and ν_2 are the dispersions n_F-n_C for the solution and solvent respectively.

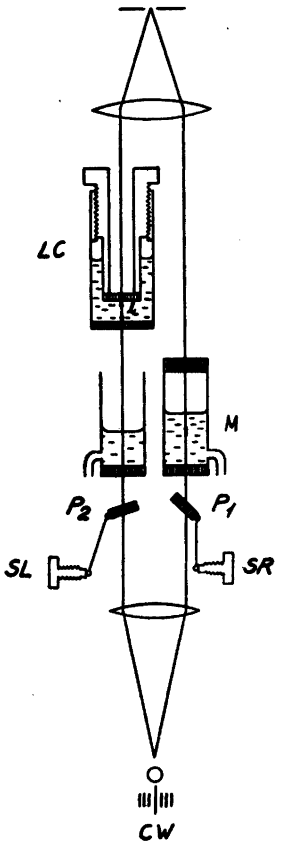

Fig. 1.

P is a function of the angle of incidence at the compensating glass plate and is easily expressed in terms of this angle. Using (3) and (8) in Adams's paper [3], we obtain

$$P(\Theta) = 2n^g \frac{\dfrac{1}{\sqrt{H + \sin 2\Theta}} - \dfrac{1}{\sqrt{H + \sin 2\Theta_0}}}{\sqrt{H + \sin 2\Theta} - \cos\Theta - \sin\Theta - \sqrt{H + \sin 2\Theta_0} + \cos\Theta_0 + \sin\Theta_0} \tag{15}$$

$(45° + \Theta)$ being the angle of incidence and $H = 2\,(n^g)^2 - 1$. The initial position of the plate, Θ_0, is in our case about 35°, whereas in Adams's case $\Theta_0 = 0$. For $5° < \Theta < 35°$, *i.e.* the interval in which Θ varies in our case, $P(\Theta)$ is a monotone function varying very slowly. As a matter of fact

$$1.12 < P(\Theta) < 1.24. \tag{16}$$

(For the glass plate used $n^g = 1.5163$ and $n^g_F - n^g_C = 0.0080$.) Consequently,

Table 1 (Cyclohexane). SR is calibrated in λ_D between the screw readings 25.00—0.00. 25.00 is fringe No. 0 — 0.00 is fringe No. 820.

Reading on *LC* mm	Reading on *SR* mm	Average value *SR*	Fringe No.	N_s No. of λ_D
0.0	1.78 .75 .75 .77	1.76	738.3	
0.5	11.11 .08 .11 .09	11.10	371.1	367.2
1.0	24.64 .67 .65 .66	24.65	7.0	364.1
1.0	1.44 .42 .42 .42	1.42	753.8	
1.5	10.66 .65 .63 .63	10.64	386.9	366.9
2.0	23.89 .91 .90 .89	23.90	22.3	364.6
2.0	1.93 .91 .92 .92	1.92	730.9	
2.5	11.31 .30 .28 .28	11.29	364.6	366.3
3.0	24.98 25.00 .00 24.99	24.99	0.2	364.6

3.0	1.45 .43 .46 .43	1.44	752.8	
3.5	10.65 .66 .64 .67	10.65	386.6	366.2
4.0	23.93 .94 .96 .93	23.94	21.5	365.1
4.0	1.88 .88 .88 .89	1.88	732.8	
4.5	11.25 .26 .24 .26	11.25	366.0	366.8
5.0	24.89 .89 .90 .90	24.90	2.0	364.0

Average value $N_s = 365.6 \pm 0.4$
$l_s = (0.510 \pm 0.001)$ mm.

it is obviously justifiable to consider K_1 and K_2 as approximately constant, and the theory thus predicts the correction factor $\frac{l_s}{l}$ to be a linear function of the relative optical dispersion $\frac{n_F^l - n_C^l}{n_D^l - 1}$ of the liquid according to (11).

A brief description of the experimental arrangement and the method of measuring will be given. It is intended that the details of the apparatus will be discussed in a later publication. Fig. 1 shows the principal features of the apparatus used. The optical path through the compensating plates of glass P_1 and P_2 can be varied by moving the micrometer screws SR and SL, the screw reading being calibrated in number N_s of fringes of wave-length λ_D, D referring to the D-line ($\lambda_D = 589.3$ mμ). SR covers 820 λ_D, corresponding to about 1 mm of most liquids. The liquid compensator LC is essentially a micrometer screw threaded in an outer tube filled with liquid. The pitch of the

thread is 0.5 mm and has been accurately determined (see below). *M* is a vessel, the two halves of which are in communication with the two halves of the osmotic cell. *M* is consequently the vessel where the osmotic pressure is measured. It can be used separate from the cell by replacing the connecting tubes from the cell by tightening arrangements, and as a matter of fact it has been used in this way during this investigation. The difference in liquid level has been changed in accordance with the position of the *LC* screw; it has proved to be advantageous to use *LC* in discrete steps, *e.g.* every turn of the screw *i.e.* every 0.5 mm. The bottoms of the screw and tube in *LC*, as well as *M*, consist of optically plain glass plates. *LC* contains the same liquid as *M*.

For the study of the dispersion effect the liquid layer of known thickness l to be compensated by glass was obtained by using *LC*; *M* has been used separately as mentioned before. The distance l, equivalent to the rise of the *LC* screw (Fig. 1), was measured interferometrically as follows. The central achromatic fringe (white light) was moved by *SR* (*SL* being fixed meanwhile) until it coincides with the cross-wire *CW* of the ocular. The optical path of the left-hand beam was increased by turning the *LC* screw (l mm rise of the screw). The achromatic fringe was again brought into coincidence by moving *SR*. The difference between the two readings on *SR* gives the change in optical path due to the movement of the *LC* screw. This determination gives N_s, and consequently l_s.

The optical path on the left was increased by turning the *LC* screw in 0.5 mm steps, cross-wire settings being made for every turn as described. As the range of *SR* corresponds to about 1 mm liquid in most cases (except for large n^l), measurements for two positions of *LC* could generally be performed without changing the liquid level in *M*. In this way l_s was determined for $l = 0.500 \pm 0.002$ *, the pitch of the thread being calibrated by means of a microcomparator. The *LC* screw is made with about the same precision as the one in the comparator used. The value of l_s (and N_s), as well as l, is the average of the first ten turns of the screw. l_s was determined for ten liquids, mainly common solvents selected to cover a wide range of dispersion. As both n^l_D and $(n^l_F - n^l_C)$ were measured (with a Zeiss Abbe-Refractometer Model G) for each liquid, no special efforts were made to purify the liquids. The purest available substances were of course used. All measurements were carried out at $25.00° \pm 0.01$.

To give an idea of the accuracy in the N_s determination, a series of measurements on one of the liquids (*cyclo*hexane) is given (Table 1). The accuracy in

* The deviation is in all cases computed as $\sqrt{\frac{\Sigma \Delta_i^2}{n(n-1)}}$.

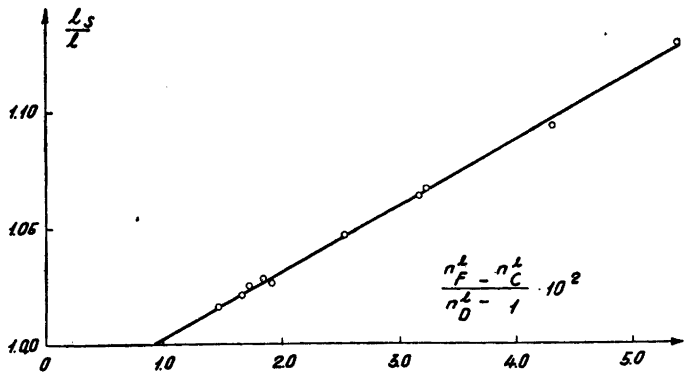

Fig. 2.

the determination is obviously about $\pm$ 0.001 mm. It should be observed from Table 1 that every second N_s value is smaller than the preceding one, due to the fact that P is not a real constant. The average is computed from all the determinations, however. Table 2 is a survey of the most pertinent experimental results, and Fig. 2 shows graphically $\frac{l_s}{l}$ as a function of the relative dispersion $\frac{n_F^l - n_C^l}{n_D^l - 1}$. Within the limits of experimental error, a straight line is obtained in agreement with the theoretical findings. The slope of the

Table 2.

Liquid	N_s	n_D^l	$n_F^l - n_C^l$	$\frac{n_F^l - n_C^l}{n_D^l - 1} \cdot 10^2$	$\frac{l_s}{l}$
Methanol	281.3	1.3262	0.00486	1.46	1.016
*Cyclo*hexane	365.6	1.4222	0.00703	1.66	1.021
Water	288.8	1.3321	0.00570	1.72	1.025
Carbon tetrachloride	398.4	1.4571	0.00842	1.84	1.028
Chloroform	382.0	1.4388	0.00840	1.91	1.026
Acetylene dichloride	392.7	1.4421	0.01117	2.53	1.047
Toluene	445.5	1.4933	0.01567	3.18	1.064
Benzene	450.8	1.4975	0.01609	3.23	1.067
Styrene	504.7	1.5442	0.02349	4.32	1.093
Carbon disulphide	597.3	1.6234	0.03369	5.39	1.130

line, K_1, and the intercept K_2 [(12) and (13)] have been computed by the method of least squares, giving

$$K_1 = 2.84 \pm 0.07 \text{ and } K_2 = 0.974 \pm 0.002$$

The corresponding values of P are

$$P = 2.5 \pm 1.1 \text{ and } P = 1.13 \pm 0.08$$

It is obvious that (12) does not give a reliable value for P. The uncertainty in the value from (13) is considerably less, and the value is furthermore in fair agreement with the theoretical value (16).

The correction to be made in the determination of l can be obtained from Fig. 2 for any liquid with known dispersion. These corrections are obviously in most cases very large, but as will be seen, can be determined very accurately. They are essential in many other applications of white light interferometry where large differences in optical path have to be compensated [*cf.* (14)]. Chromatography with interferometric recording is just one example that may be mentioned.

SUMMARY

A Rayleigh interferometer with a white light source has been used to measure the thickness of liquid layers. Differences in optical path due to the passage of one of the light beams through liquid has been compensated by glass. The effects arising from the difference in the relative optical dispersion of liquid and glass have been studied. The correction factors have been determined and plotted in a diagram against the relative optical dispersion of the liquid. This diagram gives the correction for any liquid with known dispersion. The theory developed on this subject has been verified within the limits of experimental error.

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Studies on the Chemistry of Lichens

IV.* Investigation of the Low-molecular Carbohydrate Constituents of Different Lichens

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Much work has been devoted to the study of the lichen acids, but the carbohydrate constituents of lichens, however, have been rather neglected. The occurrence of erythritol in *Rocella* species, of D-arabitol and D-mannitol in different lichens and of D-volemitol in *Dermatocarpon miniatum* is mentioned in Asahina's surveys [1]. Following the discovery of an arabitol galactoside, umbilicin, in *Umbilicaria pustulata* [2], we became interested in this field and in the present paper an investigation of the low molecular carbohydrate constituents of some 60 different lichens is reported.

Most lichens have been investigated by paper chromatography, by which method the sugar alcohols arabitol, mannitol and volemitol are easily detected. In several cases these substances were also isolated and characterized. Paper chromatography is not suitable for the detection of umbilicin, which gives rather weak spots with the silver nitrate-sodium ethoxide reagent [3], nor for the disaccharides α,α-trehalose and sucrose, previously found in Umbilicaria pustulata (Part III), as they generally occur in rather low concentrations and the usual reagents are not very sensitive to these sugars. Hydrolysis of umbilicin yields galactose, and the disaccharides yield glucose, both sugars being easily detected even in low concentrations, and by this indirect method the presence of these substances has been indicated in several lichens. This method is of course unsatisfactory, and for some lichens the carbohydrate components have been fractionated on a column of animal charcoal, as described in Part III. The results of the investigation are summarized in Tables 1 and 2.

* Part III. *Acta Chem. Scand.* **7** (1953) 140.

Table 1. Carbohydrate constituents of lichens, found by paper chromatography.

Species	Arabitol	Mannitol	Volemitol
PYRENOCARPEAE			
Dermatocarpon lachneum (Ach.) A.L.Sm.	−	+	++
» *miniatum* (L.) Mann	−	+	++
» » var. *complicatum* (Lightf.) Th. Fr.	−	+	++
» *fluviatile* (Web.) Th.Fr.	−	(+)	++
Endocarpon adscendens (Anzi) Müll. Arg.	−	+	+
GYMNOCARPEAE			
Sphaerophorus fragilis (L.) Pers.	+	+	−
Crocynia spec.	+	+	−
Lobaria pulmonaria (L.) Hoffm.	+	+	−
Nephroma arcticum (L.) Torss	+	++	−
Peltigera aphthosa (L.) Willd.	+	++	−
Rhizocarpon spec.	+	++	−
Cladonia alpestris (L.) Rabh.	+	+	−
» *uncialis* (L.) Web.	+	+	−
» *rangiferina* (L.) Web.	+	+	−
Stereocaulon spec.	+	+	−
Umbilicaria pustulata Hoffm.	++	++	−
» *papulosa* (Ach.) Nyl.	+	+	−
» *pennsylvanica* Hoffm.	+	+	−
» *polyrrhiza* (L.) Ach.	+	+	−
» *cylindrica* (L.) Del.	+	+	−
» *arctica* (Ach.) Nyl.	+	+	−
» *vellea* (L.) Ach.	+	+	−
» *hyperborea* (Ach.) Hoffm.	+	+	−
» *spodochroa* (Ach.) Frey	+	++	−
» *crustulosa* (Ach.) Frey	+	+	−
» *deusta* (L.) Baumg.	+	+	−
» *proboscidea* (L.) Schrad.	+	+	−
» *hirsuta* (Sw.) Ach.	+	+	−
» *torrefacta* (Lightf.) Schrad.	+	+	−
» *polyphylla* (L.) Hoffm.	+	+	−
» *virginis* Schaer	+	+	−
» *decussata* (Vill.) Frey	+	+	−
» *rigida* (DR.) Frey	+	+	−
Pertusaria amara (Ach.) Nyl.	+	++	−
» *corallina* (L.) Arn	+	+	−
Lecanora spec.	++	+	−
Ochrolechia tartarea (L.) Mass.	+	+	−
Haematomma ventosum (L.) Mass.	+	(+)	−

Parmelia furfuracea (L.) Ach.	++	+	−
» *caperata* (L.) Ach.	+	(+)	−
» *physodes* (L.) Ach.	+	(+)	−
» *conspersa* (Ehrh.) Ach.	++	+	−
» *omphalodes* (L.) Ach.	+	+	−
Cetraria glauca (L.) Ach.	+	(+)	−
» *islandica* (L.) Ach.	+	+	−
Letharia vulpina (L.) Vain	+	+	−
Alectoria sarmentosa Ach.	+	+	−
» *implexa* (Hoffm.) Nyl.	+	+	−
Ramalina polymorpha Ach.	+	++	−
» *farinacea* (L.) Ach.	+	+	−
Thamnolia vermicularis (Sur.) Ach.	++	+	−
Xanthoria spec.	+	+	−
Anaptychia ciliaris (L.) Kbr	+	+	−
» *fusca* (Huds.) Vain	+	+	−
Lepraria chlorina Ach.	+	+	−

++ Isolated.
\+ Identified by paper chromatography.
(+) » » » » , but only as traces.
− Not found by paper chromatography.

Table 2. Carbohydrate constituents of lichens, isolated by separation on a charcoal column. (The numbers are given in per cent of dry lichen and are very approximative.)

Species	Trehalose	Sucrose	Umbilicin
Cladonia rangiferina (L.) Web.	0.025	0.06	0
Umbilicaria pustulata Hoffm.	0.2	0.2	2.5
Umbilicaria rigida (DR) Frey	0.1	0.05	0.2
Haematomma ventosum (L.) Mass.	0.015	0.2	0.15
Cetraria islandica (L.) Ach.	0.025	0.07	0.05
Lecanora atra (Huds.) Ach.	0.04	Identified but not isolated	0

DISCUSSION

Arabitol occurs in all lichens of the order *Gymnocarpeae* which have been investigated, but not in the lichens of the genera *Dermatocarpon* and *Endocarpon*. These, on the other hand, contain *volemitol*, which was not, however, detected in *Gymnocarpeae*. Thus there seems to be a sharp chemical difference between the order *Gymnocarpeae* and the genera of the order *Pyrenocarpeae*. Because the classification of the lichens is not natural, and the order *Pyreno-*

carpeae is rather heterogenous, no generalizations can be drawn from these observations.

Mannitol has been found in all the lichens investigated and appears to be a generally occurring constituent.

α,α,-Trehalose and *sucrose* have been found in all six lichens investigated by separation of the carbohydrate fraction on a charcoal column. The presence of glucose in the hydrolysate of the acetone extract from most lichens, is evidence of the general occurrence of *α,α*-trehalose and sucrose.

Umbilicin has been isolated from four of the six more carefully investigated lichens. The percentage, however, varies between wide limits, and the occurrence of umbilicin in concentrations of 0.01 % or less might have escaped detection. The presence of galactose in many hydrolysates of carbohydrate fractions from lichens of the order *Gymnocarpeae*, indicates that umbilicin is a substance frequently occurring in lichens belonging to this order.

Spots due to unidentified substances have been found frequently. Some of these spots could be developed with ninhydrin and thus contain amines, whereas others gave phenol reactions and were probably due to lichen acids. Some spots, however, could only be developed with the silver nitrate-sodium ethoxide reagent, but nothing is known of the nature of the substances giving such spots. For example the presence of erythritol in very small concentrations in some of the lichens is not definitely excluded.

An interesting question is whether the substances discussed above are formed by the algae or by the fungi of the lichen or are products of the symbiosis. The solution of that problem necessitates the separate investigation of the isolated fungi and algae.

EXPERIMENTAL

The air-dried, ground lichen (2—25 g) was extracted continuously with ether for three days. Small amounts of arabitol were sometimes found in the ether extract of lichens rich in this substance. The extraction was then continued for 3 days with acetone. Volemitol and mannitol have low solubilities in acetone and often separated as crystals and it was found that almost pure arabitol separated from the extract of lichens very rich in arabitol. These sugar alcohols were purified by recrystallization and characterized. The acetone extract was concentrated to dryness, extracted with water and the water extract investigated by paper chromatography.

The chromatograms were prepared on Whatman No. 1 paper, using butanol-ethanol-water (4 : 1 : 5). The chromatograms were run for about 20 hours and developed with silver nitrate-sodium ethoxide and with aniline hydrogen phtalate or *p*-anisidine phosphate [4]. Generally no reducing sugars were found with the latter reagents, but in some cases small amounts of hexoses were found, probably indicating that partial hydrolysis of disaccharides and of umbilicin had occurred. Part of the extract was hydrolysed

with 0.1 *N* hydrochloric acid at 100° over night and then investigated for reducing sugars by paper chromatography as described above. The results are summarized in Table 1.

For the more carefully investigated lichens larger amounts (100—200 g) were extracted and the extracts worked up as described in Part III. The results are summarized in Table 2.

SUMMARY

The low-molecular carbohydrate constituents of a number of lichens have been investigated. All the substances found, arabitol, mannitol, volemitol, α,α-trehalose, sucrose and umbilicin, previously have been occasionally observed, but they have now been shown to be either lichen constituents of general occurrence or to be characteristic for specific taxonomic groups.

The authors are indebted to Docent Ove Almborn, Universitetets Botaniska Museum, Lund, for valuable discussions, for the supply of several species of lichens and for help with the examination of others. We also wish to thank Mr. J. Paju for skilful assistance.

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Synthetic Plant Hormones

IV. Preparation and Physiological Activity of some α-Phenoxy fatty Acids, especially Isobutyric Acids *

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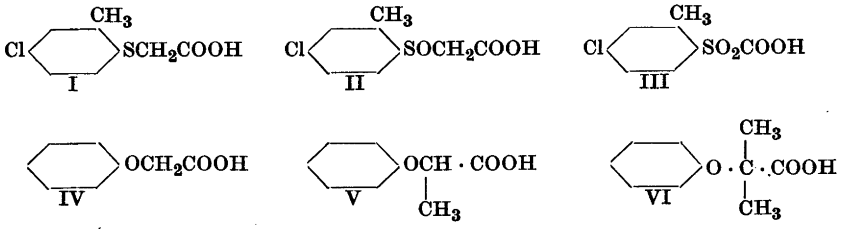
Investigations of the growth promoting properties of 4-chloro-2-methylphenylsulphideacetic acid (I) and related compounds have shown that oxidation of (I) to the corresponding sulphoxide (II) does not cause any fundamental alteration in the auxin activity of the compound. However, the sulphone (III) was inactive. Using a somewhat different test, 2,4-dichlorophenylsulphideacetic acid was found to be active but both the oxidation products inactive [2].

It appeared possible that the lack of activity of the oxidised compounds might be due to a sterical blocking effect and a number of methyl substituted phenoxyacetic acids belonging to the α-phenoxypropionic and α-phenoxy*iso*-butyric acid series were therefore prepared for comparative studies of their auxin properties.

The physiological properties of these compounds have been investigated by Burström and Hansen [6] and are reported on in detail elsewhere. It was found that the phenoxyacetic (IV) and α-phenoxypropionic acid (V) derivatives



* Part III. *Acta Chem. Scand.* **6** (1950) 993.

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have an activity resembling that of 3-indoleacetic acid, causing inhibition of the cell elongation of wheat roots [2,3]. (Phenoxyacetic acid itself was inactive at concentrations up to 10^{-5} *M*. In a 10^{-4} *M* solution, however, it accelerated root growth.) The *α-phenoxyisobutyric acid* (VI) *derivatives strongly increased the cell elongation* [4,5]. For example, in 10^{-6} *M* solution *p*-chlorophenoxy*iso*butyric acid caused a cell (and root length) elongation of about 75 per cent [4]. Further, it was found that by using mixtures of *α-p*-chlorophenoxy*iso*butyric acid and 3-indoleacetic acid [4] or *α*-phenoxy*iso*butyric and D,L-*α*-phenoxypropionic acid [3] in suitable proportions *it was possible to alter the effect on cell elongation gradually from acceleration to inhibition.* The ratio of *α*-phenoxy*iso*butyric acid to D,L-*α*-phenoxypropionic acid which results in normal cell length was approximately 2.5 for all concentrations of the acids between 0 and 10^{-5} *M*. This indicates that phenoxy*iso*butyric acids act as true "auxin antagonists".

Table 1 summarises the results obtained with these and several other acetic acid derivatives. It appears that the introduction of *one* alkyl group into the

Table 1. Type of activity of some α-aryloxy fatty acids.

(+ = inhibits cell elongation
0 = inactive
− = accelerates cell elongation)

Acid	Type of activity	Acid	Type of activity
Phenoxyacetic acid	0[1]	*α*-Phenoxyisobutyric acid	−
p-Chloro-	+	*p*-Fluoro-	−
2.4-Dichloro-	+	*o*-Chloro-	−
2.4-Dimethyl-	+	*m*-Chloro-	−
		p-Chloro	−
D,L-*α*-Phenoxypropionic acid	+	2.4-Dichloro-	−
D,L-*p*-Chloro-	+	2.4.5-Trichloro-	−
D,L-2.4-Dichloro-	+	2.4.6-Trichloro-	−
D,L-2.4-Dimethyl-	+	2.3.4.5.6-Pentachloro-	−
		p-Bromo-	−
D,L-*α*-*p*-Chlorophenoxycaproic acid	+	*p*-Iodo-	−
		o-Methyl-	−
α-1-Naphtoxy*iso*butyric acid	−	2.4-Dimethyl-	−
α-2-Naphtoxy*iso*butyric acid	−	*p*-Amino-	0
		p-Acetamino-	0
		p-Carboxy-	0
*α-Cyclo*hexyloxy*iso*butyric acid	−	*p*-Nitro-	0

[1] In normal concentrations (up to 10^{-5} *M*). Accelerates root elongation in higher concentrations (10^{-4} *M*).

side chain of a nuclear substituted phenoxyacetic acid does not cause any reversal of the auxin activity, but that the introduction of a *gem*dimethyl group in general gives rise to compounds possessing antiauxin activity. A reservation must be made, according to Burström [3], for the possibility that several physiologically different activities are included in the general concept of "auxin activity".

Substituents in the nucleus merely modify the auxin or antiauxin activity of the parent compound and this substantiates the deduction that it is the structure of the side chain which is of paramount importance for producing the auxin or antiauxin effect. It is interesting to note that *α-cyclo*hexyloxy-*iso*butyric acid is a powerful auxin antagonist, or at least causes elongation [6] of root cells. No compounds, which does not contain at least one double bond in the nucleus has ever been found to exhibit auxin activity [7].

It would seem reasonable to assume that the *iso*butyric acids act by preventing indoleacetic acid or the auxinlike acids such as the phenoxyacetic acids or the α-phenoxypropionic acids from carrying out their normal function in the cell. The mechanism is quite obscure but it is attractive to assume that there is competition between the *iso*butyric acid and the acetic acid derivatives for an apoenzyme. Several other explanations of the effect, however, are possible.

Although the auxinlike properties of phenoxyacetic, propionic and *n*-butyric acids and related compounds, have been extensively studied in the past, the *iso*butyric acid analogues have received very little attention. Recently, however, Osborne and Wain reported that phenoxy*iso*butyric acids cause no growth responce in a number of standard tests for auxin activity [8,9]. Åberg [10] has investigated the activity of 1-naphthylmethylsulphide acetic, α(1-naphthylmethyl-sulphide) propionic and α-(1-naphthylmethyl-sulphide) *iso*butyric acid and the corresponding 2-naphthyl compounds and found, that they all antagonize 2,4-D. He has also found [11], that (+)-α-(2-naphthoxy)-propionic and (−)-α-(1-naphthoxy)propionic acid act like indole acetic acid in depressing root growth, whereas their enantiomorphs behave as auxin antagonists.

The phenoxyacetic, α-phenoxypropionic and α-phenoxycaproic acids were prepared *via* the ethyl esters in the usual way from the ethylesters of the α-chloro acids and the appropriate sodium phenolate.

The α-phenoxy *iso*butyric acids were prepared by a modification of Galimberti and Defrancheschi's [9] method from phenol, chloroform, acetone and sodium hydroxide using excess of acetone as solvent. Sometimes neutral, crystalline compounds were obtained as by-products. In a few cases they were isolated and found to be phenyl orthoformates.

2,4,6-Tribromophenol and 2,4,6-triiodophenol failed to react and these trihalogenated ethyl α-phenoxy*iso*butyrates could not be obtained by reacting ethyl α-bromo-*iso*butyrate with the dry sodium phenolates. This is obviously due to steric hindrance.

α-*p*-Aminophenoxy*iso*butyric acid was obtained by reduction of the nitro acid.

α-*p*-Bromophenoxy*iso*butyric acid was converted into its nitrile *via* the acid chloride and the amide.

EXPERIMENTAL *

Phenoxyacetic, α-phenoxypropionic and α-phenoxycaproic acids.

These acids were prepared by heating the dry sodium phenolate with an equimolecular amount of the ethyl ester of the halogenated aliphatic acid at 100° in a sealed tube for about twelve hours, followed by alkaline hydrolysis of the crude ester. With the exception of α-chlorophenoxy caproic acid they have all been reported earlier.

D,L-*α-p-chlorophenoxy caproic acid* forms colourless needles from petroleum ether: M.p. 74—75° C. (Found: Cl 14.5 %; equiv. weight 242.7 · $C_{12}H_{15}ClO_3$ requires: Cl 14.6 %; equiv. weight 243.6).

α-Aryloxy*iso*butyric acids

General procedure: Phenol (0.15 mole), sodium hydroxide (0.85 mole, pellets) and dry acetone (2.6 mole) were mixed in a three-necked flask equipped with a mechanical stirrer, a dropping funnel and a reflux condenser, and heated to boiling on a water bath. The bath was removed and chloroform (0.21 mole) was run in with stirring at such a speed that the mixture boiled gently. If the chloroform is added too rapidly the reaction may become uncontrollable. After the addition the mixture was heated under reflux with stirring for four hours. The excess acetone was destilled off, and the salt cake dissolved in water. The solution was filtered (in some instances solid, crystalline by-products remained on the filter) saturated with carbon dioxide, and extracted with ether to remove unreacted phenol. A stream of air was blown through the solution to remove dissolved ether and the acid was precipitated by acidification with dilute sulphuric acid. Most of the acids separate as crystallising oils in yields of 40—85 per cent of the theoretical. Analyses and melting points for the compounds not previously described are given in Table 2.

In the case of 2,4-dichloro- and 2,4-dimethylphenoxy*iso*butyric acids which have not been obtained crystalline the crude acid was dissolved in ether, the solution was dried and the acid was precipitated as its *cyclo*hexylamine salt by the addition of excess *cyclo*hexylamine.

Cyclohexylammoniumsalt: a) Of α-2,4-dichlorophenoxy*iso*butyric acid, M.p. 170—171° C. (Found N 4.02. $C_{16}H_{23}Cl_2NO_3$ requires N 4.02 %); b) Of α-2,4-dimethylphenoxy-*iso*butyric acid, M.p. 183—185° C. (Found C 69.8, H 9.49. $C_{18}H_{29}NO_3$ requires C 70.3, H 9.51 %.)

* All melting points uncorrected.

Table 2. Analytical data for some α-phenoxyisobutyric acids not previously described.

α-Phenoxy*iso*butyric acid	M.p. °C	Equiv. weight Found	Equiv. weight Calc.	Halogen Found	Halogen Calc.
o-Chloro-	73—74	215	214.6	16.6	16.5
m-Chloro-	58—59	216	214.6	16.4	16.5
2,4,5-Trichloro-	90—91	284	283.5	37.9	37.5
2,4,6-Trichloro-	68—70	284	283.5	37.7	37.5
2,3,4,5,6-Pentachloro-	142—143	353	352.3	50.5	50.3
p-Bromo-	132—133	259	259.0	30.7	30.9
p-Fluoro-	83—84	198	198.1	C 60.9 H 5.7	C 60.9 H 5.6
p-Iodo-	137—138	306	306.0	C 39.9 H 3.75	C 39.2 H 3.63

The neutral by-products were recrystallised from glacial acetic acid and formed long, thin needles.

Tri-o-chlorophenyl orthoformate, M.p. 129—130° C. (Found Cl 26.7 %; mol.weight 417 (Rast). $C_{19}H_{13}Cl_3O_3$ requires Cl 26.9 %; mol. weight 396.)

Tri-2,4-dichlorophenyl orthoformate, M.p. 201—202° C. (Found C 45.8; H 2.00; Cl 42.1 %; mol.weight 476 (Rast). $C_{19}H_{10}Cl_6O_3$ requires C 45.7; H 2.02; Cl 42.6 %; mol.weight 499.)

Tri-2,4,5-trichlorophenyl orthoformate, M.p. 230—231° C. (Found Cl 52.5 % mol. weight 583 (Rast). $C_{19}H_7Cl_9O_3$ requires Cl 53.0 % mol.weight 602.)

α-*p*-Carboxyphenoxy*iso*butyric acid

The methyl ester of this acid was prepared by the general procedure from methyl *p*-hydroxybenzoate and hydrolysed directly with aqueous sodium hydroxide. Prisms (from water). M.p. 170—171° C. (Found C 59.6, H 5.4 %; equiv.weight 112.1. $C_{11}H_{12}O_5$ requires C 58.9, H 5.4 %; equiv.weight 111.5.)

α-*p*-Aminophenoxy*iso*butyric acid

α-*p*-Nitrophenoxy*iso*butyric acid (22.5 g, 0.10 mole) was dissolved in a mixture of ammonia (50 ml) and water (50 ml) and reduced with ferrous hydroxide, prepared from $FeSO_4 \cdot 7H_2O$ (175 g) dissolved in water (400 ml) and precipitated with concentrated aqueous ammonia (100 ml). The reaction proceeds rapidly. The ferric hydroxide sludge was filtered off and washed thoroughly with dilute ammonia. The combined filtrate and washings were acidified with hydrochloric acid to pH 6. After cooling the crystalline precipitate was collected by filtration (11 g). Additional material was obtained by concentrating the mother liquor under reduced pressure. The total yield of sandy crystals was 15 g (77 %). M.p. 217—218° C (decomp.). Purification *via* the hydrochloride did not raise the melting point. (Found N 7.0 %, equiv.weight 195.1. $C_{10}H_{13}NO_3$ requires N 7.2 %, equiv.weight 196.3.)

N-Acetylderivative M.p. 169—170° C (from water). (Found N 5.9 %, equiv.weight 238.2. $C_{12}H_{15}NO_3$ requires N 5.9 %, equiv.weight 237.1.)

α-*p*-Bromophenoxy*iso*butyramide

α-*p*-Bromophenoxy*iso*butyric acid (26 g) was refluxed with thionylchloride (75 ml) for 2 hours on a water bath. The excess thionylchloride was distilled off, the last traces being removed in a vacuum. The residue in the flask was dissolved in dry benzene (100 ml) and saturated with dry ammonia. The precipitated ammonium chloride was filtered off and washed with benzene, and the combined filtrate and washings were evaporated to dryness, yielding a crystalline product (19.5 g) which was repeatedly recrystallised from chloroform-petroleum ether. M.p. 128° C. (Found Br 30.8 %. $C_{10}H_{12}BrNO_2$ requires Br 31.0 %.)

α-*p*-Bromophenoxy*iso*butyronitrile

α-*p*-Bromophenoxy*iso*butyramide (10.5 g) was refluxed with thionylchloride (50 ml) on a water bath for 1 hour. The excess thionylchloride was distilled off and the residue was poured on to ice. After some hours the oil was dissolved in ether, the solution washed with sodium bicarbonate solution and water, dried with potassium carbonate and fractionated under reduced pressure. Yield 6.9 g (71 %). Colourless oil. B.p. 131—132° C/10 mm (uncorrected). (Found Br 33.1 %. $C_{10}H_{10}Br$ NO requires Br 33.3 %.)

SUMMARY

Following our earlier studies on the auxin activity of aryl thiophenoxy acids and their "branched" oxidation products (sulphoxides and sulphones) a series of α-aryloxy-propionic and *iso*butyric acids have been prepared for a study of the effect of branching in the acetic acid section. As expected the compounds of the type D,L-aryl-$OCH(CH_3)COOH$ were shown to exhibit normal type of auxin activity (inhibition of root cell elongation), but those of the type aryl—$O—C(CH_3)_2COOH$ possessed marked antiauxin activity (acceleration of root cell elongation).

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The Preparation of Hyaluronic Acid and the Determination of the Viscosity and Optical Rotation

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Several preparations of hyaluronic acid have been prepared which by analysis have been found to be rather pure. Some of these preparations exhibit unusually high viscosities in aqueous solution. The optical rotation of these highly viscous and pure preparations seems to be much smaller than that usually accepted.

The characteristics of the hyaluronic acid vary very much according to the sources and the methods of isolation (Meyer [1], Jensen [2]). The substance has been prepared from synovial fluid from cutting cattle, and from human umbilical cords, according partly to known methods and partly to methods modified by the author.

PREPARATION OF HYALURONIC ACID

A. From Umbilical Cords

The umbilical cords * free from placental tissue are stored under acetone after thorough rinsing with water so that all blood is removed. The cords are cut into small fragments (two to three cm), put into acetone and carefully minced in a Waring Blendor. On account of the production of heat the Waring Blendor must work only for a minute at a time, and it must be cooled constantly. After four to six treatments the pulp is extracted with acetone at room temperature for four days, the acetone being replaced every twenty-

* The author wishes to thank Prof. Erik Rydberg, M. D., Obstetrical Department of the Rigshospital, Copenhagen, for kindly placing extraordinary well rinsed human umbilical cords at his disposal.

four hours; then the pulp is filtered through gauze, and the rest of the acetone is removed in a vacuum desiccator containing silica gel. The dried powder is extracted with two to three volume glacial acetic acid at room temperature for a week under occasional stirring, the glacial acetic acid being replaced every twenty-four hours. By doing so remnants of blood are removed, and the protein to which the hyaluronic acid is attached is split off. The filtrate from the treatment with glacial acetic acid gives an ample precipitation upon boiling, and the protein-like precipitate also gives the ordinary reactions for proteins *e.g.* the biuret test, the test for sulphur and Millon's test. The bulk of the glacial acetic acid is removed by repeated quick rinsings with water through eight layers of gauze with suction, and the remainder is neutralized to pH 7 by adding saturated potassium hydroxide. Now the pulp is extracted with three to four volume water at about 4° C, and toluene is added in order to avoid bacterial growth. The first extraction lasted a week, and the next four weeks (occasional stirring). The hyaluronate is precipitated with one and a half volume alcohol. At this time the preparation is greyish; it is purified through ten rinsings (5 ml every time) with absolute alcohol on a Buchner funnel with weak suction. It is treated in the same way with ether distilled over sodium. This preparation is called Prep. 1.

Three preparations were prepared after the foregoing chemical procedure but at different pH values and a meatchopping machine for mincing the cords. Prep. 2a was precipitated with alcohol after extraction at pH 5, and Prep. 2 b and 2 c were precipitated with alcohol saturated with potassium acetate after extraction at pH 8.5 and pH 11, respectively.

B. From Synovial Fluid

The synovial fluid is withdrawn into a 30 ml syringe through a coarse canula from the ankle joints of the hindlimbs of cutting cattle, 15—20 minutes after slaughtering. The canula is provided with a stilett at the introducing. The canula is of the same kind as that used at bone marrow biopsy. If one introduces the canula into the lateral posterior tibio-tarsal joint capsule just anterior to the saphenous vein which is here crossing the capsule joint it is possible to obtain the synovial fluid as a colourless fluid without contamination with blood and tissue fragments. The withdrawing is done most easily when the animal is lying on its back and the Achilles tendon is cut. If the animal is suspended from hooks the withdrawing is difficult on account of the tightening of the joint capsule. The cows give about 15 ml, the bulls about 50 ml synovial fluid per ankle joint.

Just after the withdrawing the synovial fluid is cooled to 1° C, and the working up of the hyaluronic acid is started at once. The synovial fluid is diluted with about one volume of water, and the mucin is precipitated with about one volume per cent glacial acetic acid under vigorous stirring with a glass rod (Meyer [3], Blix [4], Lundquist [5]). The protein is separated from the hyaluronic acid after three different principles.

1. Enzymatic Digestion. The mucin is dissolved in water, and the pH is adjusted to about 8. Pancreatin (B.D.H.) is added together with 0.5 per cent sodium bicarbonate (Lundquist [5]). Incubation at 37° C for twenty-four hours destroys the protein. The subsequent filtration is rather difficult, preferably a folded filter or a layer of Hyflo-Super-Cel (Johns Manville) combined with weak suction is used. After cooling 10 per cent trichloroacetic acid is added, and the mixture is filtered again. The resulting solution is neutralized with potassium hydroxide and a saturated solution of potassium hydroxide in alcohol is added. This preparation is marked 3 A.

2. The Method of Sevag [6]. The aqueous mucin solution is shaken with ½ volume chloroform and 1/20 volume *iso*amylalcohol (Meyer [3], Blix [4]); we shook vigorously for half an hour by hand, in a nitrogen atmosphere to protect the hyaluronic acid against air (oxygen). After two shakings no protein is precipitated at the interface. The mixture is centrifuged at 0° C (3 500 r.p.m.), and the hyaluronic acid is now precipitated with alcohol saturated with potassium hydroxide. This preparation is marked 3 B.

3. The Glacial Acetic Acid Method. The mucinclot was chopped into small fragments and extracted with 4 volumes glacial acetic acid for eight days, the glacial acetic acid being replaced every twenty-four hours (Meyer and Palmer [7]). The glacial acetic acid is removed and the remainder neutralized to pH 7 with saturated potassium hydroxide. The pulp is extracted with water for a week, and the hyaluronic acid is precipitated with about 1 ½ volume alcohol. This preparation is marked 3 C.

All the preparations are nearly white or white and very hygroscopic, they dissolve quickly in water and the solutions are clear; they do not contain glycogen.

Nitrogen Determination

The nitrogen content was determined after Kjeldahl, modified by Blom [8]. The method of Blom is distinguished by requiring only one standard liquid, the acid. The ammonia set free is distilled into a receiver containing a nickel-ammoniumsulphate solution (for details see Blom in a forthcoming paper). The method was used as a micromethod. 8—10 mg hyaluronate were destruc-

Table 1. Nitrogen content and viscosity of diffe-

Source of hyaluronate	Method of preparation
Umbilical cords	Prep. 1. Mincing in a Waring Blendor, acetone + extraction with glacial acetic acid. Precipitation with ethanol at pH = 7.
	Prep. 2. Mincing with a chopping machine. Extraction with glacial acetic acid. Precipitations with a) ethanol at pH = 5 b) ethanol + potassium acetate at pH = 8.5 c) ethanol + potassium acetate at pH = 11
Synovial fluid	Prep. 3. Precipitation of mucin with glacial acetic acid. Separation of protein by A) Enzymatic digestion [1] B) Chloroform + *iso*amylalcohol [1] C) Treatment with glacial acetic acid [2]

[1] The synovial fluid contaminated with blood and tissue debris.
[2] The synovial fluid appeared to be pure, a preparation prepared in quite the same way showed a relative viscosity of 4.93, but the source of material was impure.

ted with 1 ml sulphuric acid, about 1 g potassium sulphate and about 100 mg mercuric oxide added. The ammonia is liberated with sodium hydroxide containing sodium sulfide and distilled into about 2 ml nickelammoniumsulphate solution. Eight nitrogen analyses on ammoniumchloride yielded the theoretical nitrogen content $\pm$ 1 %, and five analyses on pyridinhydrochloridezinc-chloride, which Dr. Blom kindly placed at our disposal, yielded nitrogen contents of 7.598, 7.606, 7.612, 7.601, 7.609 % respectively (theoretically 7.626%). The time of destruction of the hyaluronic acid was about two hours, the time of distillation about ten minutes. We titrated with a 0.1 *N* hydrochloric acid from a burette of 1 ml, divided into 0.01 ml. The colour change from green to purple was seen distinctly (indicator: a mixture of methyl red and methylene blue). All determinations were double determinations which agreed within a half per cent relatively. All volumetric flasks, the pipette and the burette had been standardized. The nitrogen per cents found are seen in Table 1. The theoretical nitrogen content calculated from the formula $(C_{14}H_{20}NO_{11}K)_n$ is 3.36 %.

rent preparations of potassium hyaluronate.

Yield	% N	Relative viscosity Conc. 1 g/l
From 12 cords		
1. extraction: 465 mg stringy hyaluronate	3.36	11.80
1. extraction: 480 » flocculent »	3.35	11.91
2. extraction: 145 » stringy »	3.31	12.19
2. extraction: 60 » flocculent »	3.34	12.12
From 3 × 6 cords		
60 mg hyaluronate	2.90	5.46
100 » »	2.88	68.46
745 » » [3]	2.82	5.29
From 3 × 500 ml synovial fluid		
205 mg stringy + 65 mg flocculent hyaluronate	1.16	5.75
460 » » + 250 » » »	5.78	(4.62)[4]
190 » hyaluronate	2.99	76.39

[3] The preparation was only partly soluble in water.
[4] The aqueous solution became clear only after centrifugation.

Determination of the Viscosity of Hyaluronic Acid

The relative viscosity, that is the viscosity of the solution divided by the viscosity of water, was determined by the Kvorning and Dalgaard-Mikkelsen [9] viscosimeter, a modification of the Ostwald viscosimeter. The apparatus has a capacity of 0.5 ml, the volume between the two marks being 0.2 ml. The flow time of our apparatus was 43.0 seconds at 20° C for distilled water. The concentration of hyaluronic acid was 100 mg in 100 ml solution. On account of its hygroscopicity the substance was weighed in a stoppered weighing bottle. The results of the measurements are in Table 1.

Measurement of the Optical Rotation of Hyaluronic Acid

The optical activity of hyaluronic acid was determined in a Schmidt and Haensch polariscope with a tubuslength of 4 dm. The readings were accurate to 0.01 angular degrees. The source of light was sodium light and the tem-

perature 20.0° C. The concentration of hyaluronic acid was 20 to 25 mg in 25 ml solution. The rotation angles are small, varying from 0.04° to 0.32°, and the corresponding $[\alpha]_D^{20}$-values are therefore to be considered with some reservation. When ferrous sulphate was added to the solutions to a concentration of 0.01 *M* the angles of rotation increased appreciably during six hours. The results of the measurements are in Table 2.

Table 2. The optical rotation of three preparations of potassium hyaluronate and its change by the influence of ferrous ions.

Time		0		1	2	6
Sample	Conc. mg/ml	α	$[\alpha]_D^{20}$	α	α	α
Prep 1	0.80	−0.08	− 25	−0.20	−0.24	−0.26
Prep 3 A	0.77	−0.32	− 104	−0.32	−0.32	−0.32
Prep 3 C	0.84	−0.04	− 12	−0.20	−0.24	−0.25

The times indicated are the times in hours from the addition of ferrous sulphate. The optical rotation, α, was measured in 4 dm tubes.

DISCUSSION

The great majority of viscosity determinations of hyaluronic acid vary from 1 to 8, most of them are beneath 4 as seen in a table by Hadidian and Pirie [10]. More recent reports indicate figures of a similar size (Jeanloz [11], Blix [12]). In the literature only one determination is rather high: 39 (Ogston [13]). This is a preparation from synovial fluid prepared by filtering the fluid or the dissolved mucin through membranes of collodion and filters of sintered glass into an evacuated reservoir. Ogston's preparation, however, contains protein, and the nitrogen content is about 7 %.

The measurements reported are of preparations of hyaluronic acid of different origin: umbilical cords, synovial fluid, exudates, the vitreous body, the cock's comb, peritoneal fluid and mesothelioma; the methods of preparation also differ considerably. The author is of the opinion that the much higher viscosity reported here is due to: 1) pure sources of material, 2) quick working

up, 3) effective treatment with glacial acetic acid and, as to the synovial fluid, 4) immediate cooling. Seeing that ferrous and ferric ions depolymerise hyaluronic acid [14] great care was taken to obtain human umbilical cords and synovial fluid free from blood, and by vigorous treatment with glacial acetic acid to remove every trace of blood from the material. The umbilical cords employed were rinsed so effectively that all the blood seemed to be removed. From the ankle joints of cutting cattle, just at the spot mentioned above, we succeeded in withdrawing a synovial fluid which was quite free from blood and tissue debris. Great importance was attached to this fact because the tissue round the synovial capsule contains relatively much ascorbic acid (Robertson [15], McClean [16], Daubenmerkl [17]) which also depolymerises the hyaluronic acid. The quick withdrawing and cooling of the synovial fluid certainly effect a decrease of the activity of depolymerising substances possibly present. The vigorous mechanical treatment in the Waring Blendor may be the reason why the viscosity of the preparation denoted Prep. 1 is lower than the highest ones obtained (see Mogilevskii and Klyuchareva [18]). The viscosity of the best preparations is stable in time.

Meyer [1] reports $[\alpha]_D^{20}$ to be $-70°$ and Rapport *et al.*[19] $[\alpha]_D^{25}$ to be $-74°$. Much the same ($-64°$) was found in preparations prepared after Meyer. As seen in Table 2, however, we find much smaller values in two preparations. It seems that the highly viscous preparations exhibit a small optical rotation and those of low viscosity an appreciably larger one.

CONCLUSION

As a result of the experiments the method of preparation denoted Prep. 1 is considered to be the better one. The yield was good, the nitrogen content equalled the theoretical content, and the viscosity was relatively high. The preparations were entirely white and very hygroscopic. They dissolved in water very quickly, and the solutions were quite clear.

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The Crystal Structure of Boron Oxide

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The crystal structure of B_2O_3 is of particular interest, because it is one of the most difficult inorganic substances to crystallize. Until 1937 the oxide had been known only in the vitreous state. Crystallization was first accomplished by McCulloch [1] and apparently independently also by Kracek, Morey and Merwin [2]. The determination of the crystal structure was, however, first accomplished by the present author in 1950–1951. The results, which have been summarized in a preliminary note [3], are reported in more detail in this paper.

PREPARATION OF CRYSTALLINE B_2O_3

Crystalline B_2O_3 was prepared by the methods of McCulloch [1] and of Kracek, Morey and Merwin [2]. A closer study of the conditions under which crystalline B_2O_3 can be formed gave results, which were in agreement with the report of the latter authors.

The boric acid was dehydrated in open Pyrex glass vessels at about 240° C (McCulloch [1]). After heating for twenty-four hours the preparation was a highly viscous liquid with a water content of nearly 20 mole per cent. The subsequent dehydration and crystallization process then proceeded very slowly. Small spheroidal aggregates of crystalline B_2O_3 – identified as such by powder photographs – appeared at the glass walls after about a week. However, the crystallization of the whole mass, leading to a white stony substance, was in most cases completed only after several months. In a few cases, however, an extremely rapid dehydration, immediately accompanied by crystallization, occurred after heating for about two weeks. This phenomenon is also reported by McCulloch, who speaks of the "volcanic violence" of the reaction. A rapid crystallization could always be effected by seeding a boric acid melt, which still contained water, with a small amount of crystalline B_2O_3.

A simple and relatively rapid method of obtaining crystalline B_2O_3, which does not require any seeds, makes use of the fact that the stable modification of metaboric acid – denoted by Kracek, Morey and Merwin as HBO_2 I – can readily be dehydrated with the formation of crystalline B_2O_3. If a melt of boric acid is kept at about 175° C one obtains a viscous liquid with a slowly increasing precipitation of crystalline HBO_2 I. This phase gives off water forming crystalline B_2O_3 and this process can be accelerated by raising the

temperature to slightly below the melting point of HBO_2 I (236° C). It is probable that the violent reaction mentioned above also takes place via HBO_2 I. The temperature may in these cases have been below the melting point of HBO_2 I.

Single crystals could not be isolated from the products of crystalline B_2O_3 obtained by the above methods. In order to obtain larger crystals experiments were carried out in which boric acid melts containing different amounts of water were heated in sealed Pyrex tubes, enclosed in an autoclave. These experiments were not successful, probably owing to the fact that the autoclave did not permit heating at temperatures above 300° C.

As a consequence, the structure determination had to be carried out from powder data only, which considerably increased the difficulties.

UNIT CELL

All powder photographs were taken with CuK radiation. The reflections in the range $\sin^2\Theta \leq 0.510$ were obtained in a camera of the Guinier type, using monochromatized Cu$K\alpha$ radiation. The range $\sin^2\Theta \geq 0.510$ was covered by means of cameras of the Seeman-Bohlin type.

The crystalline products gave — after separation from small amounts of glassy substance — excellent powder photographs with sharp lines. They could be completely interpreted by means of a hexagonal unit cell with the axial lengths $a = 4.334$ Å and $c = 8.334$ Å. According to Hendricks [4], McMurdie has indexed powder diffraction patterns of crystalline B_2O_3 based on a hexagonal lattice having $a = 4.33$ Å and $c = 8.392$ Å, which is in good agreement with the above result.

The density of the crystalline B_2O_3 was found to be 2.44 g/cm³. McCulloch and Kracek *et al.* give the values 2.42 and 2.46 g/cm³ respectively. The density calculated on the basis of 3 B_2O_3 per unit cell with a volume of 135.6 Å³ is 2.56 g/cm³.

The intensities of the diffraction lines in the Guinier photograph were obtained from a microphotometer record. Values of $p|F|^2$ were calculated from the intensities by means of an intensity expression derived for Guinier cameras by Hägg (private communication).

The exact intensity expression is rather cumbersome but Hägg has shown that it can be approximated with good accuracy by the following formula, valid for the relative intensities in a given photograph without air absorption (exposure in vacuum):

$$I = C \frac{1 + \cos^2 2\Theta_m \cdot \cos^2 2\Theta}{\sin^2\Theta \cdot \cos\Theta} \cdot \frac{1}{\cos(2\Theta - \alpha)} \cdot e^{-\frac{\mu t}{2\cos(2\Theta-\alpha)}} \left[1 - e^{-\frac{\mu_f h}{\cos(2\Theta-\alpha)}}\right] p|F|^2$$

Here Θ_m = the glancing angle in the monochromator crystal, α = the angle between the beam incident on the plane specimen and the normal of the latter, μ = absorption coefficient of specimen, t = thickness of specimen, μ_f = absorption coefficient of the emul-

sion layer, h = thickness of the emulsion layer. For Kodak x-ray film and Cu$K\alpha$ radiation $\mu_f h = 0.13$. The product μt must be determined separately for each specimen.

The intensities of the diffraction lines in the Seeman-Bohlin photographs were visually estimated and based on the relation $I_\beta : I_{\alpha 2} : I_{\alpha 1} = 2 : 5 : 11$ as the relative values for CuK radiation. Values of $p|F|^2$ were calculated from the intensities by means of the intensity expression given by Hägg-Regnström [5]:

$$I = C \frac{1 + \cos^2 2\Theta}{\sin^2\Theta \cdot \cos\Theta} \frac{1}{\sin(2\Theta - \alpha) + \sin\alpha} A_a \cdot A_p \cdot A_f \cdot p|F|^2$$

Provided the experimental conditions are constant all factors preceeding p are functions of Θ only. The intensities and the powder diffraction data are given in Table 1.

SPACE GROUP AND STRUCTURE

Two different methods (a dynamical method and a Giebe-Scheibe method) were tried in order to test the crystalline B_2O_3 for piezoelectricity, but no effect could be detected. Because of the small size of the crystallites this negative result is not conclusive.

Of the absences required in the trigonal and hexagonal space groups only the absence of $00l$ for $l \neq 3n$ or for $l \neq 6n$ (the line observed at $\sin^2\Theta = 0.0761$ may as well be 102 as 003) is compatible with the observed interferences. This makes space groups containing three- or sixfold screw-axes probable.

Among the non-rhombohedral space groups one can immediately exclude the ones which do not contain threefold positions as such a position is necessary for placing the nine oxygen atoms in the cell. Furthermore the space groups C_{3v}^1—$C3m$, C_{3v}^2—$C31m$, C_6^1—$C6$ and C_{6v}^1—$C6mm$, where all points in the sixfold positions have the same c-coordinate, can be excluded. From spatial considerations six oxygen atoms can not be accomodated in such a position and as a consequence all oxygen atoms have to be placed in positions of lower multiplicity. As a result the a and b coordinates of all oxygen positions are governed by a maximum of two parameters. The contribution of the oxygen atoms to reflections $hk0$ could, therefore, easily be calculated and showed the impossibility of these four space groups.

It can be assumed that the structure of B_2O_3 is either built up of BO_3 triangles or BO_4 tetrahedra or a combination of both of these coordination figures. Among the remaining space groups are some (namely C_{3i}^1—$C\bar{3}$, D_{3d}^1—$C\bar{3}1m$, D_{3d}^3—$C\bar{3}m$, C_{6h}^1—$C6/m$ and D_{6h}^1—$C6/m\ mm$) in which practically all positions have such point symmetries, that one can decide whether they can accomodate the centre of a BO_3 triangle or a BO_4 tetrahedron. The orientation of the symmetry elements also indicates the orientation of the coordina-

tion figure in question. The knowledge of the approximate dimensions of a BO_3 triangle and a BO_4 tetrahedron then makes it fairly simple to decide whether the available space or the approximate intensity distribution permit these structures. It was found that they could all be excluded.

The following space groups then remain: C_3^1-C3, $C_3^2-C3_1$ $(= C_3^3-C3_2)$, D_3^1-C312, D_3^2-C32, $D_3^3-C3_112$ $(= D_3^5-C3_212)$, $D_3^4-C3_12$ $(= D_3^6-C3_22)$, $C_{3h}^1-C\bar{6}$, $C_6^4-C6_2$ $(= C_6^5-C6_4)$, $D_{3h}^1-C\bar{6}m2$, $D_{3h}^3-C\bar{6}2m$, D_6^1-C62, $D_6^4-C6_22$ $(= D_6^5-C6_42)$.

Of these space groups C_3^1-C3, D_3^1-C312, D_3^2-C32, $C_{3h}^1-C\bar{6}$, $D_{3h}^1-C\bar{6}m2$, $D_{3h}^3-C\bar{6}2m$, D_6^1-C62 are less probable as they do not require the absence of $00l$ for $l \neq 3n$. It was also found impossible to base a structure on any of these groups.

The remaining five enantiomorphic pairs all require the absence of $00l \neq 3n$. As it seemed hardly possible to obtain acceptable structures in any of the four more symmetrical of these pairs, the efforts were finally concentrated on $C_3^2-C3_1$ $(= C_3^3-C3_2)$, which is contained in the others as a subgroup.

The only position in $C_3^2-C3_1$ is general and threefold. The placing of six boron and nine oxygen atoms on positions of this kind implies the determination by trial-and-error methods of fifteen parameters (of which fourteen are independent). As a result a set of parameters has been found, which give good intensity values as well as an acceptable distribution in space. The parameters are as follows:

O_I	:	0.20,	0.15,	0.00,
O_{II}	:	0.46,	0.79,	0.07,
O_{III}	:	0.51,	0.23,	0.56,
B_I	:	0.54,	0.15,	0.02,
B_{II}	:	0.59,	0.77,	0.26.

Observed and calculated $p|F|^2$ values are given in Table 1.

DISCUSSION OF THE STRUCTURE

The structure of B_2O_3 thus found is built up of two sets (a and b) of BO_4 tetrahedra, reproduced in Fig. 1. The projection of the unit cell is given parallel to [001]. Each type (a and b) of tetrahedra forms a spiral chain, which is seen from Fig. 2, showing a stereoscopic view of the arrangement of the tetrahedra. The spirals are connected in a way that each oxygen atom belongs to two spiral chains. If the function of an oxygen atom is characterized by the types of tetrahedra to which it belongs the oxygen atoms of an a tetrahedron may be

Table 1. Powder diffraction data of B_2O_3 · CuK radiation.

Int.	*hkl*	$p\|F\|^2_{Obs.}$	$p\|F\|^2_{Calc.}$	$\sin^2\Theta_{Obs.}$	$\sin^2\Theta_{Calc.}$
		Guinier camera			
vw	100	12	34	0.0420	0.0420
m	101	305	234	.0506	.0505
vs	{102 / 003}	946	{804 / 157}	.0761	{.0761 / .0767}
s	103	762	652	.1188	.1187
w	110	120	192	.1261	.1261
vs+	111	1 000	1 014	.1348	.1346
w+	112	230	177	.1603	.1602
vw	200	66	73	.1686	.1681
w+	{201 / 104}	398	{ 79 / 401}	.1780	{.1766 / .1784}
m	{202 / 113}	526	{561 / 54}	.2024	{.2022 / .2028}
w	203	186	137	.2450	.2448
w+	105	274	239	.2551	.2550
—	114	—	171	—	.2624
w	210	148	98	.2943	.2942
m	{211 / 204}	845	{494 / 226}	.3034	{.3027 / .3044}
—	006	—	71	—	.3068
w	212	202	305	.3281	.3282
w	115	218	149	.3391	.3391
w	106	295	347	.3488	.3488
w	213	138	274	.3710	.3708
w	{300 / 205}	330	{171 / 215}	.3797	{.3782 / .3811}
vw	301	102	179	.3873	.3867
m	302	339	378	.4125	.4123
m	{214 / 116}	525	{478 / 65}	.4307	{.4305 / .4328}
m	303	308	230	.4557	.4549
w	107	176	135	.4604	.4596
vw	206	98	62	.4751	.4748
m	{220 / 215}	418	{ 57 / 451}	.5070	{.5043 / .5072}
		Seeman-Bohlin cameras			
m	{220*a* / 215*a*}	418	{ 57 / 451}	.5070	{.5043 / .5072}
vw	{221*a* / 304*a*}	53	{ 94 / 179}	.5140	{.5128 / .5145}

vw	$222a$	57	128	.5387	.5383
vw	$\begin{Bmatrix}117a\\310a\end{Bmatrix}$	118	$\begin{Bmatrix}69\\188\end{Bmatrix}$	.5462	$\begin{Bmatrix}.5436\\.5463\end{Bmatrix}$
w+	$\begin{Bmatrix}311a_1\\311a_2\end{Bmatrix}$	423	$\begin{Bmatrix}228\\105\end{Bmatrix}$	.5561	$\begin{Bmatrix}.5540\\.5566\end{Bmatrix}$
w +	$\begin{Bmatrix}312a_1\\223a_1\\312a_2\\223a_2\\207a_1\end{Bmatrix}$	455	$\begin{Bmatrix}185\\126\\85\\58\\136\end{Bmatrix}$	.5818	$\begin{Bmatrix}.5794\\.5800\\.5823\\.5829\\.5846\end{Bmatrix}$
—	$\begin{Bmatrix}108a_1\\207a_2\end{Bmatrix}$	—	$\begin{Bmatrix}9\\61\end{Bmatrix}$	—	$\begin{Bmatrix}.5864\\.5875\end{Bmatrix}$
—	$\begin{Bmatrix}108a_2\\305a_1\end{Bmatrix}$	—	$\begin{Bmatrix}4\\91\end{Bmatrix}$	—	$\begin{Bmatrix}.5893\\.5902\end{Bmatrix}$
—	$305a_2$	—	42	—	.5931
w	$\begin{Bmatrix}216a_1\\216a_2\end{Bmatrix}$	164	$\begin{Bmatrix}198\\91\end{Bmatrix}$	.6006	$\begin{Bmatrix}.5999\\.6029\end{Bmatrix}$
w	$\begin{Bmatrix}313a_1\\313a_2\end{Bmatrix}$	354	$\begin{Bmatrix}289\\133\end{Bmatrix}$	.6231	$\begin{Bmatrix}.6220\\.6251\end{Bmatrix}$
—	$224a_1$	—	109	—	.6395
—	$224a_2$	—	50	—	.6427
vw +	$\begin{Bmatrix}118a_1\\400a_1\end{Bmatrix}$	148	$\begin{Bmatrix}78\\23\end{Bmatrix}$	.6708	$\begin{Bmatrix}.6703\\.6713\end{Bmatrix}$
—	$\begin{Bmatrix}118a_2\\400a_2\end{Bmatrix}$	—	$\begin{Bmatrix}36\\11\end{Bmatrix}$	—	$\begin{Bmatrix}.6736\\.6747\end{Bmatrix}$
—	$401a_1$	—	13	—	.6798
w +	$\begin{Bmatrix}314a_1\\401a_2\\306a_1\\314a_2\end{Bmatrix}$	370	$\begin{Bmatrix}146\\6\\53\\67\end{Bmatrix}$	.6830	$\begin{Bmatrix}.6815\\.6832\\.6839\\.6849\end{Bmatrix}$
—	$306a_2$	—	24	—	.6873
—	$009a_1$	—	25	—	.6891
—	$009a_2$	—	12	—	.6925
—	$402a_1$	—	173	—	.7052
—	$402a_2$	—	80	—	.7087
vw	$\begin{Bmatrix}217a_1\\208a_1\\217a_2\end{Bmatrix}$	177	$\begin{Bmatrix}209\\157\\96\end{Bmatrix}$	.7129	$\begin{Bmatrix}.7105\\.7122\\.7140\end{Bmatrix}$
—	$\begin{Bmatrix}208a_2\\225a_1\end{Bmatrix}$	—	$\begin{Bmatrix}72\\77\end{Bmatrix}$	—	$\begin{Bmatrix}.7158\\.7161\end{Bmatrix}$
—	$225a_2$	—	35	—	.7197
vw	$\begin{Bmatrix}109a_1\\109a_2\end{Bmatrix}$	93	$\begin{Bmatrix}195\\90\end{Bmatrix}$	.7311	$\begin{Bmatrix}.7310\\.7347\end{Bmatrix}$
—	$403a_1$	—	58	—	.7478
—	$403a_2$	—	27	—	.7519
vw	$\begin{Bmatrix}315a_1\\315a_2\end{Bmatrix}$	14	$\begin{Bmatrix}47\\22\end{Bmatrix}$	.7599	$\begin{Bmatrix}.7580\\.7618\end{Bmatrix}$
—	$307a_1$	—	93	—	.7944

vw	$\left\{\begin{matrix}320a_1\\307a_2\\320a_2\end{matrix}\right.$	95	$\left\{\begin{matrix}68\\43\\31\end{matrix}\right.$	.7984	$\left\{\begin{matrix}.7971\\.7984\\.8011\end{matrix}\right.$
—	$321a_1$	—	110	—	.8056
—	$404a_1$	—	42	—	.8074
vw	$\left\{\begin{matrix}321a_2\\226a_1\\404a_2\\226a_2\end{matrix}\right.$	104	$\left\{\begin{matrix}51\\39\\19\\18\end{matrix}\right.$	.8120	$\left\{\begin{matrix}.8096\\.8097\\.8114\\.8137\end{matrix}\right.$
—	$119a_1$	—	17	—	.8150
—	$119a_2$	—	8	—	.8191
vw	$\left\{\begin{matrix}322a_1\\322a_2\end{matrix}\right.$	129	$\left\{\begin{matrix}88\\40\end{matrix}\right.$	.8321	$\left\{\begin{matrix}.8311\\.8353\end{matrix}\right.$
vw	$218a_1$	114	185	.8376	.8381
—	$218a_2$	—	85	—	.8423
vw	$\left\{\begin{matrix}316a_1\\316a_2\\209a_1\end{matrix}\right.$	73	$\left\{\begin{matrix}77\\35\\25\end{matrix}\right.$	.8551	$\left\{\begin{matrix}.8516\\.8559\\.8569\end{matrix}\right.$
—	$209a_2$	—	12	—	.8612
vw	$\left\{\begin{matrix}323a_1\\323a_2\end{matrix}\right.$	67	$\left\{\begin{matrix}121\\56\end{matrix}\right.$	.8752	$\left\{\begin{matrix}.8737\\.8781\end{matrix}\right.$
—	$410a_1$	—	22	—	.8810
—	$405a_1$	—	120	—	.8839
—	$410a_2$	—	10	—	.8854
—	$405a_2$	—	55	—	.8883
vw	$\left\{\begin{matrix}411a_1\\411a_2\end{matrix}\right.$	103	$\left\{\begin{matrix}169\\78\end{matrix}\right.$	.8922	$\left\{\begin{matrix}.8895\\.8939\end{matrix}\right.$

designated by the symbols *aab*, *aab*, *abb* and *ab*. The first two atoms link the tetrahedra within an *a* chain but also connect this chain with a *b* chain. The third atom also serves as a connection between an *a* and a *b* chain but links the tetrahedra of a *b* chain. The fourth atom only connects two different chains.

A formal description of the arrangement in the unit cell is of a certain interest. When projected on (010) the structure is found to be constructed of two-dimensional oxygen layers connected by the boron atoms and indefinitely extended parallel to $(\bar{1}03)$. This is also clear from a projection on a plane — close to (102) — perpendicular to $(\bar{1}03)$. The two projections are given in Fig. 3 and Fig. 4. A single layer — there are three within the height of the unit cell — may be described as built up of close-packed oxygen spheres. A projection of such a layer on the plane $(\bar{1}03)$ is illustrated in Fig. 5, from which the unit area of the layer — marked with dashed lines — is shown to consist of two regions with different densities and extended parallel to [010]. The density

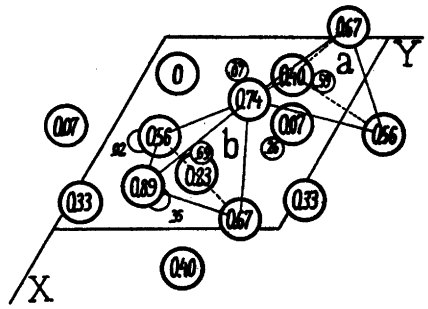

Fig. 1 a. A basal projection of atoms in and about the hexagonal unit of B_2O_3. The large circles are the oxygen atoms. The numbers denote the heights above the basal plane.

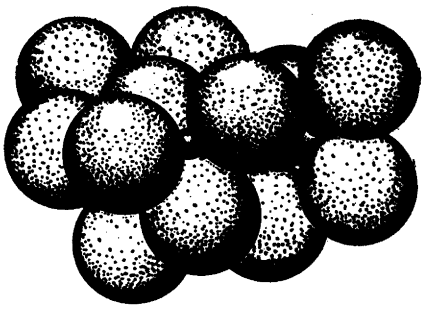

Fig. 1 b. A drawing of the B_2O_3 structure showing how the atoms in the Figure 1a pack together to provide the two sets of tetrahedra.

variation is caused by the gradual change between triangular and quadratic close-packing. Because of the trigonal symmetry the layer is repeated parallel to $(1\bar{1}3)$ and (013).

Calculated from the atomic parameters given in the previous chapter the interatomic distances are found to be:

a tetrahedron		*b* tetrahedron	
$O'_I - O'_{II}$	= 2.49 Å	$O_I - O_{II}$	= 2.41 Å
$O'_I - O'_{III}$	= 2.37	$O_I - O_{III}$	= 2.41
$O'_{II} - O'_{III}$	= 3.06	$O_{II} - O_{III}$	= 2.37_5
$O_{II} - O'_I$	= 2.65	$O'_{III} - O_I$	= 2.74
$O_{II} - O'_{II}$	= 2.93	$O'_{III} - O_{II}$	= 2.96
$O_{II} - O'_{III}$	= 2.48	$O'_{III} - O_{III}$	= 2.96
$B_I - O'_I$	= 1.31	$B_{II} - O_I$	= 1.48
$B_I - O'_{II}$	= 1.90	$B_{II} - O_{II}$	= 1.48
$B_I - O'_{III}$	= 1.60	$B_{II} - O_{III}$	= 1.37
$B_I - O_{II}$	= 1.71	$B_{II} - O'_{III}$	= 2.14_5

The denomination of the different sets of atoms as given in the previous section, is O_I, O_{II}, O_{III} and B_I, B_{II}, respectively. However, in this section a comma is added within each set when moving one step according to the screw axes.

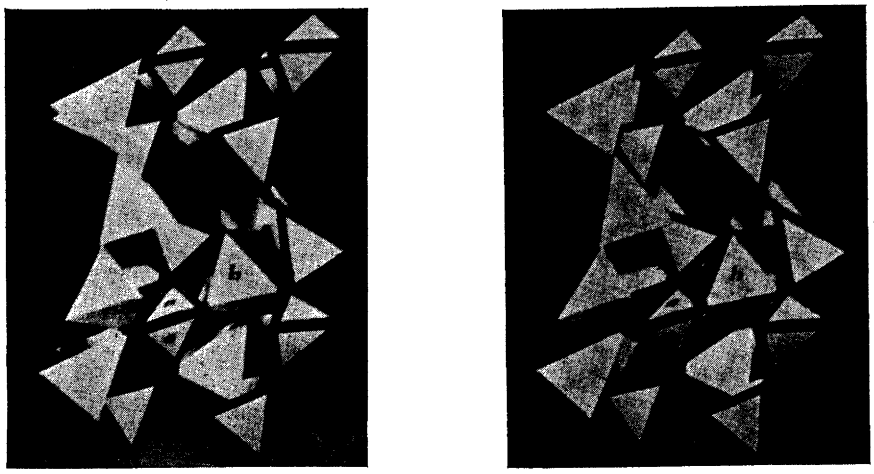

Fig. 2. A stereoscopic view showing the arrangement of the BO_4 tetrahedra of the structure of B_2O_3. Only some tetrahedra near the centre of the model (e.g. the two marked a and b) have the surroundings corresponding to a complete lattice.

Pauling's electrostatic valence rule is fulfilled if the strength of the bond $B-O_I$ is 1 and the strengths of the other B—O bonds are 2/3. In the tetrahedron *a* these bond strengths are in accord with the short distance $B_I-O'_I$, which has practically the same value as the B—O distance corresponding to

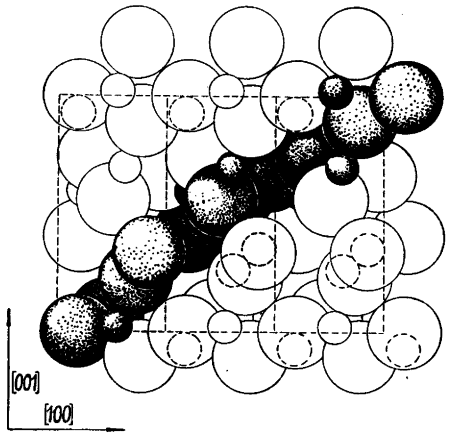

Fig. 3. A projection of the structure on the plane (010) showing oxygen layers extended parallel to the plane ($\bar{1}03$) and connected by boron atoms.

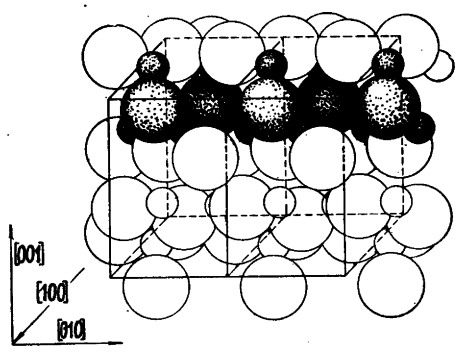

Fig. 4. A projection of the structure on a plane — close to (102) — perpendicular to ($\bar{1}03$).

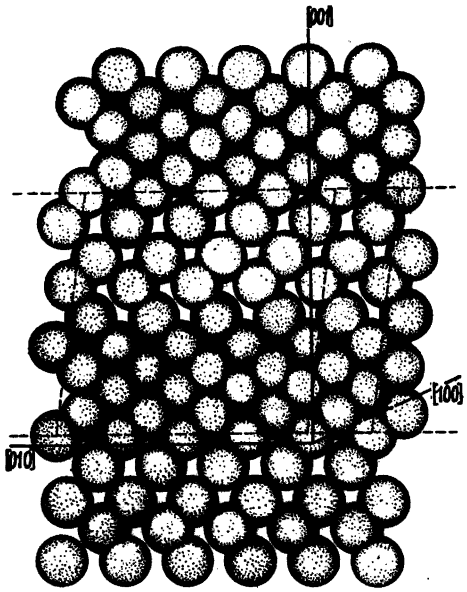

Fig. 5. A projection of one oxygen layer on the plane ($\bar{1}03$).

the strength 1 in the BO_3 triangles of known borate structures. In tetrahedron *b*, however, O_I forms part of a highly contracted oxygen triangle with the edges 2.37_5, 2.41 and 2.41 Å, respectively. The boron atom is placed only 0.44 Å from the plane of this triangle. The distance to the fourth oxygen O'_{III} is as long as 2.14_5 Å, and consequently the configuration around B_{II} is actually a hybrid between a triangular and a tetrahedral one. Attempts have been made to adjust the parameters in a way that the boron atom in tetrahedron *b* could be placed either in the centre of the oxygen triangle or in the centre of the tetrahedron but without success. The distribution of the bond strengths is evidently not so simple as could be supposed from Pauling's rule.

On account of the fact that B_2O_3 tends to crystallize spontaneously from a viscous liquid of partially dehydrated H_3BO_3 in the presence of crystals of cubic HBO_2 (designated HBO_2 I by Kracek, Morey and Merwin), it seems reasonable to suppose that a structural relationship exists between B_2O_3 and HBO_2 I. However, the crystal structure of HBO_2 I has not yet been crystallographically worked out. The monoclinic modification (designated HBO_2 II) has recently been investigated by Zachariasen [6]. Because Kracek, Morey and Merwin did not find that HBO_2 II initiated the spontaneous crystallization of B_2O_3 it is of interest in the present case to establish that the structure is built up of endless chains, the constituents of which are both BO_4 tetrahedra and BO_3 triangles, the latter forming B_2O_5 groups. The marked difference

between the two modifications of the metaboric acid, HBO_2 I and HBO_2 II, in promoting crystallization is in favour of the cubic form HBO_2 I being structurally analogous to B_2O_3.

A study of the structure of B_2O_3 and of α-(β-) SiO_2 — the last of which is a more specialized form of the α-modification only — shows that certain structural analogies exist. The symmetry is trigonal in both cases and the quartz structures are built up of SiO_4 tetrahedra forming spiral chains by coupling of corners. But in quartz the oxygen atoms are only common to two tetrahedra. Two oxygen atoms in one tetrahedron only form links to adjacent tetrahedra in the same chain. The two other oxygen atoms form connections with other chains.

In a recent paper Fajans and Barber [7] make some assumptions as to the structure of boron oxide. From various physical properties said not to be reconcilable with a structure in which a coordinative network extends, they state that boron oxide (both vitreous and crystalline) below about 300° C consists of units of molecular dimensions with a probable composition of B_4O_6. This structure with the groups held together by "weak" forces is said to change gradually with increasing temperature to a "strong" one, approaching more nearly the ideal coordinative type.

These assumptions as regards the molecular structure of B_2O_3 can, however, hardly be understood in the light of the remarkable reluctance of the glass to crystallize. Actually there is experimental evidence from Fourier analysis of vitreous B_2O_3 undertaken by Warren, Krutter and Morningstar [8], which is in complete agreement with Zachariasen's [9] predictions, showing a random network structure to exist. In this each boron is triangularly bonded to three oxygens and each oxygen is bonded to two borons and the interatomic distances found are in good agreement with the bond lengths experimentally derived for BO_3 triangles in known crystalline borates. The reluctance of the oxide to crystallize seems possible to explain by the difficulty to transform one three-dimensional network into another, which difficulty ought to be more pronounced as the coordination number of boron has to change at the same time.

It is not easy to see why a glass, built up according to Fajans and Barber from relatively small and loosely combined B_4O_6 molecules, should not transform quite easily into a crystal with principally the same structure.

SUMMARY

The crystal structure of B_2O_3 has been determined by trial-and-error methods. The unit cell contains three formula units and belongs to the space group $C_3^2-C3_1$ ($C_3^3-C3_2$). Dimensions and atomic parameters are given.

The structure is built up of two sets of BO_4 tetrahedra forming interconnected spiral chains of two types running parallel to the trigonal axis. Within each tetrahedron the strength of one B—O bond equals unity and the strengths of the remaining three bonds equals two thirds of unity. A stereoscopic view of the tetrahedral arrangement is given in Fig. 2.

I should like to express my sincere gratitude to Professor G. Hägg not only for having suggested this problem but also for the kind and helpful hospitality shown me during two years of work at his Institute. I am also indebted to Mr. E. Blomgren of the same Institute, who has kindly undertaken the piezoelectric tests.

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The Molecular Weight Distribution in Polymethyl Methacrylate Prepared by Redox Polymerisation in Water Phase. II

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In an earlier article [1], the kinetic aspects of the redox polymerisation of methyl methylacrylate in aqueous solution in the presence of a ferrous sulphate-hydrogen peroxide initiator were considered. The theoretical molecular weight distribution was derived on the assumption of the deactivation of active radicals either by disproportionation or by combination, and in each case it was compared with the distributions determined experimentally for different polymers by fractionation and viscosity measurements. In that connection, the degree of reproducibility with which the molecular weight distribution of polymethyl methacrylate can be determined by this method was also investigated. This paper gives an account of some sedimentation and diffusion measurements, and of viscosity determinations carried out on a fractionated polymer prepared by the method mentioned above, and conclusions are drawn regarding the polymolecularity of both the individual fractions and the original unfractionated product, as well as the shape of the molecules in the dissolved state. Reference may be made to the earlier paper for details concerning the conditions of polymerisation [1].

SEDIMENTATION MEASUREMENTS

A detailed account of the ultracentrifuge process is given in the monograph by Svedberg and Pedersen [2]. Only certain technical data will be mentioned here. A Svedberg oil-turbine ultracentrifuge was used. The distance between the meniscus and the axis of rotation was 5.9 cm. The speed of the rotor was in most cases 45 000 revs./min. Under these conditions the centrifugal field at the actual meniscus is 134 000 g. Lamm's scale method [3] was used for the observation of the sedimentation velocity. The temperature of the rotor was

regulated by cooling the turbine oil, and the temperature recorded by means of a thermocouple immediately adjacent to the rotor. The mean value of the difference between the highest and lowest temperatures during any one running period was 0.23° C. The highest temperature measured was 21.8° C, and the lowest 19.2° C. The sedimentation constants have been corrected to a temperature of 20.0° C according to the relation:

$$s_{20} = s_t \frac{\eta_t}{\eta_{20}} \frac{(1-V_{20}\varrho_{20})}{(1-V_t\varrho_t)}$$

where s_{20} and s_t are the sedimentation constants at 20° and t° respectively, and η_{20}, η_t and ϱ_{20}, ϱ_t the viscosities and densities of the solvent at the corresponding temperatures. V is the partial specific volume of the solute. The last factor can be put equal to 1 for the temperature interval used. Since the hydrostatic pressure varies from about 1 atm. at the meniscus to 200—400 atm. at the bottom of the cell, depending on the density of the solvent, changes in the viscosity and density of the solvent at different cell levels must be taken into account. The sedimentation constant has been reduced to the value at 1 atm. by means of the formula [3]

$$s_1 = s_p \frac{\eta_p\ (1-V_1\varrho_1)}{\eta_1\ (1-V_p\varrho_p)}$$

where all symbols have their usual significance. The indexes 1 and p refer to pressures of 1 and p atm. There are no available data about the variation of the partial specific volume of polymethyl methacrylate with pressure, but as it is generally assumed that this variation is small, V_p has been put equal to V_1. The solvent used was ethyl acetate. For the part of the cell used the correction factor varies from 1.02 to 1.10.

In order to test the reliability of the correction formula, $s_1 = s_p \frac{\eta_p\,(1-V_1\varrho_1)}{\eta_1\,(1-V_p\varrho_p)}$, the sedimentation rate of a polymethyl methacrylate fraction was determined for three different rotor speeds and three different dilutions. Table 1 gives the values of s_{20} which were obtained. The uncorrected value of s_{20} shows a definite trend for all concentrations, while the corrected value shows no systematic variation with rotor speed. This is most clearly indicated by the figures in the last two columns, where $\Sigma\, s_{20}$ for all three concentrations is given for the three rotor speeds. The table also gives the magnitude of the mean deviation from the mean $\bar{s}$ defined as $\frac{\Sigma|s-\bar{s}|}{n}$, for the different sedimentation constants. The deviation thus defined is of the order of 1 % for the corrected s_{20} values.

Table 1. The effect of the speed of rotation on the sedimentation constant.

	Conc. 0.1629 g/ml		Conc. 0.1091 g/ml		Conc. 0.0561 g/ml		All three dilutions	
Rotor speed	s_{20} uncorr.	s_{20} corr.	s_{20} uncorr.	s_{20} corr.	s_{20} uncorr.	s_{20} corr.	Σs_{20} uncorr.	Σs_{20} corr.
600 revs/s	15.8	16.3	17.0	17.8	18.0	18.6	50.8	52.7
800 revs/s	15.0	16.2	16.0	17.3	17.2	18.8	48.2	52.3
1 000 revs/s	13.9	15.8	15.6	18.1	15.5	18.5	45.0	52.4
Mean	14.9	16.1	16.2	17.7	16.9	18.6		
Mean deviation	4.5 %	1.2 %	3.3 %	1.7 %	5.5 %	0.5 %		

Measurements were made on six different concentrations for each fraction. In extrapolating the sedimentation constants to infinite dilutions, the following relationship was used [5]

$$s_{20} = s^0_{20} - K \cdot s_{20} \cdot c$$

where s_{20} is the sedimentation constant measured at the concentration c, and s^0_{20} the extrapolated value for zero concentration. The method of least squares has been used for the determination of s^0_{20} and K. Figure 1 shows how s_{20} and $s_{20} \times c$ vary with the concentration. The sedimentation constant for polymethyl methacrylate exhibits a remarkably low concentration dependence in ethyl acetate, when compared with benzene for instance [6]. For the first three fractions K is of the order of magnitude 5—6, whereas K for the lower fractions lies between 0.8 and 2.5. The concentration arbitrarily used here is that at the boundary between the solvent and the solution which has been put equal to half the initial concentration in the extrapolation formula (see Table 2).

CALCULATION OF THE HETEROGENEITY OF THE FRACTIONS

Gralén [7] introduced the width of the sedimentation curve as a measure of the polymolecularity of a substance. As the measure of the width (B) he used the ratio of the area of the curve to its maximum height

$$B(x_m) = \frac{\int_{x_0}^{\infty} \left(\frac{dc}{dx}\right) dx}{\left(\frac{dc}{dx}\right)_{max}}$$

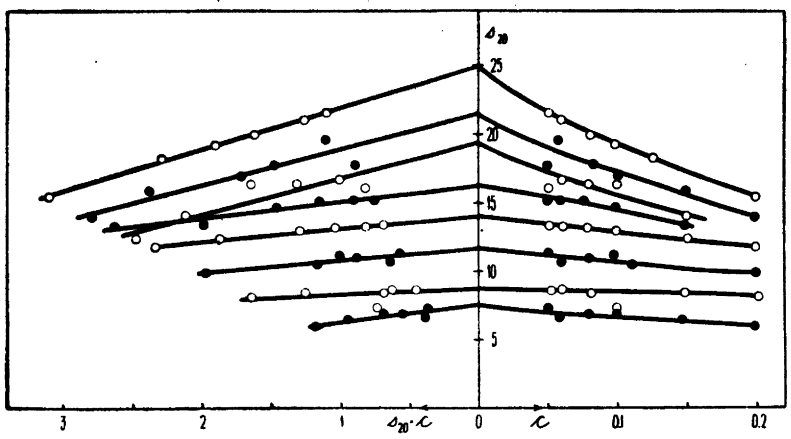

Fig. 1. The sedimentation constant s_{20} both as a function of concentration c and as a function of the product $s_{20} \times c$. The solvent used was ethyl acetate.

The width varies with the position of the maximum point in the cell (x_m). The derivative $\left(\frac{dB}{dx_m}\right)$ is in its turn a function of the solute's concentration (c), as long as this is not so low that the macromolecules sediment independently of one another. In determining $\left(\frac{dB}{dx_m}\right)$, the measured value of B is plotted against x_m, and the slope at the origin gives the value of $\lim_{x_m \to x_0}\left(\frac{dB}{dx_m}\right) = \lim_{t \to 0}\left(\frac{dB}{dx_m}\right) = \left(\frac{dB}{dx}\right)_c$. As this slope depends on the concentration, $\left(\frac{dB}{dx}\right)_c$ is determined for several dilutions, whereupon a value of $\left(\frac{dB}{dx}\right)_{c=0}$ may be obtained by extrapolation. The diffusion constant was so low for the substances for which Gralén [7] and Rånby [8] previously used this method of estimating the polymolecularity, that the broadening of the sedimentation curve could be attributed entirely to the different sedimentation rates of the solute particles (different s values). But the contribution of diffusion to the width of the curve is so large for the fractionated polymethyl methacrylates concerned here, that a correction is necessary.

The following assumptions are made:

1) Diffusion and sedimentation occur independently in the centrifuge cell. This is not quite true for higher concentrations of polymethyl methacrylate, but since $\left(\frac{dB}{dx}\right)_c$ is always extrapolated to 0, the assumption is probably justified.

Table 2. The sedimentation constant and $\left(\frac{dB}{dx}\right)$ *for the different fractions in ethyl acetate solutions.*

Concentration	s_{20} (corr.)	$\frac{dB}{dx}$	$\frac{dB}{dx}$ (corr.)	Concentration	s_{20} (corr.)	$\frac{dB}{dx}$	$\frac{dB}{dx}$ (corr.)
Fraction L(6) F(2) I				Fraction L(6) F(2) II			
0.2004	15.5	0.22	0.17	0.1995	14.0	0.26	0.17
0.1254	18.3	0.29	0.26	0.1499	15.9	0.30	0.24
0.0988	19.3	0.30	0.25	0.1012	17.0	0.30	0.27
0.0811	20.0	0.29	0.29	0.0833	17.8	0.30	0.25
0.0600	21.1	0.37	0.22	0.0578	19.6	0.35	0.30
0.0511	21.6	0.34	0.28	0.0502	17.8	0.42	0.37
0	25.0		0.27	0	21.5		0.30
$K = 6.0$				$K = 5.2$			
Fraction L(6) F(2) III				Fraction L(6) F(2) IV			
0.2000	12.4	0.14		0.1984	13.3	0.19	
0.1502	14.1	0.26		0.1487	13.4	0.29	0.24
0.1005	16.4	0.28	0.23	0.0994	14.7	0.37	0.31
0.0799	16.4	0.24	0.16	0.0760	15.2	0.30	0.20
0.0604	16.7	0.23	0.19	0.0595	15.2	0.32	0.25
0.0508	16.1	0.26	0.21	0.0499	15.2	0.30	0.23
0	19.4		0.21	0	16.3		0.27
$K = 5.2$				$K = 2.4$			
Fraction L(6) F(2) V				Fraction L(6) F(2) VI			
0.1986	11.8	0.25	0.14	0.1992	9.92	0.34	0.21
0.1512	12.4	0.31	0.17	0.1113	10.5	0.39	0.24
0.0995	13.0	0.33	0.22	0.0982	11.2	0.39	0.26
0.0787	13.2	0.30	0.24	0.0803	11.0	0.41	0.32
0.0615	13.3	0.32	0.24	0.0601	10.7	0.48	0.35
0.0513	13.4	0.37	0.31	0.0502	11.4	0.37	0.29
0	14.1		0.26	0	11.7		0.32
$K = 1.9$				$K = 1.8$			
Fraction L(6) F(2) VII				Fraction L(6) F(2) VIII			
0.2017	8.15	0.43	0.16	0.1989	5.97	0.72	0.27
0.1487	8.45	0.41	0.21	0.1468	6.48	0.69	0.22
0.1001	7.35	0.43	0.21	0.0999	6.92		
0.0815	8.41	0.46	0.17	0.0796	6.89	0.72	0.35
0.0606	8.70	0.42	0.27	0.0585	6.65	0.55	0.11
0.0525	8.64	0.41	0.17	0.0503	7.36	0.55	
0	8.8		0.22	0	7.6		0.25
$K = 0.8$				$K = 2.5$			

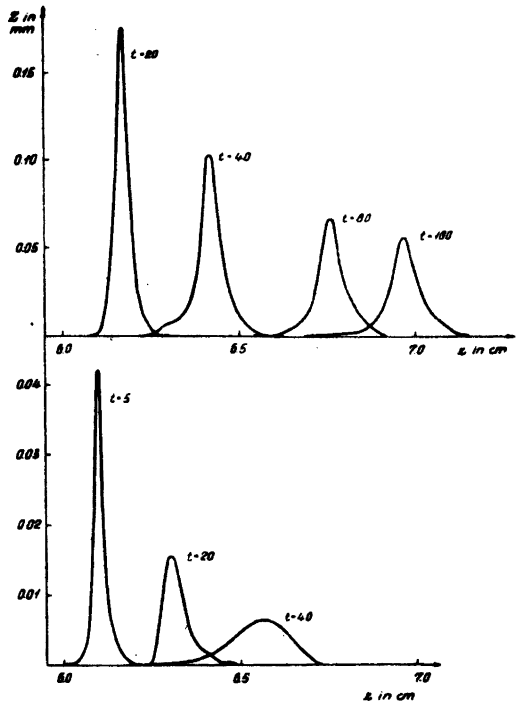

Fig. 2. Sedimentation diagram for the sample L (6) F (2) III, dissolved in ethyl acetate at a concentration of 0.200 g/100 ml (upper diagram) and 0.051 g/ml (lower diagram). The times given for the exposures are times after the rotor attained full speed. The distance from the scale to the cell was 2.0 cm.

2) Each fraction is supposed to be such that the mass is distributed over the different s values in accordance with a Gaussian frequency curve with first moment $\bar{s}$, second moment p^2 and width

$$b = \frac{\int_0^\infty \left(\frac{dc_0}{ds}\right) ds}{\left(\frac{dc_0}{ds}\right)_{max}}$$

This assumption is rather arbitrary, but may be regarded as permissible in view of the fact that it is only an estimation of the fraction's polymolecularity which is required, and in view of the actual appearance of the experimental curves (Fig. 2).

Symbols used:

σ^2_{total} = the total second moment of the experimental sedimentation diagram,
$\sigma^2_{sed.}$ = the second moment if only sedimentation occurred,
$\sigma^2_{diff.}$ = the second moment if only diffusion occurred,
s = the sedimentation constant,
D = the diffusion constant,
ω = the angular velocity of the rotor,
t = time,
x = position coordinate in the centrifuge cell,
dc_0 = the concentration of substance with sedimentation constants in the interval s to $s + ds$ initially,
dc = the concentration of substance with sedimentation constants in the interval s to $s + ds$ at time t and at the point x,
x_0 = the distance of the meniscus from the axis of rotation.

At time t, molecules with sedimentation constant s are at the level x given by

$$x = x_0 \, e^{\omega^2 st} \tag{1}$$

On account of the cell's sector form there is a dilution [2], so that the concentration of molecules with sedimentation constants in the interval s to $s + ds$ at the level x and time t is

$$dc = dc_0 \, e^{-2\omega^2 st} \tag{2}$$

On differentiating eq. (1) we get

$$dx = x_0 \, e^{\omega^2 st} \, \omega^2 t ds \tag{3}$$

Furthermore, according to eq. (2),

$$dc = \left(\frac{dc}{dx}\right) dx = \left(\frac{dc_0}{ds}\right) (ds) e^{-2\omega^2 ts} \tag{4}$$

For the second moment we have

$$\sigma^2_{sed} = \int_0^\infty (x_0 e^{\omega^2 \bar{s} t} - x_0 e^{\omega^2 st})^2 \left(\frac{dc}{dx}\right) dx \tag{5}$$

or

$$\sigma^2_{sed} = \int_0^\infty (x_0 e^{\omega^2 (\bar{s} - s) t} - x_0)^2 \left(\frac{dc_0}{ds}\right) ds \tag{6}$$

Expansion in series gives

$$\sigma^2_{sed} = \int_0^\infty x_0^2 \left\{ \omega^2(\bar{s}-s)t + \frac{\omega^4(\bar{s}-s)^2t^2}{2!} e^{\Theta\omega^2(\bar{s}-s)t} \right\}^2 \left(\frac{dc_0}{ds} \right) ds \qquad (7)$$

and if we neglect the higher terms,

$$\sigma^2_{sed} = x_0^2\omega^4t^2 \int_0^\infty (\bar{s}-s)^2 \left(\frac{dc_0}{ds} \right) ds = x_0^2\omega^4t^2p^2 \qquad (8)$$

According to assumption 1)

$$\sigma^2_{total} = \sigma^2_{diff} + \sigma^2_{sed} \qquad (9)$$

According to assumption 2) p^2 is the second moment of a Gaussian distribution; and under the conditions for which it can be justified to neglect the higher terms in equation (7) σ^2_{sed} can also be considered to correspond to a Gaussian distribution. σ^2_{diff} is the second moment of a Gaussian distribution according to the accepted theory of free diffusion [3]. Therefore, σ^2_{total} must be the second moment of a Gaussian distribution. For such a parameter,

$$B^2 = 2\pi\sigma^2 \qquad (10)$$

or, according to (9) and (10),

$$B^2_{total} = B^2_{diff} + B^2_{sed} \qquad (11)$$

and according to eqs. (8), (10) and (11)

$$B_{sed} = \sqrt{B^2_{total} - B^2_{diff}} = x_0\omega^2tb \qquad (12)$$

For B_{diff} we have

$$B^2_{diff} = 4\pi Dt \qquad (13)$$

so that

$$B_{sed} = \sqrt{B^2_{total} - 4\pi Dt} \qquad (14)$$

If eq. (12) is differentiated we get

$$\left(\frac{dB}{dx} \right) = \left(\frac{dB}{dt} \right) \left(\frac{dt}{dx} \right) = x_0\omega^2b \frac{1}{x_0\omega\bar{s}e^{\omega^2\bar{s}t}} \qquad (15)$$

and

$$\lim_{\substack{x=x_0 \\ t=0}} \left(\frac{dB}{dx} \right) = \frac{b}{\bar{s}} \qquad (16)$$

Formula (14) has been used for correcting the width, t being taken as time after the rotor attained its full speed. Table 2 gives both the corrected and uncorrected values of $\left(\frac{dB}{dx}\right)_c$ for all concentrations and fractions, for comparison purposes.

DIFFUSION MEASUREMENTS

The diffusion constants were determined at 20° C. Unless otherwise stated, the diffusion cell was of the Claesson type [9]; in some cases a Svedberg cell was used [3]. The diffusion was followed by means of Lamm's scale method [3]. The diffusion constant was as a

Table 3. Diffusion constants for sample L(6) F(2) V in ethyl acetate solutions.

Concentration Higher	Concentration Lower	Diffusion cell	D_A	D_m
0.40	0	Claesson (1′)	4.22	4.56
		» (1″)	4.14	4.18
		» (2)	3.29	3.23
		» (2)	3.76	3.35
		Svedberg	*3.39*	*3.27*
			3.76	3.72
0.30	0	Claesson (1′)	3.96	4.00
		» (1″)	4.13	4.21
		» (2)	3.75	4.22
		Svedberg	*3.46*	*3.25*
			3.83	3.92
0.25	0	Claesson (1′)	3.36	3.13
		» (1″)	3.68	3.59
		» (2)	*3.45*	*3.49*
			3.50	3.40
0.60	0.40	Claesson	3.78	3.60
0.50	0.30	Claesson	4.15	4.23
0.30	0.10	Claesson	3.67	3.67
Mean		$\bar{D}$	3.75	3.73
Mean deviation $= \frac{\lvert D-\bar{D}\rvert}{n}$			0.26	0.40

Table 4. Diffusion constants for sample L(6) F(2) VI in ethyl acetate.

Concentration		D_A	D_m
Higher	Lower		
0.60	0.40	4.48	4.10
0.50	0.30	4.65	4.52
		5.10	4.50
0.40	0.20	4.69	4.30
Mean $\bar{D}$		4.73	4.36

rule calculated for three or four different concentrations. In some indicated cases the differential diffusion constant was measured. As may be seen from Tables 3, 4 and 5, the reproducibility was poor, probably due to the fact that no suitable sealing substance was found. As no systematic trend of the diffusion constant with concentration could be detected, the mean value of the diffusion constants for all concentrations used in the experiments was used in the calculation of the molecular weights *.

Table 5. Diffusion constants for different fractions of polymer L(6) in ethyl acetate solutions.

Concentration	L(6)F(2) I		L(6)F(2) II		L(6)F(2) III		L(6)F(2) IV		L(6)F(2) V		L(6)F(2) VI		L(6)F(2) VII		L(6)F(2) VIII	
	D_A	D_m	D_A	D_m	D_A	D_m	D_A	D_m	D_A	D_m	D_A	D_m	D_A	D_m	D_A	D_m
0.40	2,41	2.41	2.37	2.33	3.44	3.38	3.51	3.56					5.68	5.84		
0.30	2.38	2.35	2.58	2.61			—	—					5.59	5.08		
0.25	2.23	2.25	—	—			3.87	4.46	See Table 3		See Table 4		6.09	5.75		
0.20	1.99	—	2.75	2.69			3.36	3.34					5.90	5.97	8.10	7.21
Mean value	2.25	2.34	2.57	2.54	3.44	3.38	3.58	3.79	3.75	3.73	4.73	4.36	5.82	5.66	8.10	7.21

DETERMINATION OF THE PARTIAL SPECIFIC VOLUME

It is necessary to know the partial specific volume of the substance dissolved in the solvent used when calculating the molecular weight from measurements of sedimentation and diffusion. For this purpose two 30 ml pychnometer flasks as near as possible identical

* This decision also takes into account a large number of diffusion measurements which have not yet been published.

were used, the solution and the solvent being introduced into the respective flasks by means of a syringe. One of the flasks was used just as a counterweight, and was filled with the pure solvent. The other pychnometer contained either pure solvent to begin with, in which case the concentration of polymer was increased by removing some of the solution and replacing with concentrated solution, or concentrated solution initially, in which case the concentration was diminished in an analogous manner. In measurement series 3, the dry polymethyl methacrylate was introduced into the pychnometer through an extra side tube, which was then sealed off.

The calculation of the partial specific volume V_1 of the solute involves the relation

$$V_1 = V + \omega_2 \frac{\partial V}{\partial \omega_1}$$

where V is the specific volume of the solution, and ω_1, ω_2 the weight fractions for solute and solvent respectively. The derivative $\frac{\partial V}{\partial \omega_1}$ may be taken to be constant in the concentration interval concerned and was determined by the method of least squares (Table 6).

Table 6 a. The partial specific volume of polymethyl methacrylate dissolved in ethyl acetate.

Measurement series	$-\frac{dV}{dc}$	Partial spec. volume
1.	0.236	0.785
2.	0.316	0.795
3.	0.330	0.781
	Mean	0.787

Table 6 b. The partial specific volume of polymethyl methacrylate dissolved in benzene.

Measurement series	$-\frac{dV}{dc}$	Partial spec. volume
1.	0.321	0.818

MOLECULAR WEIGHT DETERMINATIONS

The molecular weights M_{Ds} of the different fractions were calculated with the aid of Svedberg's formula [2]

$$M_{Ds} = \frac{RTs}{D\ (1-V\varrho)}$$

where T = the absolute temperature,
R = the universal gas-constant,
V = the partial specific volume of the polymer,
ϱ = the density of the solvent.
See Table 7.

VISCOSITY MEASUREMENTS

The intrinsic viscosity $[\eta]$ was determined by means of an Ostwald viscometer at 20.0° C. The solvent was either ethyl acetate or benzene. The reduced viscosity $\frac{\eta_{sp}}{c}$ was determined for the following concentrations (c): 0.40, 0.30, 0.20, and 0.10 g of dry solute in 100 ml solution. The intrinsic viscosity was obtained by extrapolating $\frac{\eta_{sp}}{c}$ to infinite dilution. This extrapolation was facilitated by the fact that $\frac{\eta_{sp}}{c}$ is a linear function of the concentration:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2\ c$$

Besides $[\eta]$, the constant k' was also determined. The molecular weight $(M)_\eta$ corresponding to the intrinsic viscosity $[\eta]$ was calculated by means of the formula [10] *

$$(M)_\eta = 2.81 \cdot 10^5\ [\eta]^{1.32}$$

See Table 7.

THE MASS DISTRIBUTION OF THE UNFRACTIONATED POLYMER

In constructing a curve of mass distribution for the unfractionated polymer product, it is possible to apply Schulz's method [11] and assume that half the mass of a fraction has a s_0 value lower than the sedimentation constant determined for the fraction, the other half having a higher value, and that the fractions do not overlap. In this method it is not possible to take the polydispersity of the fractions into account, and it therefore gives an uncertain result when the fractions are broad. A construction more satisfactory in principle has been

* The constants in this formula are determined at 25.0° C and the molecular weights calculated from them here are consequently 2—3 % too low.

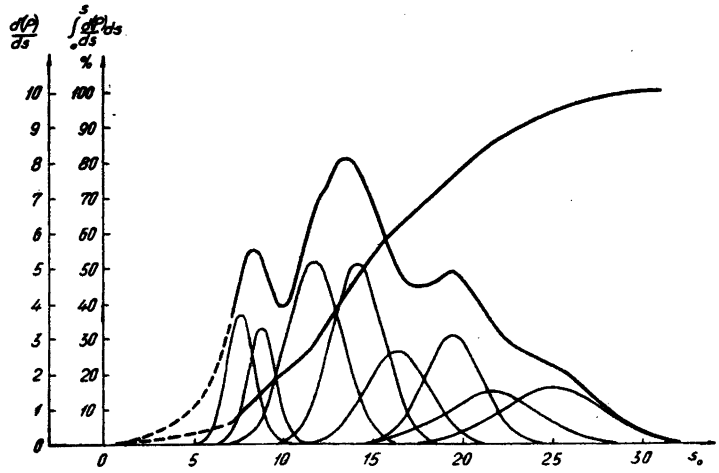

Fig. 3. Frequency curve and mass distribution curve for the unfractionated polymer together with the frequency curves for the separate fractions.

given by Rånby [8] for the case where the fractions' $\left(\frac{dB}{dx}\right)$ values are known. In this method each fraction's frequency curve is approximated to by a symmetrical triangular curve where the s_0 value for the apex of the triangle is put equal to the s_0 value determined for the fraction. In accordance with the definition of b, the base of the triangle is $2b = 2\bar{s}_0\left(\frac{dB}{dx}\right)$ (formula 16). The value adopted for the height of the triangle is such that the area of the triangle is proportional to the weight of the fraction. $\sigma = \frac{-\,s\frac{dB}{dx}}{\sqrt{2\pi}}$, according to formulas (10) and (16). It is therefore also possible to construct a Gaussian frequency curve for which the area is proportional to the weight of the fraction and σ equal to the σ value calculated for the fraction. The frequency curves constructed in this way for the various fractions are given in Fig. 3. The figure provides a good illustration of the polydispersity of the different fractions. It will also be seen from the figure that the different fractions overlap one another almost everywhere. Addition of all the ordinates for each of a series of s_0 values gives the frequency curve for the original unfractionated polymethyl methacrylate. As no measurements were made for fraction IX, which was merely an evaporation residue, the left of the frequency curve for the original product is indicated by a broken line. The three methods for obtaining the

Table 7.

Fraction	Weight in %	$M_{Ds}10^{-3}$	$[\eta]_{benzene}$	$k'_{benzene}$	$M_{\eta\ benzene}10^{-3}$	$[\eta]_{ethyl\ acetate}$	$k'_{ethyl\ acetate}$	$\frac{M_{Ds}}{M_\eta}$
L(6)F(2) I	10.75	931	2.08	0.32	739	1.24	0.37	1.26
L(6)F(2) II	9.36	701	1.72	0.28	575	1.10	0.35	1.22
L(6)F(2) III	12.56	473	1.43	0.34	451	0.96	0.39	1.05
L(6)F(2) IV	11.50	381	1.30	0.30	397	0.77	0.54	0.96
L(6)F(2) V	18.75	315	1.00	0.42	281	0.64	0.64	1.12
L(6)F(2) VI	19.23	207	0.71	0.31	179	0.49	—	1.16
L(6)F(2) VII	6.39	127	—	—	—	0.37	—	
L(6)F(2) VIII	7.00	79	0.26	—	48	0.27	—	1.65
L(6)F(2) IX	4.45							

mass distribution of the unfractionated polymer product give results in close agreement in this case (Table 8).

It will be seen from columns 3, 6 and 9 of Table 7 that the two methods for the determination of the molecular weight do not give the same result (*cf.* also footnote p. 634), as they give different kinds of averages for a polymolecular fraction. Neither method gives a well-defined average, the nature of the average in each case being very complex and difficult to interpret. Determination of the molecular weight by means of the ultracentrifuge and diffusion measurements assigns greatest significance to the molecules for which the concentration by weight is largest. This uncertainty with regard to the nature of the average naturally makes it difficult to compare the mass distri-

Table 8. The mass distribution of the original sample calculated by a) Schulz's method, b) by Rånby's triangle method, and c) with the aid of Gaussian frequency curves.

Fraction	$\bar{S}_0$	Distribution according to		
		a)	b)	c)
L(6)F(2) I	25.0	94.6 %	93.6 %	93.5 %
L(6)F(2) II	21.5	84.6	84.3	84.2
L(6)F(2) III	19.4	73.6	75.4	75.0
L(6)F(2) IV	16.3	61.6	60.6	61.0
L(6)F(2) V	14.1	46.5	46.7	46.5
L(6)F(2) VI	11.7	27.5	28.6	28.5
L(6)F(2) VII	8.8	14.7	14.8	14.5
L(6)F(2) VIII	7.6	8.0	8.4	8.2

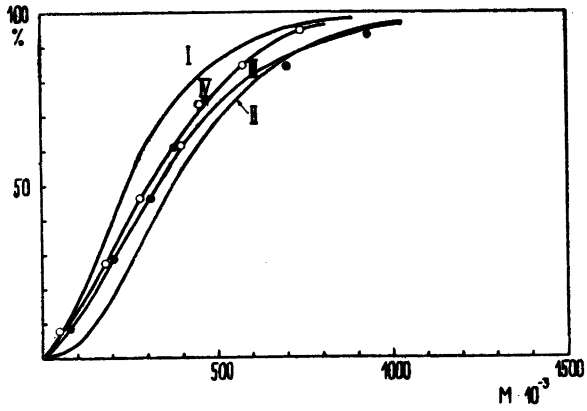

Fig. 4. Mass distributions for the unfractionated polymer.

Curve I: theoretical, calculated on the basis of deactivation of the growing molecule through disproportionation [1].

Curve II: theoretical, calculated on the basis of deactivation of the growing molecule through combination [1].

Curve III: calculated from the sedimentation and diffusion constants on the assumption of Gaussian distributions for the separate fractions.

Curve IV: calculated from the intrinsic viscosity of the fractions in accordance with Schulz's method.

bution of the unfractionated product as determined experimentally and as determined theoretically. However, it does appear that the weight average for a polydisperse fraction is to be preferred to the number average. Curve IV in Fig. 4 represents the mass distribution obtained from viscosity data according to Schulz's method, while curve III gives the corresponding distribution calculated from diffusion and sedimentation data. Curves I and II are theoretical curves based on the assumption that the growing radicals are rendered inactive by either disproportionation (I) or combination (II). As regards the theoretical calculations, see [1].

THE MOLECULAR SHAPE AND FLEXIBILITY OF POLYMETHYL METHACRYLATE

The experimental data also give us some information about the shape and flexibility of the polymethyl methacrylate molecule in the dissolved state. The intrinsic viscosity of polymethyl methacrylate depends to a great extent on the particular solvent. In the poor solvent isobutyl acetate, for instance, it is about one eight of what it is in chloroform [6]. This close dependence on the

Table 9. The dependence of the

Polymethyl methacrylate (I) in ethyl acetate					Polymethyl methacrylate (II) in ethyl acetate			
Temperature °C	20	30	40	Per cent/ degree	20	30	40	Per cent/ degree
Concentration c	0.3014	0.2970	0.2930		0.3971	0.3914	0.3861	
Specific viscosity η_{sp}	2.448	2.501	2.567	0.24	0.338	0.343	0.349	0.16
Reduced viscosity $\frac{\eta_{sp}}{c}$	8.12	8.42	8.76	0.39	0.85	0.88	0.90	0.29
Concentration c	0.1990	0.1962	0.1935		0.2985	0.2942	0.2902	
Specific viscosity η_{sp}	1.356	1.390	1.425	0.25	0.247	0.251	0.255	0.16
Reduced viscosity $\frac{\eta_{sp}}{c}$	6.81	7.09	7.36	0.40	0.83	0.85	0.88	0.30
Concentration c	0.1117	0.1101	0.1086		0.1984	0.1955	0.1929	
Specific viscosity η_{sp}	0.649	0.666	0.681	0.25	0.158	0.161	0.164	0.19
Reduced viscosity $\frac{\eta_{sp}}{c}$	5.81	6.05	6.27	0.40	0.80	0.82	0.85	0.31
Concentration c	0.0490	0.0483	0.0476		0.0995	0.0981	0.0968	
Specific viscosity η_{sp}	0.256	0.262	0.267	0.21	0.076	0.077		
Reduced viscosity $\frac{\eta_{sp}}{c}$	5.23	5.42	5.61	0.36	0.76	0.78		
			Mean	0.24 0.39			Mean	0.17 0.30

nature of the solvent indicates that the polymethyl methacrylate molecules are very flexible, assuming a shape which is more or less extended according as the affinity for the solvent is greater or less than the affinity between the different molecule segments of the polymer. Theoretical considerations [12, 14] show that for a homologous series of polymers the intrinsic viscosity and the molecular weight M are related by the equation $[\eta] = KM^a$, where K and a are constants. In the limiting case where the molecules adopt a long, rigid form, $a = 1$, while in the other extreme case, where $a = 0.5$, the molecules assume a coiled shape. If the above relation is applied to the values obtained for M_{Ds} $[\eta]_{\text{benzene}}$ and $[\eta]_{\text{ethyl acetate}}$ given in Table 7, the following values for K and a result:

$K = 2.11 \cdot 10^{-4}$ $\quad a = 0.64$ for ethyl acetate

$K = 1.51 \cdot 10^{-4}$ $\quad a = 0.70$ for benzene

viscosity on temperature.

Polymethyl methacrylate (I) in benzene					Polymethyl methacrylate (II) in benzene			
Concentration c	0.3007	0.2969	0.2935		0.4011	0.3961	0.3915	
Specific viscosity η_{sp}	4.768	4.758	4.856	0.09	0.562	0.559	0.558	− 0.04
Reduced viscosity $\frac{\eta_{sp}}{c}$	15.86	16.03	16.54	0.21	1.40	1.41	1.43	0.10
Concentration c	0.2093	0.1991	0.1968		0.3033	0.2995	0.2961	
Specific viscosity η_{sp}	2.571	2.586	2.617	0.09	0.411	0.407	0.407	− 0.05
Reduced viscosity $\frac{\eta_{sp}}{c}$	12.76	12.99	13.30	0.21	1.35	1.36	1.37	0.07
Concentration c	0.1500	0.1481	0.1464		0.2009	0.1984	0.1961	
Specific viscosity η_{sp}	1.685	1.688	1.706	0.06	0.261	0.261	0.261	0
Reduced viscosity $\frac{\eta_{sp}}{c}$	11.24	11.40	11.65	0.18	1.30	1.32	1.33	0.12
Concentration c	0.0994	0.0981	0.0970		0.1004	0.0992	0.0980	
Specific viscosity η_{sp}	0.979	0.982	0.991	0.06	0.125	0.125	0.126	+ 0.04
Reduced viscosity $\frac{\eta_{sp}}{c}$	9.85	10.01	1.021	0.18	1.25	1.26	1.28	0.12
Concentration c	0.0498	0.0491	0.0486					
Specific viscosity η_{sp}	0.443	0.447	0.448	0.06			Mean	− 0.01
Reduced viscosity $\frac{\eta_{sp}}{c}$	8.89	9.10	9.24	0.20				0.10
			Mean	0.08 0.19				

Meyerhoff and Schulz [13] give the values $a = 0.73$ for benzene and $a = 0.80$ for chloroform. It is therefore clear that the molecules are more tightly coiled in ethyl acetate than in benzene. This conclusion is also in agreement with the fact that the dependence of $[\eta]$ on temperature is 2 to 3 times larger in the former solvent than in the latter (see Table 9).

The relation between s and M_{Ds} gives us further information about the shape of the polymethyl methacrylate molecule. According to Kirkwood and Riseman [14, 15], a statistically coiled molecule experiences a frictional resistance to a translational movement which may be expressed by

$$f_T = \frac{Z\ \xi}{1 + \frac{8}{3} Z^{1/2} \lambda_0} \qquad \lambda_0 = \frac{\xi}{\sqrt{6\pi^3 \eta_0 b}}$$

where ξ = the frictional coefficient describing the friction between the chain element and the surrounding liquid,
b = the effective length of the segment,
Z = the number of segments,
η_0 = the viscosity of the solvent.

Furthermore,

$$s = \frac{\frac{dx}{dt}}{\omega^2 x} = \frac{M\ (1-V_\varrho)}{N f_T}$$

so that

$$s = \frac{M\ (1-V_\varrho)}{NZ\xi}\ (1 + \frac{8}{3}\ Z^{1/2}\lambda_0)$$

Or, if half the molecular weight of the monomer is taken as the mean value of the molecular weight of the segment,

$$M = M_0 Z = 50\ Z$$

$$s = \frac{M_0\ (1-V_\varrho)}{N\xi}\ (1 + \frac{8}{3}\ Z^{1/2}\lambda_0) = \frac{M_0\ (1-V_\varrho)}{N\xi}\ (1 + \frac{8}{3}\ \frac{\lambda_0 M^{1/2}}{M_0^{1/2}})$$

Table 10 gives the experimentally established relation between the sedimentation constant and the molecular weight, and the values of b and ξ calculated from them. For comparison, the corresponding relation has also been calculated for Meyerhoff and Schulz's [13] data for acetone in the molecular weight interval 77 000—611 000, this being the interval which most nearly corresponds to that for the ethyl acetate solutions. The effective segment length is somewhat smaller in ethyl acetate than in acetone, but on the other hand ξ is larger.

Finally the partial specific volume for polymethyl methacrylate in ethyl acetate is only 0.787, while in benzene it is 0.818; in the solid state it is 0.84.

Table 10.

Solvent	$s = A + B\sqrt{M}$	b	ξ
Ethyl acetate	$s = -0.06 + 0.026\sqrt{M}$	$5.6\ 10^{-8}$	$> 24.2 \cdot 10^{-10}$
Acetone	$s = 1.64 + 0.0441\ \sqrt{M}$	$5.9\ 10^{-8}$	$1.87 \cdot 10^{-10}$

SUMMARY

The sedimentation and diffusion constants were determined for a complete series of polymethyl methacrylate fractions. The sedimentation constant for the solution in ethyl acetate is comparatively little dependent of the concentration. Values of s_{20} for zero concentration were obtained by extrapolation in accordance with the formula $\frac{1}{s_{20}} = \frac{1}{s^0_{20}} + \frac{K}{s^0_{20}} c$. No dependence on concentration was found for the diffusion constant, though the experimental error was large in this case.

The polydispersity of the separate fractions and of the unfractionated polymer were determined. A formula is suggested for the correction of the experimental $\left(\frac{dB}{dx}\right)$ values, to take the effect of diffusion into account. Different methods for determination of the molecular weight give somewhat different experimental mass distributions.

Polymethyl methacrylate molecules are very flexible, and assume a compact coiled shape in ethyl acetate solution.

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Microwave Investigation of Tertiary Butyl Fluoride

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Determinations of the molecular structure of tertiary butyl chloride, bromide, and iodide have earlier been made by means of electron-diffraction technique [1] and microwave measurements [2]. All three molecules are symmetric tops, the threefold axis of symmetry being the carbon-halogen bond.

The electron-diffraction studies resulted in a model in which the carbon-halogen bond length was "normal", *i. e.* the same as in the methyl halides, while the angle C—C—C was found to be about 2° greater than the tetrahedral (109° 28′). The microwave measurements seemed, however, to indicate that the valence angles did not deviate from 109° 28′ and that the carbon-halogen bond length was "normal" inspite of the "tertiary" position of the halogen. In order to see whether the well-known differences in chemical reactivity of primary and tertiary bonded halogen may be partly or wholly contributed to a difference in bond length the present investigation of *tertiary butyl fluoride* was undertaken. In so doing one has, of course, abandoned to say anything definitely about the halides hitherto investigated, but probably the result for the fluoride does not deviate essentially from the correct configuration of other halides. It was decided to investigate the *fluoride* because its microwave spectrum to be expected is far more simple than the spectra of the other halides. The nuclear spin of chlorine (3/2), bromine (3/2), and iodine (5/2) deviates from 0 and $\frac{1}{2}$ which gives rise to a complicated hyper-fine structure in the molecular microwave-spectra. A detailed interpretation of these spectra has, therefore, not yet been possible. Furthermore, molecules in low-lying, torsionally excited states (rotation of the methyl groups) contribute with several extra lines, each with their hyperfine structure. As a result it is somewhat uncertain exactly what microwave absorption frequency should be used at the calculation of the moment of inertia [2]. Since the spin of the fluorine nucleus is $\frac{1}{2}$ the only complication left is the appearance of lines corresponding to molecules in vibrationally excited levels. Among the lines thus to be expected the absorption corresponding to molecules in the ground state must be by far the largest.

THE MICROWAVE SPECTRUM. MOLECULAR STRUCTURE

Experience fully confirmed the above expectations. The microwave absorption spectrum of tertiary butyl fluoride (TBF) consists of one strong line and three weaker satellites the frequencies of which are given below in order of decreasing intensity.

18 849.8 Mhz ("main" line)
18 836.3 »
18 825.3 »
18 817.5 »

Relative intensities are given in the paragraph "internal rotation". Consequently the line at 18 849.8 Mhz must correspond to a transition for molecules in their ground state. By means of the formula

$$E_{\text{rot}} = \frac{h^2}{8\pi^2 c I_B} J (J+1)$$

and the Bohr frequency rule (E_{rot} is the rotational energy, h is Planck's constant, c the velocity of light, J the rotational quantum number, and I_B the moment of inertia about the principal axis perpendicular to the C—F bond) one calculates for the transition $J = 1 \rightarrow 2$

$$I_B = 107.25 \text{ A.M.U. Å}^2 = 178.03\ 10^{-40} \text{ gcm}^2.$$

Of course this information is insufficient for a determination of the five geometrical parameters of the molecule. Therefore, the electron-diffraction pattern was studied simultaneously in a collaboration with the Chemical Department of the University of Oslo, Blindern [3]. This part of the investigation is reported in the following paper by Bastiansen, and Smedvik [4]. If the reasonable assumption is made that the length of all the C—C bonds is 1.54 Å and that 1.093 Å is the length of all C—H bonds the following combinations of the geometrical parameters are compatible with the observed microwave absorption frequency at 18849.8 Mhz:

CCF-angle	CCH-angle	C—F Å
107°28′	107°28′	1.311
107°28′	109°28′	1.339
107°28′	111°28′	1.367 *
109°28′	107°28′	1.321
109°28′	109°28′	1.349
109°28′	111°28′	1.377 *
111°28′	107°28′	1.331
111°28′	109°28′	1.359
111°28′	111°28′	1.389

This shows that if the valence angles are assumed to be 109° 28′ the C—F distance (1.349 Å) will deviate significantly from the distance in CH_3F (1.384 Å).

From the electron-diffraction pattern it follows, however, that the C—F distance is 1.38 ± 0.02 Å and that the CCF-angle is 108° ± 1.5°. The only combinations of the table above which agree with this are the two marked with an asterisk. The agreement between these two combinations is very satisfactory. It is, therefore, concluded, that the geometrical parameters of tertiary butyl fluoride are as given in Table 1.

Table 1. Geometrical parameters of tertiary butyl fluoride.

	Distances in Å	Angles
Found	C—F: 1.37 ± 0.01	CCF: 108° ± 1.5° CCH: 111°.5 CCC: 111° ± 1°.5
Assumed	C—C: 1.54 C—H: 1.093	

The result is independent of whether an 'eclipsed' or 'staggered' position of the methyl groups is assumed — and consistent with the electron-diffraction work by Beach and Stevenson [1] for the other halides.

INTERNAL ROTATION. POTENTIAL BARRIER

Since no hyperfine structure due the nuclear spin is to be expected and since the so-called K-type splitting is negligible at the low J-values involved it is necessary to interpret the lines other than 18 849.8 Mhz as due to molecules in vibrationally excited states. Due to its intensity the line at 18 836.3 Mhz could be due to molecules in the first vibrationally excited level, the line at 18 825.3 to molecules in the second vibrationally excited level etc. If all energy levels were non-degenerate this interpretation would be self-evident. Since this is not the case for TBF further evidence is necessary for this assignment.

In a molecule like TBF several low-lying, torsionally excited vibrational levels (rotation of the methyl groups) exist and two or three of these levels must be expected to lie lower than any other vibrationally excited level. According to this we shall show that the microwave lines at 18 836.3 and 18 825.3 Mhz may be interpreted as originating from molecules in the first and

second torsionally excited level, respectively. To check the validity of this, relative intensity measurements were carried out at 297° K and 213° K. Due to experimental conditions this latter temperature is not too well-defined.

	Intensity ratios.	
	18 836.3/18 849.8	18 825.3/18 836.3
At 297° K	0.54	0.61
» 213° K	0.35	0.38

Since one torsional vibrational quantum may be introduced in three ways (there are three methyl groups) and two torsional quanta may be introduced in six ways the ratios of the statistical weights of the ground level, the first and the second torsionally excited level are 1:3:6. The situation is pictured in Fig. 1.

	Concentration of molecules:	Statistical weights:
Second torsionally excited level	ΔE_{21} c_2	6
First » » »	ΔE_{10} c_1	3
Ground »	c_0	1

Fig. 1. Showing statistical weights and 'population density' of ground and torsionally excited levels. Energy differences ΔE_{10} and ΔE_{21}.

From Boltzmann statistics it follows that

$$\frac{c_1}{c_0} = \frac{3}{1} \exp. \left(\frac{-\Delta E_{10}}{kT} \right) \text{ and } \frac{c_2}{c_1} = \frac{6}{3} \exp. \left(\frac{-\Delta E_{21}}{kT} \right)$$

At 297° K, $c_1/c_0 = 0.54$ while $c_2/c_1 = 0.61$. It follows that

$$\Delta E_{10} = 352 \text{ cm}^{-1} \text{ and } \Delta E_{21} = 245 \text{ cm}^{-1}.$$

At 213° K, $c_1/c_0 = 0.35$ while $c_2/c_1 = 0.38$. It follows that

$$\Delta E_{10} = 316 \text{ cm}^{-1} \text{ and } \Delta E_{21} = 246 \text{ cm}^{-1}.$$

Considering the difficulties involved in cooling the wave-guide with the gas in a well-defined way and the usual difficulties in accurate determinations of intensity the agreement between the two values of ΔE_{10} is satisfactory (the agreement in the two ΔE_{21}-values is accidental). It is, therefore, concluded that the torsional frequency, ν_t, of TBF is approximately $\frac{316 + 352}{2} = 334 \text{ cm}^{-1}$.

It seemed reasonable first to try to verify this directly by photographing the Raman spectrum of TBF which is particularly valuable for the identification of low-lying levels. This proved to be impossible due to the readiness with which the reaction

$$(CH_3)_3CF \rightarrow CH_2 = C(CH_3)_2 + HF$$

takes place. The infrared absorption spectrum was recorded in the region from 3 to 15 μ but, as one would expect, the spectrum was too complicated and the region investigated too narrow to permit any assignment of the torsional frequency. As a substitute for this, the Raman spectrum of the more stable $(CH_3)_3CCl$ was photographed. Here, a non-polarized line appeared at 305 cm^{-1} (medium intensity).

Since a direct verification of the assumption that the torsional frequency is 334 cm^{-1} proved impossible an indirect method was attempted. The height of the barrier, hindering the internal rotation, was estimated and compared with other estimated barrier heights.

Any moment of inertia, I, can be associated with a *rotational constant*, R, given by

$$R = \frac{h}{8\ \pi^2\ cI} = \frac{27.9865\ 10^{-40}}{I\ (gcm^2)}\ cm^{-1}.$$

If a cosine form of the barrier hindering the rotation is assumed its height, V_0, is given by

$$V_0 = \frac{R^{CC}_{TBF}}{R^{CC}_{CH_3}\ R^{CC}_{FC(CH_3)_2}}\ \frac{\nu_t^2}{9}$$

Here, R^{CC}_{TBF} means the rotational constant corresponding to rotation of the entire molecule about the C—C bond while the quantities in the denominator correspond to rotations of the methyl and the $FC(CH_3)_2$ groups, respectively, about the same C—C bond.

In polar coordinates the equation of the inertial ellipsoid of TBF is

$$r^2 = \frac{1}{I_B \sin^2 \Theta + I_C \cos^2 \Theta}$$

Here, I_B is the magnitude of the two 'small' moments of inertia, I_C the magnitude of the great moment. Θ is the angle between radius vector, r, and the C—F bond. In A.M.U.Å^2, $I_B = 107.25$ and $I_C = 113.89$. TBF is, therefore, almost a spherical top. Its center of mass is close to the central carbon atom. An approximately correct value of the moment of inertia about the C—C bond is, therefore, found by setting $\Theta = 109°28'$ and ignoring that the axis

so considered deviates a little from the C—C bond. It is calculated that this moment of inertia is 107.99 A.M.U.Å^2, so that $R^{CC}_{TBF} = 0.1561$ cm^{-1}. Since $I^{CC}_{CH_3} = 5.40$ gcm^2 and $I^{CC}_{TBF} = 179.25$ gcm^2, $I^{CC}_{FC(CH_3)_2} = 173.85$ gcm^2. Consequently, $R^{CC}_{CH_3} = 5.21$ cm^{-1} and $R^{CC}_{FC(CH_3)_2} = 0.1610$ cm^{-1}. The height of the barrier, V_0, is therefore

$$V_0 = 2\,100 \text{ cm}^{-1} \text{ or } 6\,000 \text{ cal/mole}$$

The height of corresponding barriers in $(CH_3)_3CH$, $(CH_3)_4C$, and $(CH_3)_3N$ has been estimated to 3 870, 4 200, and 4 270 cal/mole, respectively. It seems as if such barriers increase if fluorine is introduced, since the barrier height in CH_3CH_3 and CH_3CF_3 is 2 750 and 3 450 cal/mole [5], respectively. From these numbers it is estimated that the barrier height in TBF should be about 5 000 cal/mole. Considering the assumption made (cosine form of potential curve) and the above-mentioned experimental imperfectness the agreement with the value found is not unsatisfactory.

As further evidence for the above interpretation of the lines at 18 836.3 and 18 825.3 Mhz it can be mentioned that both lines definitely split into two components when observed under conditions of maximum resolving power, *i. e.* at low temperature and pressure. The splitting is due to the tunneling effect to be expected in such cases. The smallness was taken as a sign that hindered rotation takes place but that the barrier must be high.

The line at 18 817.5 Mhz probably belongs to the third torsionally excited level but the line was too weak and diffuse to be of value at the calculations of the torsional frequency.

DIPOLE MOMENT

In the case of TBF it is particularly important that the dipole moment can be found by microwave technique. Due to the instability of the molecule the ordinary methods meet with serious difficulties. Actually an attempted determination has had to be abandoned [10]. In the spectroscope, isobutylene and hydrofluoric acid do not interfere with the measurements of the displacements of the Stark components at various field intensities.

In the electric field (intensity F abs.volt cm^{-1}) a rotational energy level of a symmetric top is displaced and split according to the equation

$$\Delta E_{rot}(\text{cm}^{-1}) = \frac{-\mu E}{hc} \frac{KM}{J(J+1)} + \frac{E^2\mu^2}{2Bh^2c^2}\left[\frac{(3K^2 - J(J+1))(3M^2 - J(J+1))}{J^2(J+1)^2(2J-1)\ (2J+3)} - \frac{M^2K^2}{J^3\ (J+1)^3}\right]$$

μ is the dipole moment, J the rotational quantum number, K is the component of J in the direction of the C—F bond, M is the magnetic quantum number, and B is the rotational constant (0.15721 cm^{-1}). Since the selection rules are $\Delta J = 1$, $\Delta K = 0$, $\Delta M = 0$ we get that the Stark displacement, $\Delta S(cm^{-1})$, corresponding to $(K,M) = (1.0)$ or (0.1) for the transition $J = 1 \rightarrow 2$, is

$$\Delta S(cm^{-1}) = \frac{E^2\mu^2}{2B\ h^2c^2}\left(\frac{6 \times 3}{4 \times 9 \times 3 \times 7} - \frac{-2}{1 \times 4 \times 1 \times 5}\right) = \frac{13E^2\mu^2}{210Bh^2c^2}$$

$$\Delta S(Mhz) = \frac{13E^2\mu^2}{210Bh^2c}$$

For the Stark component corresponding to $(K, M) = (0.0)$ one derives

$$\Delta S(Mhz) = \frac{-16E^2\mu^2}{105Bh^2c}$$

Thus the latter Stark component falls on the low-frequency side of the main line where all the satellites and their Stark components are situated while the former component lies at the high-frequency side where its observation is undisturbed. Measurements of the Stark displacement have, therefore, been carried out on this component. Table 2 gives the experimental results.

Table 2. Measurements of the transition $(J, K, M) = (1, 1, 0) \rightarrow (2, 1, 0)$ at various field intensities.

Field intensity F (abs.volts cm^{-1})	F^2	ΔS Mhz Undisplaced line at 18 849.8 Mhz
2.357	5.555	7.6 ± 0.1
2.525	6.375	8.7 ± 0.1
2.693	7.252	10.0 ± 0.1

From this it follows that the dipole moment of the molecule in its ground vibrational state in the ideal gas phase (the pressure being 0.003 mm at the measurements) is 2.15 ± 0.02 D. In Table 3 the dipole moments of the methyl and the tertiary *iso*-butyl halides are summarized.

Table 3. Dipole moments of methyl halides and tertiary butyl halides (D.U.).

	Fluoride	Chloride	Bromide	Iodide
Methyl halides	1.83 [6]	1.87 [7]	1.80 [8]	1.66 [7]
Tertiary butyl halides	2.15 [9]	2.04 [10]	2.17 [10]	2.20 [10]

PREPARATION

The procedure followed was a modification of the method given by Cooper and Hughes [11]. 55 g tertiary butanol (0.75 mole) and 30 g 40 % hydro-fluoric acid (0.60 mole) was heated in a platinum container on a water-bath for 1 hour during which period the temperature was raised from 20 to 100° C. The gases liberated were lead through a copper tube, partly filled with a mixture of finely powdered NaF and coarse CaF_2 for removal of excess hydrofluoric acid, and finally condensed in a glass trap at $-10°$ — $-15°$ C. After cooling in a dry-ice-ether mixture the condensed gases were treated with bromine (about 16 g) which was added dropwise until the bromine color no more disappeared. In this way unsaturated compounds (such as *iso*-butylene) were converted into high-boiling liquids. After three fractionations at 0° C where low- and high-boiling fractions were discarded, 10 g of a sample boiling at 465—468 mm Hg was obtained. Yield: 22 %. The fractionations were carried out in a glass apparatus. TBF may be kept in glass containers at dry-ice temperature for months without any serious decomposition. At atmospheric pressure the boiling point is 12° C.

SUMMARY

Because of the simplicity of its microwave spectrum TBF was chosen as an object for a combined electron-diffraction and spectroscopic investigation in order to decide whether the distances from carbon to primary and tertiary bonded halogen in simpler organic compounds differ. It was found that the C—F distance in TBF does *not* deviate significantly from the C—F distance in methyl fluoride. Small deviations from the regular tetrahedral value (109°28′) were found in the valence angles of TBF. The deviations are in the direction to be expected [12]. Observations of intensity relationships at two temperatures for lines corresponding to molecules in torsionally excited levels permitted the torsional frequency to be calculated. The interpretation was verified by calculation of the approximate height of the barrier, hindering the internal rotation of the methyl groups. The barrier height proved to be of the order of magnitude to be expected. The dipole moment of the molecule in its ground level was measured in the ideal gas state.

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Electron Diffraction Studies of Tertiary Butyl Chloride and Tertiary Butyl Fluoride

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The structures of derivatives of methane are of considerable interest. Because of their simplicity they allow a relatively accurate determination of parameter values and permit a study of the relationships between them and various substituents. The result of a joint electron diffraction-microwave investigation on tertiary butyl fluoride has already been published [1]. In the present paper we shall give a brief description of the electron diffraction studies of as well the tertiary butyl chloride as the tertiary butyl fluoride.

The upper curve of Fig. 1 is a $\frac{\sigma(r)}{r}$ -curve of tertiary butyl chloride, the lower curve that of the fluoride. We have calculated two $\frac{\sigma(r)}{r}$ -curves for the chloride and three for the fluoride. The first curves of both compounds were based upon intensity curves ranging from $s = 3$ to $s = 26$ Å^{-1}. The best molecular models obtained from these curves were used to calculate theoretical intensity curves, from which the data from $s = 0$ to $s = 3$ were taken and together with the experimental data were used to calculate the second $\frac{\sigma(r)}{r}$ -curves. The third $\frac{\sigma(r)}{r}$ -curve for the fluoride was based upon a better calculated intensity curve for $0 < s < 3$. Since the third curve was only slightly different from the second one, we felt it unnecessary to continue the calculation.

The first peak of the $\frac{\sigma(r)}{r}$ -curves corresponds to the C-H bond distance. The average value of the position of this maximum for all the calculated $\frac{\sigma(r)}{r}$ -curves is 1.10 Å, with a mean deviation of somewhat less than 0.02 Å. For

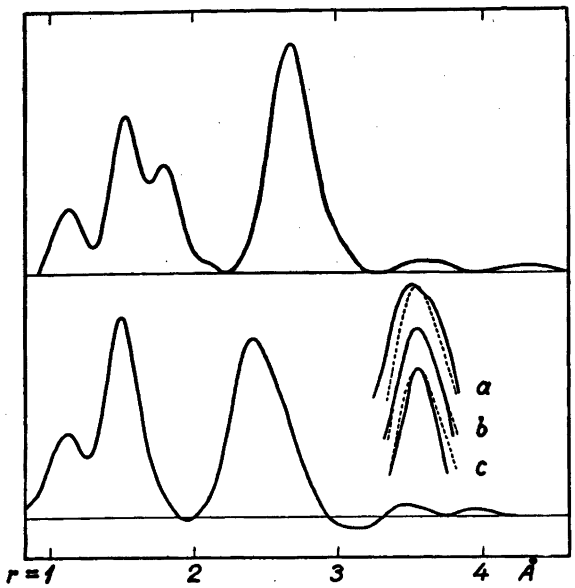

Fig. 1. $\frac{\sigma(r)}{r}$ *curves of tertiary butyl chloride (upper-curve) and tertiary butyl fluoride (lower curve). The curves are calculated from intensity functions multiplied with the modification factor* $e^{-0.007s^2}$.

the chloride the C-C and the C-Cl distances give rise to two separate maxima in the $\frac{\sigma(r)}{r}$ -curve. The position of the C—C bond distance peak is 1.54 Å for both the calculated $\frac{\sigma(r)}{r}$ -curves of the chloride. From the curve of Fig. 1 the C—Cl bond distance is found to be 1.80 Å. The error of these values is estimated to be approximately 0.01 Å, probably somewhat less for the C—C distance.

By an analysis of the peak at $r = 2.69$ Å of the upper curve of Fig. 1 we can find the C—C—Cl angle. The position of the peak is mainly determined by the C ... Cl distance. Normal curves were used to calculate theoretical $\frac{\sigma(r)}{r}$ -curves. This procedure led to a C—C—Cl angle of $107.0° \pm 1°$ corresponding to a C—C—C angle of $111.8° \pm 1°$. This is in good agreement with the results of Beach and Stevenson [2], who from a visual electron diffraction investigation found the following parameters for the molecule: C—C (assumed) 1.54 Å, C—Cl = 1.78 ± 0.03 Å, and $< \text{C—C—C} = 111.5° \pm 2°$.

Since for the tertiary butyl fluoride the contribution from the C—C and C—F bond distances results in one unresolved peak, the C—F distance cannot be determined directly. If we calculate the C—F bond distance simply from the maximum position by giving the distances their usual weight factors $\left(\frac{Z_i Z_j}{r_{ij}} n_{ij}\right)$ and assume the C—C distance equal to 1.54 Å, we find a C—F distance of 1.38—1.40 Å. A more satisfactory approach to the problem of finding the C—F bond distance is based upon the assumption that the contributions of the C—H terms and of the C—C terms are the same for the two compounds. If this assumption is correct we should end up with the same form of the $\frac{\sigma(r)}{r}$ curve in the range 1—2 Å, when the C-halogen bond contributions are subtracted. The contribution of the C—Cl bond can easily be allowed for since the value of this distance is known. The normal curve of the C—Cl bond distance is subtracted from the $\frac{\sigma(r)}{r}$ -curve of the tertiary chloride. The result is given as the upper curve of Fig. 2. This curve is now subtracted in turn from the inner part of the $\frac{\sigma(r)}{r}$ -curve of the fluoride. The resulting curve consists of a peak at 1.38 Å as indicated in the lower curve of Fig. 2. This must correspond to the C—F bond.

There is some uncertainty in the form of the normal curves. The form of the C—Cl normal curve used in this case does not, however, influence the position of the maximum of the C—F peak, but it will to a certain extent influence the shape of the curve in the neighbourhood of the peak. The minimum at the outer side of the C—F peak might well be the results of the use of a somewhat inaccurate normal curve. However, as this does not affect the determination of the C—F bond distance we have made no particular effort to obtain the best normal curve.

To subtract the upper curve of Fig. 2 from the $\frac{\sigma(r)}{r}$ -curve of the fluoride we have to introduce a proper normalization factor which introduces uncertainties. We have varied this normalization factor through a reasonable interval and thus obtained peaks with maxima varying from 1.375 to 1.396 Å. Our conclusion, then, based on the above information and upon consideration of calculated intensity curves is that the C—F bond distance is equal to 1.38 ± 0.02 Å.

The deviation of the carbon bond angles from the tetrahedral value in the fluorine compound cannot be determined with great accuracy from the bond distances and the position of the peak at 2.415 Å. The position of this

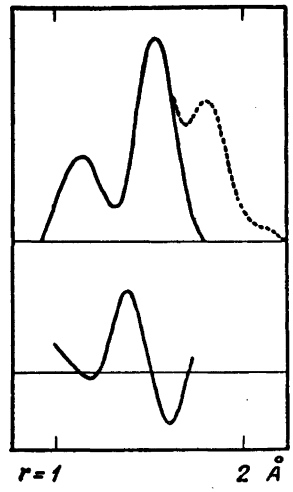

Fig. 2. Difference $\frac{\sigma(r)}{r}$ *curves for the determination of the C—F bond distance of tertiary butyl fluoride.*

peak is very little influenced by variation of the angles. The deviation from the tetrahedral symmetry will obviously not destroy the trigonal symmetry about the C—F axis. Thus, an increase in the C—C—C angle will result in a decrease of the C—C—F angle. Now, since the contribution from the long C ... C distance to the $\frac{\sigma(r)}{r}$-curve is approximately the same as the contribution of the long C ... F distance, the position of the maximum of the resulting peak will vary very little with the angle. On the other hand, the *shape* of the peak under discussion is rather sensitive to variation of the angles. To illustrate this fact three pairs of curves (a, b and c) are drawn in Fig. 1. The broken curve is the same in all three cases, namely, the experimental peak. The solid curve are theoretical peaks claculated from a C—C—F angle of a) 107°, b) 108.4° and c) 109.5°. Since the curve pair b) no doubt exhibits better agreement than the other pairs, we believe that the C—C—F angle is somewhat smaller than the tetrahedral angle. These considerations and others (the relative weights of the terms contributing to this peak were also examined) lead us to the value $108 \pm 1.5°$ for <C—C—F, corresponding to a C—C—C angle of $111° \pm 1.5°$. Although the tetrahedral value 109.5° lies just at the edge of our limit of error, we feel that our electron diffraction result is strongly suggestive of a small deviation in the same direction as for the other tertiary butyl halogenides studied [2,3].

The conclusion drawn from the joint electron diffraction microwave investigation leading to a C—F bond distance of 1.37 ± 0.01 Å is based upon the tacit assumption that the two different methods in principle give

identical values for corresponding bond distances in the same molecule. A microwave distance determination leads to a bond distance value for the molecule in a definite quantum state (here the ground state), while an electron diffraction study gives the average bond distance at the temperature in the diffraction point. One might, therefore, expect that a distance determined by the microwave method should be somewhat smaller than the distance determined by the electron diffraction method. However, the deviation is probably within the limit of the errors of the two methods, though future improvements of the methods might perhaps reveal measureable differences in the results from the two methods.

A detailed discussion of the microwave investigation of the tertiary butyl fluoride is given in the preceding paper by F. Andersen, B. Bak, and J. Rastrup-Andersen [4].

SUMMARY

Electron diffraction studies of tertiary butyl chloride and tertiary butyl fluoride leads to the following molecular parameters of the two compounds: Tertiary butyl chloride: C—H = 1.10 ± 0.02 Å, C—C = 1.54 ± 0.01 Å, C—Cl = 1.80 ± 0.01 Å and — C—C—Cl = 107 ± 1°. Tertiary butyl fluoride: C—F = 1.38 ± 0.02 Å and C—C—F = 108 ± 1.5°.

The authors wish to express their gratitude to Dr. B. Bak, Copenhagen, who suggested this work and supplied the compounds, and to Ing. E. Risberg for having carried out the experimental part of the work.

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The Variation of ADH and Catalase Activity during the Germination of the Green Pea *(Pisum sativum)*

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Earlier work by different authors *e.g.*[1,2] has shown that during the germination of the pea, great variations in the activity of the enzymes ADH (alcohol dehydrogenase), formic acid dehydrogenase and catalase occur.

The reasons for these variations are obscure. In this paper, the above mentioned variations in enzymatic activity are confirmed. The variations of protein, nonprotein nitrogen, alcohol production and of readily titrated SS and SH groups are followed, and a possible connection between these variations in the cotyledon during the germination is discussed.

METHODS

The experiments were performed with green peas of the trade mark "Fenomen". The peas were placed in water overnight, and then sowed out in a box with soil, and placed in a window. The temperature averaged 15° C. For obtaining samples from the first few days of germination, peas were also germinated between moist filter paper in Petri dishes. Every day, between 3 and 10 seedlings were taken for analysis. After washing and weighing, they were stored at − 15° C until the analyses were performed.

All analyses were made on extracts of the seedlings, either whole or dissected into embryos, cotyledons and seed shells, made by grinding in a mortar with $M/100$ K_2HPO_4. The amount of phosphate solution added, was approximately ten times the fresh weight of the sample. After standing an hour, the suspension was centrifuged for ten minutes at 3 500 r.p.m. in a small angle centrifuge, and the clear, yellowish or greenish solution used for analysis.

Protein in the extract was determined by coagulation by heating after the addition of 1 ml 0.1 N HAc per 5 ml of solution, filtering and careful washing on a small filter paper, drying at 110° C for half an hour, and weighing.

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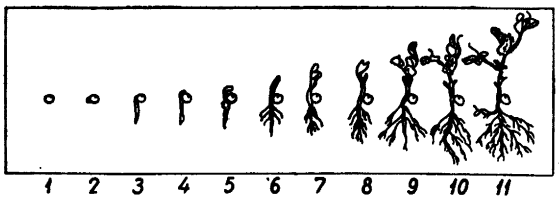

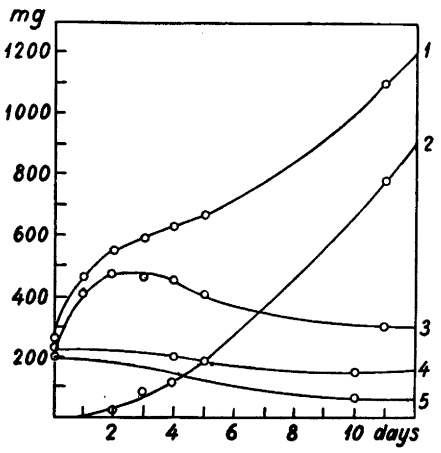

Fig. 1. The development of the pea plant during germination. Curve no. 1, shows total fresh weight, curve 2, the fresh weight of the plants without the cotyledons, curve 3, the fresh weight of the cotyledons, curve 4, the dry weight of the whole plant, and curve 5, the dry weight of the cotyledons.

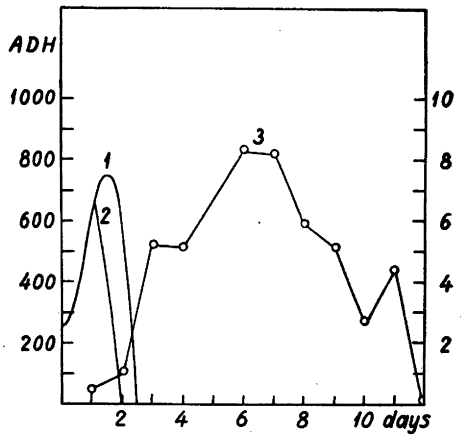

Fig. 2. ADH and catalase activity during germination. Curve no. 1, shows the ADH activity in the cotyledons, units per gram dry matter, curve 2, the ADH activity in the embryos, and curve 3, the catalase activity as Kat.f.

Total N was determined according to Kjeldahl, on 1 ml samples of the solution. Non-protein N was determined as the difference between the total N and the protein N, assuming the N content of the protein to be 17 %.

Total readily titratable SS plus SH groups in the extract were determined amperometrically according to Kolthoff and Stricks [3], and SH amperometrically according to Kolthoff and Stricks [4]. The former method involves a titration with copper salt and the latter an argentometric titration.

The activity of ADH was determined spectrophotometrically with ethanol and DPN at pH 9.7 [5,6], and the alcohol content in the peas enzymatically with ADH and DPN [7]. The catalase activity was determined by titration with permanganate [8].

By means of the results from the analyses of the extracts, the content per gram fresh weight was calculated, and by means of the curves for fresh and dry weight in Fig. 1, the content per gram dry weight found.

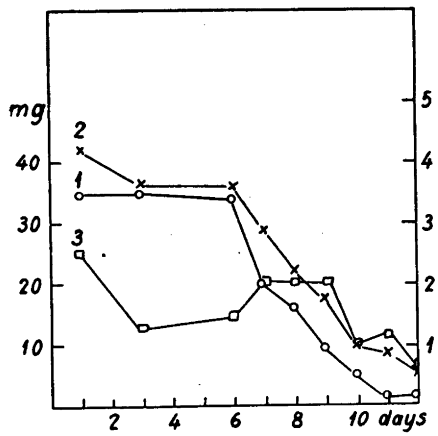

Fig. 3. Nitrogen in the cotyledons during germination. Curve no. 1, the protein content, scale to the left, curve 2, total N scale to the right, and curve 3, nonprotein N, scale to the right. The amounts are given in mg per pea (mean values).

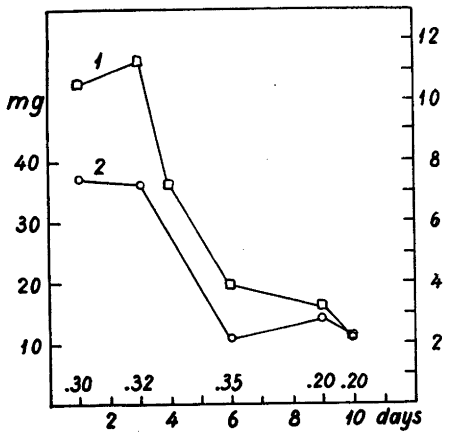

Fig. 4. Readily titratable SS and SH groups in the cotyledons during germination. Curve no. 1, RSH, scale to the right, and curve 2, RSSR, scale to the left. The values are given as µmol per gram dry weight. The figures above the abscissa show the quotient RSH/RSSR.

RESULTS

Fig. 1. shows the physical development of the peas during the experimental period.

On Fig. 2 is seen the ADH activity and the catalase activity in the pea extracts. These results show good conformity with earlier works. It might be mentioned that the ADH activity showed great variability from pea to pea, as is seen in Table 1.

Table 1. The ADH activity in single, whole peas during germination.

At start of germination (dry peas)	1st day		2nd day		3rd day		
Act./pea	Act./g	Act./pea	Act./g	Act./pea	Act./g	Act./pea	Act./g
93	279	135	245	203	335	0	0
108	240	110	205	51	103	0	0
104	207	81	160	17	29		
46	87	106	265	54	87		
47	111	164	315	155	259		

Table 2. The ethanol content in peas, kept for 12 hours in nitrogen at room temperature, at the germination stage indicated. The alcohol content given as pro mille of the fresh weight of the peas.

1st day	2nd day	4th day
0.80	1.96	0
0.74	3.50	0
0.93	2.74	0
	3.22	

The reason for the great differences between individual results may be due to a not quite simultaneous development of the peas. Thus it is quite evident that this must be the reason for the variations in the results from the second day, during which there must be a very rapid fall of ADH activity. The curves given in Fig. 2 are, we believe, the most probable ones for the ADH activity during the germination. We see from these curves also that ADH first disappears from the embryo, and thereafter from the cotyledon. The seed shell does not at any time contain any ADH.

The ability of the pea to produce alcohol under anaerobic conditions is closely correlated with the ADH activity. This is seen from Table 2. The alcohol concentrations given there, were obtained in single peas by keeping them in nitrogen for 12 hours at the development-stage indicated. It should be stressed that the alcohol contents are determined enzymatically, and thus are presumably true values of the ethanol concentration, not obtainable by non-specific methods such as the Widmark technique.

In Fig. 3 are given the curves for the variations in the cotyledons of total N, protein dry weight and nonprotein N.

It seems as if the pea during the first six days of germination, utilizes the non-protein components of the cotyledon. Then, there is a rapid fall in the protein content, together with a slight rise in the non-protein N, showing that after the sixth day the hydrolysis of proteins has started, and that the products of the hydrolysis are being removed from the cotyledon. In this period of germination an increase in the activity of proteases and peptidases should be expected. After the 12th day, the store of nitrogen, soluble in $M/100$ K_2HPO_4 in the cotyledons, is practically depleted.

In Fig. 4, are seen the changes in the titratable RSSR and RSH groups in the cotyledon extracts during the germination. It is seen that both SS and SH groups disappear quickly during the first 6 days after which time the amount

becomes more constant. The ratio SH/SS is slightly decreasing after the sixth day of germination, but not to such an extent that it would mean a significant change in the redox potential of the extract.

DISCUSSION

The connection between the alcohol producing ability of the pea and the ADH activity, seem to leave no doubt that the enzyme ADH is responsible for the alcohol production.

The protein content of the pea keeps nearly constant till the 6th day, so the drop in ADH activity is hardly due to utilisation of protein in the cotyledon. The drop of catalase activity, however, falls together with the utilisation of protein beginning at the 6th day. The fall in the amount of SH groups as shown in Fig. 4 goes together with the disappearance of the ADH and the increase in catalase activity. In this connection it is interesting to notice that the ADH is depending upon SH groups for its activity while catalase is inhibited by SH groups [9,10] and at the same time it slowly oxidizes the SH groups to SS [11,12].

However, it should also be remembered that there is a lack of knowledge of the changes in the metabolism during the germination. A number of biologically active substances investigated, showed marked variations during the germination period. Important in this connection is the finding of Van Herk [13] that the DPN content falls rapidly between the 4th and the 7th day of germination. Changes have also been observed in the ascorbic acid content [14], and of a number of the B vitamins [15]. This might indicate a fundamental change in the metabolic pattern and thus in the enzyme composition of the pea during the germination.

From the 7th day of germination, the variation of the enzymes seems to be dependent upon the disappearance of protein as shown in Fig. 3.

SUMMARY

The variations of the enzymes ADH (alcohol dehydrogenase) and catalase, and of the concentrations of protein, total N and readily titratable SH and SS groups, extractable in $M/100$ K_2HPO_4 are followed during the first 12 days of germination of the green pea.

The ADH activity parallels the decrease in titratable SH groups during the first three days. At the same time the catalase activity is increasing.

The alcohol producing ability of the peas is closely correlated with the ADH activity. The significance of these findings is discussed.

One of us, J. G., wishes to express his thanks to the *Norwegian Research Council* for a scholarship, and to Professor Hugo Theorell for his hospitality.

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Studies on the Extraction of Metal Complexes

V. Two-parameter Equations for a Complex-formation System and their Application to the Two-phase Distribution of Metal Complexes

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In recent years much work has been done on the extraction on metal ions (M) from aqueous solutions with an organic solvent and a complex-forming group (A). In mathematical calculations on such processes, the standard approach has been to assume that the organic phase contains only the uncharged complex MA_N, and the aqueous phase only the uncomplexed metal ion M. This assumption leads to the very simple equation (7) below.

Rydberg [1] found that this simple equation certainly did not hold true for the thorium-acetylacetonate system studied very carefully by him ($M = Th^{4+}$, $A = Aa^- = (CH_3COCHCOCH_3)^-$, $N = 4$). The experimental data could be explained only by considering the formation in the aqueous phase of all the complexes, MA, MA_2, MA_3, and MA_4. The same conclusion has been reached for a number of other systems studied by our team.

In the special system studied by Rydberg, the accuracy of the data allowed the determination of five constants: the distribution constant λ_4 for MA_4, and the complexity products $\varkappa_1$, $\varkappa_2$, $\varkappa_3$, and $\varkappa_4$ for the formation of all the complexes MA_n. In other cases, however, the data may be too few or of too low accuracy to allow the determination of so many independent constants.

The present paper gives an approximation that has already proved useful for describing a number of systems where the data are accurate enough to rule out the simple equation (7) but not to determine all equilibrium constants independently.

GENERAL EQUATIONS

Let M be the metal ion and A the (negatively charged) ligand. (We shall for simplicity leave out the signs for charge.) We assume that a series of mononuclear complexes MA_n are formed. MA_N is the uncharged complex and MA_S the saturated complex; *e.g.* with $M = Th^{4+}$ and $A = X^-$, a uninegative ion, MA_N would be ThX_4, and MA_S might be ThX_6^{2-}. Polynuclear complexes and hydroxo complexes are neglected.

The equilibrium constants for the stepwise formation of the complexes are

$$k_n = \frac{[MA_n]}{[MA_{n-1}][A]} \tag{1}$$

Here, as in the following, brackets denote the concentration in the *aqueous* phase, unless the index "org" is added to indicate the organic solvent phase. We assume that, by the use of a constant ionic medium, the activity factors are kept constant so that concentrations can be used instead of activities in the law of mass action.

The complexity products $\varkappa_n$ are defined as

$$\varkappa_n = \frac{[MA_n]}{[M][A]^n} = k_1 k_2 \ldots\ldots k_n \tag{2}$$

For reasons of symmetry we shall introduce

$$\varkappa_0 = 1 \tag{2a}$$

The total concentration of M in the aqueous phase will be

$$[M]_{total} = [M] + \sum_1^S [MA_n] = [M]\,(1 + \sum_1^S \varkappa_n[A]^n) = [M] \sum_0^S \varkappa_n[A]^n \tag{3}$$

The organic phase, we assume, can dissolve M only in the form of the uncharged complex MA_N. The distribution constant of this complex is

$$\lambda_N = \frac{[MA_N]_{org}}{[MA_N]} \tag{4}$$

The quantity directly observed in the experiments is usually the (variable) net distribution ratio.

$$q = \frac{[M]_{total,\ org}}{[M]_{total,\ aq}} = \frac{[MA_N]_{org}}{[M]_{total}} \tag{5}$$

The data are conveniently recorded by plotting log q versus log [A]. It follows from the equations (2), (3), (4), and (5):

$$\log q = \log \lambda_N + \log \varkappa_N + N \log [A] - \log \sum_0^S \varkappa_n [A]^n \quad (6)$$

The curve log q versus log [A] will have 2 asymptotes, namely

$$[A] \to 0;\ \log q = \log \lambda_N + \log \varkappa_N + N \log [A] \quad (7)$$

$$[A] \to \infty;\ \log q = \log \lambda_N + \log \varkappa_N - \log \varkappa_S - (S-N) \log [A] \quad (8)$$

Equation (7) implies that in the aqueous phase M is present chiefly as uncomplexed M. This equation has been used repeatedly in literature. However, in many systems studied by our team it has been of little use even as an approximation.

Equation (8) implies that in the aqueous phase M exists practically only in the form of the saturated complex MA_S, which may be either identical with the uncharged MA_N ($S = N$) or negative ($S > N$). From the slope of the asymptote, $(S-N)$ can thus be obtained.

TWO-PARAMETER EQUATIONS FOR A COMPLEX SYSTEM

We shall now suggest a two-parameter approximation for the formation of the consecutive complexes MA_1, $MA_2 \ldots\ldots MA_S$. We assume that the complex formation stops at MA_S.

As one parameter we choose the quantity a defined by

$$\varkappa_S = 10^{Sa};\ \log \varkappa_S = Sa \quad (9)$$

It may be noted that J. Bjerrum's [2] mean complexity constant k is equal to 10^a in the present paper.

The other parameter b should determine the ratios between subsequent complexity constants. At the present state of our knowledge it seems hard to predict these ratios for a new system from arguments on, say, statistical factors and electrostatic forces. We shall make the simple approximation that the ratio k_n/k_{n+1} is equal for each step. We thus have

$$\frac{k_n}{k_{n+1}} = 10^{2b} = \beta^2;\ \log k_n - \log k_{n+1} = 2b = 2 \log \beta \quad (10)$$

From (2), (9), and (10) follows (noting that the log k_n form an arithmetical series)

$$\log k_n = a + b(S + 1 - 2n) \quad (11)$$

$$\log \varkappa_n = an + bn(S-n) \quad (12)$$

We shall introduce for convenience a variable y defined by

$$y = [A] \cdot 10^a; \quad \log y = \log [A] + a \tag{13}$$

We find from (2), (10), (12), and (13)

$$[MA_n] = \varkappa_n [M][A]^n = [M]\ y^n \beta^{n(S-n)} \tag{14}$$

To visualize the meaning of the parameters a and b one may consider the diagrams I or II in Fig. 1. They are of the well-known type giving, as a function of log [A], the percentage of the total M present as a certain complex. (See the text of Fig. 1.)

A horizontal line at 50 % will cut the boundaries of the field for MA_n approximately at the points with $-\log [A] = \log k_n$ and $\log k_{n+1}$, provided b is positive and not too small. At the first point, the amounts of MA_n and MA_{n-1} are equal, in the second point $[MA_{n+1}] = [MA_n]$. The maximum fraction of MA_n is obtained very nearly at the intermediate point, $-\log [A] = \frac{1}{2}(\log k_n + \log k_{n+1})$.

The abscissa value $\log y = 0$, thus $\log [A] = -a$, will be the mid-point of the range of complex formation; thus, for an even S, the mid-point of the area for $MA_{\frac{1}{2}S}$. The spacing between the curves at the 50 % level will be approximately $2b$, provided b is positive and not too small; in the curves in Fig. 1, the parameter b is either 0.25 or 0.5, thus the pacing 0.5 or 1.0. With decreasing b, the spacing decreases but of course never becomes negative, even for negative b.

One might add that our equations are exact (and not only approximate) for the simple cases $S = 2$ or 1. For $S = 1$, of course, only one parameter is necessary.

APPLICATION TO DISTRIBUTION EQUILIBRIA

Introducing (12), (13), and (14) into (6) we find

$$\log q = \log \lambda_N + N \log y + bN(S-N) - \log \sum_0^S y^n \beta^{n(S-n)} \tag{15}$$

It follows from (13) and (15) that a change in a or λ_N will only mean a parallel shift of the curve log q versus log [A] whereas, as we shall see, a change in b will affect the shape of the curve.

We shall consider in detail three cases I, II, and III, typical complex formation curves for which have been given in Fig. 1.

In case I no negative complexes are formed; thus the saturated complex MA_S is identical with the uncharged MA_N ($S = N$).

In case II, the saturated complex is negative ($S > N$), and the uncharged MA_N is an intermediate, whose formation is not specially favored.

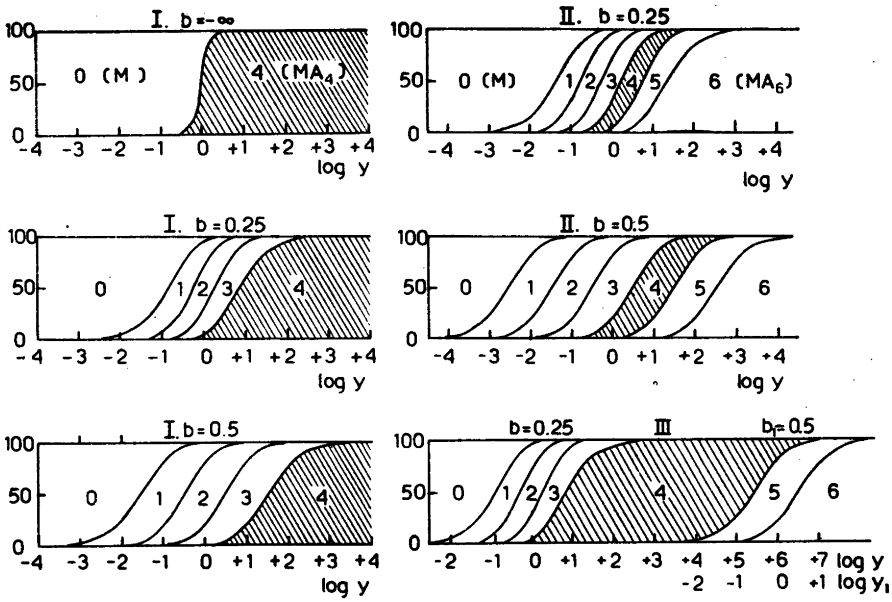

Fig. 1. Distribution of total M in the aqueous phase over the complexes MA_n, for varying log [A]. The abscissa is log y = log [A] + a. On the ordinate axis, the distance 0—100 represents the total amount of M present. If, for a given value of [A], a vertical line is drawn at the corresponding log y, the segment of this line falling in a certain area, e.g. "2" = MA_2, represents the fraction of the total amount of M present as that complex. In each diagram the area for the extractible complex MA_N has been shaded. The diagrams have been calculated with the approximation proposed in the text, that the ratio between consecutive complexity constants is a constant (= 10^{2b}). They represent the cases I, II, III considered in the text:

I. No negative complexes formed; the extractible complex is saturated (N = S). In the diagrams N = S = 4, b = − ∞, 0.25, or 0.5.

II. The extractible complex is intermediate in the series and not expecially favored. In the diagrams N = 4, S = 6, R = S−N = 2, b = 0.25 or 0.5.

III. The ranges of positive and negative complexes are separated by a broad range where MA_N predominates. In the diagram: N = 4, S = 6, R = S−N = 2, b = 0.25 (for positive complexes), b_1 = 0.5 (for negative complexes), and a = a_1 + 6, thus log y_1 = log y−6.

In case III, as in case I, the complexes up to MA_N are formed in a rather narrow log y (= log [A] + a) range, with roughly equal spacing. If log y is further increased, there is first a broad range where MA_N predominates. At still higher log y values, a group of negative complexes are formed, MA_{N+1}...MA_S; the spacing here is not necessarily the same as for the lower complexes. In case III the formation of positive and negative complexes can be treated separately.

I. No negative complexes formed

If the complex formation stops at the uncharged complex MA_N, then we have in equations (6)—(15)

$$S = N \tag{1.1}$$

and for instance (15) gives

$$\log q = \log \lambda_N + N \log y - \log \sum_0^N y^n \beta^{n(N-n)} \tag{1.2}$$

The asymptotes will be

$$y \to 0;\ \log q = \log \lambda_N + N \log y = \log \lambda_N + Na + N \log [A] \tag{1.3}$$

$$y \to \infty;\ \log q = \log \lambda_N \tag{1.4}$$

In order to grasp how the three constants λ_N, a, and b (or β) affect the experimental curves we shall consider for a moment the special case $N = 4$. We find from (12)

$$\log \varkappa_1 = a + 3b;\ \log \varkappa_2 = 2a + 4b;\ \log \varkappa_3 = 3a + 3b;\ \log \varkappa_4 = 4a \tag{1.5}$$

and from (1.2)

$$\log q = \log \lambda_4 + 4 \log y - \log (1 + y\beta^3 + y^2\beta^4 + y^3\beta^3 + y^4) \tag{1.6}$$

or in full

$$\log q = \log \lambda_4 + 4a + 4 \log [A] - \log [1 + [A] \cdot 10^{a+3b} + [A]^2 \cdot 10^{2a+4b} + [A]^3 \cdot 10^{3a+3b} + [A]^4 \cdot 10^{4a}] \tag{1.7}$$

In Fig. 2 we have plotted $\log q - \log \lambda_4$ as a function of $\log y = a + \log [A]$ for various values of b, namely $-\infty$, 0, 0.25, 0.50, 1, 1.50, and 2. All the curves go together at high values for $\log y$ (practically only MA_4 present in both phases, equation 1.4), and at low y (practically only M in the aqueous phase, equation 1.3). In an intermediate y range the curve family spreads out; the difference between the curves is greatest at $y = 1 (\log y = 0)$. Similar families of curves would be obtained for other values of N.

Of especial interest is the curve for $b = -\infty (\beta = 0)$, which corresponds to the assumption that the only species to be considered are M, MA_N and A. This curve has been drawn thicker than the others in Fig. 2. We shall denote the q function so calculated by Q; it obeys he equation

$$\log Q = \log \lambda_N + N \log y - \log(1 + y^N) \tag{1.8}$$

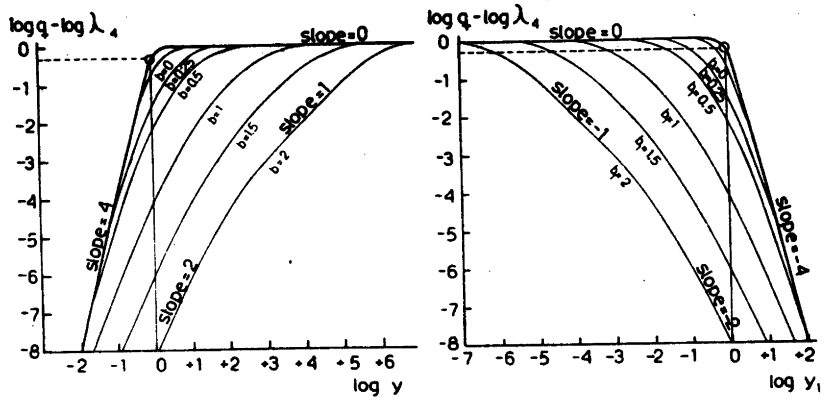

Fig. 2. Variation of the net distribution ratio q with [A] for case I with N = 4, (case III with R = 4) and various values for the parameter b (b_1). Abscissa = log y = log [A] + a. Ordinate: log q—log λ_4; λ_4 = true distribution constant for MA_4. The thick limiting curve, which may be used as a templet, corresponds to b = $-\infty$, $\beta = 0$ (no intermediate complexes formed). The circles give the points for log y = 0, log [A] = − a. The diagram to the right (for the negative complexes in case III) is the mirror image of the left diagram (for positive complexes in case I).

It can easily be shown from equation (1.2) and (1.8) that the function (log Q—log q) has a maximum at $y = 1$. This maximum difference, which we shall call $\triangle$, can be used for determining the second parameter b. We find from (1.2) and (1.8)

$$\triangle = (\log Q - \log q)_{y=1} = \log \tfrac{1}{2} \sum_{0}^{N} \beta^{n(N-n)} \tag{1.9}$$

For the N values between 1 and 6, equation (1.9) gives

$$\begin{aligned}
N &= 1 & \triangle &= 0 \\
N &= 2 & \triangle &= \log (1 + \tfrac{1}{2}\beta) \\
N &= 3 & \triangle &= \log (1 + \beta^2) \\
N &= 4 & \triangle &= \log (1 + \beta^3 + \tfrac{1}{2}\beta^4) \\
N &= 5 & \triangle &= \log (1 + \beta^4 + \beta^6) \\
N &= 6 & \triangle &= \log (1 + \beta^5 + \beta^8 + \tfrac{1}{2}\beta^9)
\end{aligned} \tag{1.9a}$$

For each N, $\triangle$ may be calculated as a function of β or of $b = \log \beta$.

Determination of a, b, and λ_N. Suppose that measurements in a wide range of [A] are available and that we have plotted log q versus log [A]. To determine a, b, and λ_N one can prepare a templet giving, for this N value, log Q versus log y according to (1.8). The templet should have a mark at log $y = 0$ ($y = 1$),

thus at an ordinate -0.3 ($q/\lambda_4 = \frac{1}{2}$) units lower than the limiting value. This templet is moved parallel with the experimental plot, $\log q$ versus $\log$ [A], until the two extreme parts of the experimental curve coincide with the templet (cf. Fig. 5).

Then $\log \lambda_N$ is, of course, the limiting value for $\log q$, and a can be read as $-\log$ [A] at the mark for $\log y = 0$ on the templet. Finally one measures the distance $\triangle$ from the templet to the curve below the mark for $\log y = 0$ and finds β or $b = \log \beta$ from (1.9).

If b is greater than 0.5, and $\log q$ values are not available over a very wide range, it may be hard to locate accurately the asymptote for low [A] by fitting a templet for $b = -\infty$ (see the lowest curves in Fig. 2). One may then prepare a series of templets with the same N and varying b values, each with a mark at $\log y = 0$. The one or those that give the best fit can be used for finding the asymptote and then b and a. One may also proceed by successive approximations, preparing a number of templets around the best b value.

If b were greater than 0.75 or 1.0, the limiting slope N would not be even approached in the measurable range of q. On the other hand, λ_N and one or two of the k_n could be calculated with a good accuracy from the curve. For instance, in the lowest curve in Fig. 2, the two almost rectilinear parts of slopes 1 and 2, corresponding to the predominance of MA_{N-1} and MA_{N-2} in the aqueous phase, could be used for calculating k_N and k_{N-1}. It is then a matter of judgment whether one should use equations (11) and (12) to calculate a and b, and to predict approximate values for the other equilibrium constants. Systems with such a great spread in the complexity constants have not yet been found by our team.

II. Negative complexes; formation of MA_N not especially favored

We shall assume that, besides the positive complexes $MA \ldots . MA_{N-1}$ and the uncharged MA_N, a series of negative complexes up to MA_{N+R} can also be formed, and that the area for MA_N in the formation diagrams has the same spacing as those for the surrounding complexes.

In equations (9)—(15) we then have

$$S = N + R \tag{2.1}$$

Equation (15) will take the form

$$\log q = \log \lambda_N + N \log y + bRN - \log \sum_0^{N+R} y^n \beta^{n(N+R-n)} \tag{2.2}$$

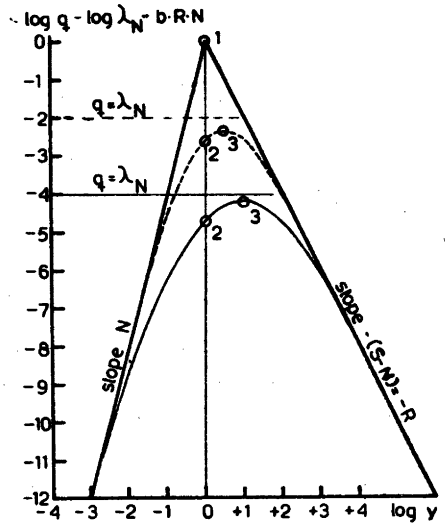

Fig. 3. Variation of net distribution ratio q with [A] for case II with (as in Fig. 1) N = 4, S = 6, and b = 0.25 (broken) or 0.5 (full-drawn curve). Abscissa: log y = a + log [A]. Ordinate = log q − log λ_N − bRN. Straight lines: asymptotes with slope N (chiefly M in aqueous phase) and slope − R (chiefly MA_S in aqueous phase). Points: 1) intersection of asymptotes, 2) point on curve at the abscissa of point 1, 3) maximum of curve. The line q = λ_N has been marked out for each curve.

For some calculations it will prove useful to transform this equation by using the new index j

$$j = N - n \tag{2.3}$$

$$\log\ q = \log \lambda_N - \log \sum_{-R}^{+N} y^{-j}\beta^{j(N-R-j)} \tag{2.4}$$

The asymptotes of the log q curve will be

$$y \to\ 0;\ \log\ q = \log \lambda_N + bRN + N\ \log\ y \tag{2.5}$$

$$y \to \infty;\ \log\ q = \log \lambda_N + bRN - R\ \log\ y \tag{2.6}$$

Fig. 3 shows curves for a system with $N = 4$, $R = 2$, and $b = 0.25$ or 0.50.

From experimental data on a system one could thus plot log q versus log [A] and find N and $-R$ as the slopes of the asymptotes. To determine the three remaining unknowns, a, b (β), and λ_N, one may use the coordinates of three points:

1) The intersection point of the asymptotes at log $y_1 = 0$. We have here

$$\log\ [A]_1 = -a \tag{2.7}$$

$$\log\ q_1 = \log \lambda_N + bRN \tag{2.8}$$

2) The point on the curve immediately below point 1, thus with log $y_2 = 0$ has

$$\log\ q_2 = \log \lambda_N + bRN - \log \sum_{0}^{N+R} \beta^{n(N+R-n)} = \log \lambda_N - \log \sum_{-R}^{+N} \beta^{j(N-R-j)} \tag{2.9}$$

3) The maximum of the log q curve will very nearly coincide with the point

$$\log [A]_3 = -a + b(N-R) \tag{2.10}$$

$$\log q_3 = \log \lambda_N - \log \sum_{-R}^{+N} \beta^{-j} \tag{2.11}$$

For β values larger than 2, only two or three terms in the sum need be taken into account:

$$\log q_3 = \log \lambda_N - \log (1 + 2\beta^{-1} + 2\beta^{-4} + \ldots\ldots) \tag{2.11a}$$

We would thus have five equations to calculate our three unknowns; however, with actual experimental data one or two of the coordinates may be so inaccurately known as to be of little use.

In the special case $N = R$, points 2 and 3 coincide. The equations for case I are obtained by setting $R = 0$, as could be expected.

III. Negative complexes formed; broad range for uncharged complex

We shall now assume that the existence range of the uncharged complex MA_N is broader than the others and so broad that one can treat the formation of positive and negative complexes separately. Thus in one (lower) range of [A], only the positive or uncharged complexes M, MA ..... MA_N need be considered: in another (higher) [A] range, only the uncharged or negative complexes, MA_N, MA_{N+1} ..... MA_{N+R} are present in appreciable concentrations. The curve log q versus log [A] will be almost horizontal, at $\log q = \log \lambda_N$, in the intermediate range where MA_N predominates (Fig. 4).

For the "positive" range one can proceed as for case I.

The formation of negative complexes by the addition of A to the core MA_N can be described by a set of complex constants k'_r and $\varkappa'_r$ defined by

$$k'_r = \frac{[MA_{N+r}]}{[MA_{N+r-1}][A]} = k_{N+r} \tag{3.1}$$

$$\varkappa'_r = \frac{[MA_{N+r}]}{[MA_N][A]^r} = k'_1 k'_2 \ldots\ldots k'_r = \frac{\varkappa_{N+r}}{\varkappa_N} \tag{3.2}$$

We may then, in analogy with (9) and (10), assume that these equilibria are described by two new parameters a_1 and b_1, or β_1.

$$\log \varkappa'_R = Ra_1 \tag{3.3}$$

$$\log k'_r - \log k'_{r+1} = 2b_1 = 2 \log \beta_1 \tag{3.4}$$

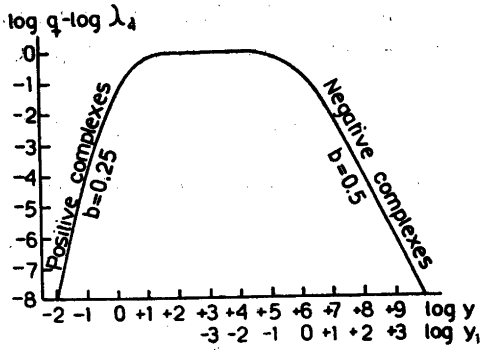

Fig. 4. Variation of net distribution ratio q with [A] for case III with N = 4, R = 2, b = 0.25, and b_1 = 0.5; a = a_1 + 6 as in Fig. 1. Abscissa: log y = log [A] + a; (log y = log y_1 + 6). Ordinate: log q − log λ_N. To the left, positive complexes predominate in the aqueous phase, to the right negative complexes; each part of the curve can be treated separately by the methods given for I.

This gives, as above,

$$\log \varkappa_r' = a_1 r + b_1 r(R-r) \tag{3.5}$$

$$\log \varkappa_{N+r} = \log \varkappa_N + \log \varkappa_r' = Na + a_1 r + b_1 r(R-r) \tag{3.5a}$$

Inserting the latter into (6), and neglecting the positive ions ($n<N$)

$$\log q = \log \lambda_N - \sum_0^R \varkappa_r' [\mathrm{A}]^r = \log \lambda_N - \log \sum_0^R y_1^r \beta_1^{r(R-r)} \tag{3.6}$$

with the variable

$$\log y_1 = \log [\mathrm{A}] + a_1 \tag{3.7}$$

We may transform (3.6) as follows:

$$\log q = \log \lambda_N - R \log y_1 - \log \sum_0^R y_1^{-(R-r)} \beta_1^{r(R-r)} =$$

$$\log \lambda_N - R \log y_1 - \log \sum_0^R y_1^{-r} \beta_1^{r(R-r)} \tag{3.6a}$$

Equation (3.6) has the same form as (1.2) except that N is replaced by R and log y by $-\log y_1 (= \log y_1^{-1})$. The family of log q curves (3.6) for negative complexes with, say, $R = 3$ and various b values is then the mirror image of the family of log q curves (1.2) for positive complexes with $N = 3$.

We may thus use the same set of templets as for positive complexes after half a turn around the vertical axis. As master templet (for a given R) we may use the curve with $\beta_1 = 0$.

$$\log Q = \log \lambda_N - \log (1 + y_1^R) \tag{3.8}$$

the shape of which is the mirror image of (1.8). The maximum difference (log Q—log q) occurs at log $y_1 = 0$ ($y_1 = 1$).

$$\triangle = (\log Q - \log q)_{y_1=1} = \log \tfrac{1}{2} \sum_0^R \beta_1^{r(R-r)} \qquad (3.9)$$

Equation (3.9) has the same form as (1.9), and can be used for finding β_1 and b_1.

APPLICATION TO THORIUM-ACETYLACETONATE COMPLEXES

It is interesting to apply this method (case I) to the Th-acetylacetone (Th—HAa) system which has been carefully studied by Rydberg [1]. From Fig. 5 we may see that the theoretical curve with $a = 6.66$ and $b = 0.45$ fits the experimental values rather well. The constants calculated in this way are given in Table 1 together with Rydberg's values. It is seen that the values of $\varkappa_4$, k_1, and k_3 are the same within reasonable limits of error: k_2 and k_4, however, deviate somewhat. The value of λ_4 is also somewhat different. A careful study of Fig. 5 shows small divergences between the two-parameter curve and the experimental values around —log [Aa] ≈ 6 and 7.7. These divergences have been accounted for by Rydberg using four parameters.

Table 1.

	Values calculated with two parameters	Values calculated by Rydberg with four parameters
log $\varkappa_4$	26.64 ($= 4a$)	26.86
log k_1	8.01 ($= a + 3b$)	7.85 ± 0.35
log k_2	7.11 ($= a + b$)	7.73 ± 0.16
log k_3	6.21 ($= a - b$)	6.28 ± 0.08
log k_4	5.31 ($= a - 3b$)	5.00 ± 0.04
log λ_4	2.43	2.52 ± 0.04

In part VI of this series [3], our equations are applied to the thorium-oxinate and thorium-cupferrate complexes.

APPLICATION TO OTHER EXPERIMENTAL METHODS

The approximate two-parameter equations may also prove useful when complex formation equilibria are studied by other experimental methods. Starting from equation (14), it is not hard to derive the necessary equations and devise methods for obtaining a and b.

For instance it is often possible, by means of an electrode of the corresponding metal or amalgam, to measure either log [M] directly or, if the E_0

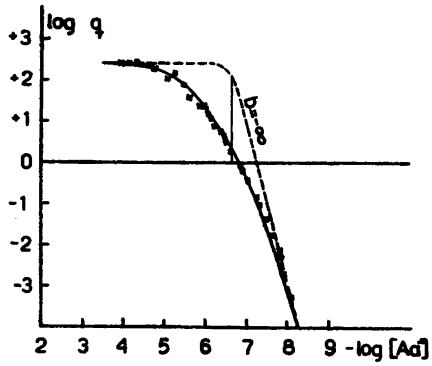

Fig. 5. Data of Rydberg [1] *treated by the approximation case I. The abscissa is here* $-\log [A] = -\log y + a$. *Crosses = Rydberg's experimental data, full-drawn curve = values calculated with* $a = 6.66$, $b = 0.45$. *Broken curve (master templet): calculated with* $b = -\infty$, $a = 6.66$, *thus neglecting all complexes in the aqueous phase besides M and* MA_4.

is not accurately known, log [M] + constant. From analytical data, $[M]_{total}$ is known. Now it follows from (3) and (14).

$$\log [M] - \log [M]_{total} = -\log \sum_{0}^{S} y^n \beta^{n(S-n)} \qquad (16)$$

The sum on the right of (16) is of the same form as the sums in (1.2) or (3.6). Thus, from a plot of log [M]—log $[M]_{total}$ versus log [A], the parameters, a and b can be determined using the same templet and method as for cases I and III; the templet should be turned as for the negative complexes in case III.

In this case it is immaterial whether A is charged or not. The charge of MA_S is also immaterial.

SUMMARY

As a reasonable approximation it has been assumed that the ratio of adjacent complexity constants is the same throughout a series of complexes of a metal ion M and a ligand A (eq. 10). The complex-formation equilibria can be described by two parameters, called a and b; sometimes it is convenient to use $\beta = 10^b$. The complexity products $\varkappa_n$ then follow eq. (12), and the concentration of each complex MA_n eq. (14); y is defined by eq. (13).

This approximation has proved helpful in systems where the data are not accurate enough to allow a separate determination of all the consecutive equilibrium constants. Equations are given and methods are devised for application to distribution equilibria, where one complex (the uncharged) MA_N can be extracted with an organic solvent. It is shown that the equations can also be applied to electrometric measurements of the concentration of free M.

For extraction, three cases are discussed in detail (see Fig. 1): (I) The extractible complex MA_N is identical with the saturated MA_S, (II) MA_N is

intermediate in the series, thus $S > N$, (III) the negative complexes ($n > N$) are formed at a much higher [A] than the positive complexes ($n < N$); in a broad intermediate range of [A], MA_N predominates. For each case it is shown how a diagram of log q versus log [A] (q = net distribution ratio) can be used for finding the parameters a and b, and the true distribution constant λ_N.

As an example (of case I), the methods are applied to Rydberg's data [1] on the thorium-acetylacetonate complexes and found to give rather good agreement.

More applications are given in a paper by Dyrssen [3].

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The Reaction between Acetylacetone and *p*-Benzoquinone

II. Some New Derivatives of Benzofuran

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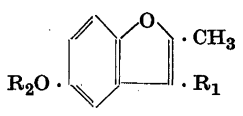
Acetylacetone and *p*-benzoquinone can under certain conditions react to give an intensely red-coloured quinone $C_{16}H_{16}O_6$[1] the constitution of which is being investigated in this laboratory.

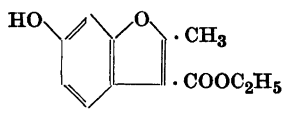
Apart from being one of the reactants acetylacetone also serves as reaction medium and is consequently present in great excess. It became therefore necessary to regenerate the unreacted part of this relatively expensive reagent. During the process of regeneration a new product, a white, crystalline substance with the composition $C_{11}H_{10}O_3$, could be isolated from the reaction mixture. It dissolved in alkali, gave a positive iodoform reaction and could be methylated and acetylated to give the mono derivatives. With 2,4-dinitrophenylhydrazine it formed a monohydrazone and with phenyl isocyanate a monourethan. Combined with the knowledge of the starting materials these results suggested the structure I



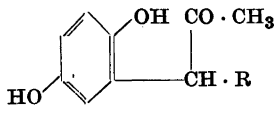
I	$R_1 = COCH_3$	$R_2 = H$
II	$R_1 = COOC_2H_5$	$R_2 = H$
III	$R_1 = COCH_3$	$R_2 = CH_3$
IV	$R_1 = COOH$	$R_2 = CH_3$

A constitutionally similar compound has been prepared by Ikuta [2] by the condensation of *p*-benzoquinone and ethyl acetoacetate in the presence of anhydrous zinc chloride. The structural formula II proposed by Ikuta for his condensation product has, however, been discussed by Graebe and Levy [3] who considered the isomeric structure V as just as probable. The two structures II and V were arrived at by supposing different paths of reaction in the following way: Ikuta presumed that the initial step was the formation of a carbon-carbon bond giving rise to the hydroquinone VI which by loss of one molecule

of water from its tautomeric form will give II. Graebe and Levy gave the structure VIII for the intermediate hydroquinone thus regarding the establishing of the ethereal bond as the first step. Elimination of water from VIII gives of course V.

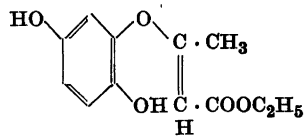


V



VI R = $COOC_2H_5$

VII R = $COCH_3$



VIII

There is good reason to believe that in basic acetylacetone solutions the reaction is initiated by the formation of carbon-carbon bonds [4] and the same seems to be true in the reaction between *p*-benzoquinone and ethyl cyanoacetate in ammoniacal alcoholic solution [5]. In alcoholic solutions of zinc chloride the reaction might or might not take another course. We have, however, been able to isolate the compound I also from the condensation of acetylacetone and *p*-benzoquinone in methanolic zinc chloride solution thus proving the identity of reaction paths in the two different media. Further the methyl ether III could be oxidized by sodium hypobromite to an acid $C_{11}H_{10}O_4$ (IV) which also could be obtained from II by methylating the hydroxyl group and hydrolyzing the ester. Consequently the hydroxyl group has the same position in I and II, and since the structural formula of Ikuta has been corroborated by the above considerations the constitution I for $C_{11}H_{10}O_3$ seems to be established with a good degree of certainty.

The hydroquinone VII which by loss of water would give I has not been isolated but is assumed to be present in the mother liquor from the quinone $C_{16}H_{16}O_6$ for the following reasons: On keeping for a long time the basic solution deposited only very small amounts of the benzofuran but after shaking with dilute sulphuric acid it was obtained readily in about five per cent yield. The fact that I also separated from the acid washings, in which it is almost insoluble, seems to indicate that the condensation product is present as the presumably more soluble hydroquinone VII which is subsequently converted into the benzofuran by action of the sulphuric acid.

The formation of this hydroquinone is obviously a base-catalyzed Michael addition followed by stabilisation through rearrangement to an aromatic structure. Of the two steps which lead from the hydroquinone to the benzofuran *viz.* the enolisation and the elimination of water, one or both must be catalyzed by acids.

EXPERIMENTAL PART

2-Methyl-3-acetyl-5-hydroxybenzofuran (I)

The dark mother liquor from the preparation of the quinone $C_{16}H_{16}O_6$[1] which contained some pyridine was shaken with 2 *N* sulphuric acid in order to remove the basic component from the mixture. Remaining traces of acid were washed out by shaking with pure water. If the reaction mixture after drying for a short time with calcium chloride was left for a couple of days, a crystalline substance separated. Usually, however, this substance was isolated from the residue remaining from the vacuum distillation of the reaction mixture. After washing with ethanol and recrystallisation from the same solvent the reaction product was obtained as greyish needles with m. p. 238°. The discolouration was very difficult to remove by recrystallisation and treatment with charcoal, but the substance could be sublimed *in vacuo* to yield white needles also of m.p. 238°. In all preparations the substance also separated from the sulphuric acid used for the removing of pyridine. The total yield of the benzofuran derivative usually amounted to about five per cent. If the before-mentioned dark mother liquor was kept for some months prior to the acid treatment the benzofuran was deposited only in very small amounts.

$C_{11}H_{10}O_3$	Calc.	C	69.45	H	5.30	
	Found	»	69.25	»	5.12	

Methyl ether of I: The benzofuran (1.0 g) was dissolved in a solution of sodium hydroxide (0.3 g) in water (50 ml). To the clear, somewhat darkish solution was added methyl sulphate (1.0 ml) and the mixture shaken thoroughly. After some time more methyl sulphate (0.4 g) and sodium hydroxide (0.2 g) were added followed by shaking as before, this process was repeated a second time. Precaution had to be taken that the temperature during the methylation did not exceed about 20°. The separated brown solid (0.65 g) was filtered off and recrystallized thrice from dilute ethanol. M.p. 72°.

$C_{12}H_{12}O_3$	Calc.	C	70.58	H	5.93	M	204
	Found	»	70.47	»	5.96	»	192 (in camphor)

Acetate of I: The benzofuran (0.2 g) was dissolved in a mixture of acetic anhydride (5 ml) and pyridine (5 ml) by gentle heating and left for six hours. On pouring on ice an oil separated which rapidly crystallized (0.11 g). After recrystallizing the substance twice from dilute ethanol it had m.p. 88°

$C_{13}H_{12}O_4$	Calc.	C	67.22	H	5.21
	Found	»	67.08	»	5.11

2,4-Dinitrophenylhydrazone of I: To a filtered solution of dinitrophenylhydrazine (1.0 g) in hydrochloric acid (60 ml, 2 *N*) was added a solution of I (0.2 g) in hot ethanol (25 ml). Heated on a water-bath for 20 minutes. Small red needles (0.19 g) separated which after recrystallisation once from ethanol and once from chloroform had m. p. 273°.

$C_{17}H_{14}N_4O_6$	Calc.	C	55.14	H	3.81	N	15.12
	Found	»	55.04	»	4.01	»	14.90

Phenylurethan of I: The benzofuran (0.3 g) dry xylene (5 ml) and phenyl isocyanate (1 ml) were refluxed for one hour. Next day the separated solid was washed with toluene and recrystallized twice from methanol. M.p. 180°

$C_{18}H_{15}NO_4$	Calc.	C	69.90	H	4.88	N	4.53
	Found	»	69.63	»	4.95	»	4.81

Condensation of acetylacetone and *p*-benzoquinone in presence of zinc chloride

p-Benzoquinone (3.0 g) and acetylacetone (3.5 ml) to which anhydrous zinc chloride (10 g) in absolute methanol (12.5 ml) was added were heated on a steam-bath for half an hour. After cooling the separated crystals (1.4 g) were filtered off, washed with dilute methanol and treated with 2*N* sodium hydroxide. The substance went partly in solution (the alkali-insoluble part of the condensation product will be dealt with on another occasion) and on acidifying this a substance separated which after recrystallizing from glacial acetic acid had m. p. 238°. Mixed melting point with I: 238°.

Hypobromite oxidation of the methyl ether III

III (2.6 g), sodium hydroxide solution (20 ml, 2*N*) and sodium hypobromite solution (from 5 g of bromine) were shaken for 10 hours at room temperature. The separated bromoform and some unreacted substance were filtered off. To the filtrate was added a solution of sodium sulphite and the mixture thereafter acidified with 2*N* sulphuric acid. A yellow substance (0.65 g) separated which after two recrystallisations from dilute ethanol was almost white, formed small needles and had m. p. 212°

$C_{11}H_{10}O_4$	Calc.	C	64.06	H	4.89
	Found	»	63.86	»	4.86

Methylation and hydrolysis of II

II (0.95 g) and sodium hydroxide (0.5 g) were dissolved in water (100 ml). Methyl sulphate (0.8 g) was added and the mixture shaken well at room temperature. The addition of methyl sulphate was repeated twice while sodium hydroxide (0.3 g) was added once. The turbid, alkaline solution was extracted twice with 75 ml ether in order to remove the methyl ether of II. The dried ethereal extracts were evaporated to dryness and the crystalline residue (0.15 g) was treated with charcoal and recrystallized twice from dilute ethanol. M. p. 38—40°

$C_{13}H_{14}O_4$	Calc.	C	66.66	H	6.02
	Found	»	66.86	»	6.13

The aqueous phase from the above ether extractions was acidified with concentrated hydrochloric acid and the separated oil extracted with ether (2 × 75 ml). After evaporation of the dried ethereal extract a brownish crystalline residue (0.26 g) was obtained. Treated with charcoal and recrystallised twice from dilute alcohol it yielded almost white needles. M. p. 212—213°. Mixed melting point with $C_{11}H_{10}O_4$ from the foregoing experiment: 212°

$C_{11}H_{10}O_4$	Calc.	C	64.06	H	4.89
	Found	»	64.06	»	4.89

SUMMARY

Acetylacetone and *p*-benzoquinone react in presence of pyridine or methanolic zinc chloride to give 2-methyl-3-acetyl-5-hydroxybenzofuran. Some

derivatives of this compound and of 2-methyl-5-hydroxybenzofuran-3-carboxylic acid are described.

The author's thanks are due to *Grosserer Alf Bjerckes legat* for a grant.

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On the Kinetics of the Oxidation of Formic Acid by Chlorine

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The kinetics of the oxidation of formic acid by chlorine has previously been investigated by Gróh in 1912 [1] and Shilov and Slyadnev in 1948 [2]. Gróh reports only a few experiments in 0.05—0.1 *M* solution of nitric acid. Gróh supposed that the reaction takes place between chlorine and formate ion in a similar way as the reaction between bromine and formic acid [3], thus

$$Cl_2 + HCOO^- \rightarrow H^+ + 2Cl^- + CO_2 \qquad (1)$$

and this mechanism is evidently in harmony with his experiments. Shilov and Slyadnev carried out experiments in 0.1—0.5 *M* solution of hydrochloric acid, and found that the reaction takes place only partly in accordance with Gróh's assumptions. Therefore the authors also claimed a reaction between free acid and chlorine:

$$Cl_2 + HCOOH \rightarrow 2H^+ + 2Cl^- + CO_2 \qquad (2)$$

which takes place simultaneously with the reaction (1). It will be shown in this paper that this claim is not substantiated by the experiments of the author.

THEORETICAL PART

To the two above mentioned reactions correspond the following two expressions for the velocity

$$-\frac{d[Cl_2]}{dt} = k_1[Cl_2][HCOO^-] \qquad (I)$$

$$-\frac{d[Cl_2]}{dt} = k_2[Cl_2][HCOOH] \qquad (II)$$

In the experiments the total concentration of chlorine was determined iodometrically. It is therefore necessary to introduce this concentration as well as the total concentration of formic acid in the expressions above.

Formic acid is dissociated in solution, thus

$$HCOOH \rightleftharpoons H^+ + HCOO^-$$

and in the equilibrium we have

$$\frac{[H^+][HCOO^-]}{[HCOOH]} = K_F$$

where K_F stands for the concentration equilibrium constant.

Formic acid has $K_S = 1.77\ 10^{-4}$ at 25° C [4]. From the expression $pK_F = pK_S + \log f_A - f_{H^+}$ in connexion with the expressions of Harned and Owen, and Bjerrum and Unmack [5] for the activity coefficients of formate ion and hydrogen ion, respectively, we get $K_F = 2.34\ 10^{-4}$ at an ionic strength of 0.98. This ionic strength was used in the experiments from which the velocity constants k_1 were calculated. In the solution the equilibrium

$$Cl_2 + HOH \rightleftharpoons H^+ + Cl^- + HOCl$$

is certainly established. Denoting this hydrolysis constant by K_H we have

$$\frac{[H^+][Cl^-][HOCl]}{[Cl_2]} = K_H$$

From the expressions above in connexion with

$$[Cl_2] + [HOCl] = [\text{total chlorine}] = C$$

$$[HCOOH] + [HCOO^-] = [\text{total formic acid}] = F$$

we find for the two cases respectively

$$-\frac{d[Cl_2]}{dt} = k_1 \frac{[H^+][Cl^-]\ K_F}{(K_H + [H^+][Cl^-])\ (K_F + [H^+])} CF \qquad (I)$$

$$-\frac{d[Cl_2]}{dt} = k_2 \frac{[H^+]^2[Cl^-]}{(K_H + [H^+][Cl^-])\ (K_F + [H^+])} CF \qquad (II)$$

The effects of the reaction $Cl_2 + Cl^- \rightleftharpoons Cl_3^-$ are negligible.

On account of the information obtained from the papers referred to above about the kinetic of the reaction, the attention was concentrated on the expression (I).

In strongly acid solution the hydrolysis of chlorine is negligible, so we may use the total concentration of chlorine directly in the expression (I). We then get

$$-\frac{d[Cl_2]}{dt} = k_1 \frac{K_F}{K_F + [H^+]} CF$$

and from this expression we get

$$\frac{dx}{dt} = k_1 \frac{K_F\ (a-x)\ (b-x)}{K_F + (h + 2x)}$$

where h represents the initial concentration of hydrogen ion, a of total chlorine and b of total formic acid.

After integration we get the following expression

$$k_1 t = \frac{1}{a-b}\left((1 + \frac{h + 2a}{K_F}) \ln \frac{a-x}{a} + (1 + \frac{h + 2b}{K_F}) \ln \frac{b}{b-x}\right) \quad \text{(III)}$$

If we in this expression denote

$$(1 + \frac{h + 2a}{K_F}) \frac{1}{a-b} = r_1$$

and

$$(1 + \frac{h + 2b}{K_F}) \frac{1}{a-b} = r_2$$

we get

$$f(x) = k_1 t = r_1 \ln \frac{a-x}{a} + r_2 \ln \frac{b}{b-x}$$

which has been used for computing k_1. As will be seen from the tables, this expression agrees rather well with the experiments.

EXPERIMENTAL PROCEDURE

All experiments were carried out at 25° C. On account of the volatility of the chlorine it was necessary to use an apparatus from which specimens could be taken with only a small loss of chlorine. The apparatus, which was used, is shown in Fig. 1. It had a volume of 1 020 ml, and was filled with one litre of a solution of chlorine. This solution was prepared by dissolving chlorine in a stock solution, which in these experiments consisted of a solution of sodium chloride and hydrochloric acid.

20 ml of the solution was taken out and used for determining the initial concentration of total chlorine. The reaction was started by adding 20 ml of a known solution of formic acid to the solution mentioned above. The time t was measured from the moment when

half of the formic acid was added. Immediately after adding the formic acid, the solution was mixed thoroughly by the 20 ml air which remained over the solution and carbon dioxide gas with a pressure of a little more than one atmosphere was put over the solution. From time to time specimens were taken out by drawing off part of the solution, about 40 g, in a tared flask containing a solution of potassium iodide. The quantity was weighed and the chlorine was titrated by 0.05 N solution of sodium thiosulphate. As the volume over the solution becomes greater during the experiments a small loss of chlorine cannot be avoided by this method.

Reagents. The formic acid used for the experiments was conc. formic acid *pro analysi* (Riedel-de Haën) which proved to have a boiling point of 100.7° C and a freezing point of 8.2° C. The literature quotes 100.75° C [6] and 8.4° C [7]. The chlorine was taken from a bomb, and washed by water before it was used for experiments. All the other chemicals were *pro analysi*. The water was redistilled before being used for experiments.

Experimental. In strongly acid solution the rate of reaction has been investigated in experiments with nearly the same initial concentrations of chlorine, formic acid and chloride ion, but the hydrogen ion concentration was varied from 0.05—0.5 M. To keep the ionic strength practically constant sodium chloride was added, so the concentration of chloride ion was 0.98. The experimental results from an experiment at 25° C in a solution which is 0.098 M as to hydrogen ion and 0.98 M as to chloride ion are in Table 1. In column 5 are the values of $f(x) = r_1 \ln \frac{a-x}{a} + r_2 \ln \frac{b}{b-x}$. These values were plotted against the corresponding t values and a straight line was fitted to the points. From its slope k_1 is calculated. In column 6 are the values of $t = \frac{1}{k_1} f(x)$. A comparison of column 1 and 6 shows that the assumed mechanism agrees with the kinetic experiment. The disagreement at 140 and 150 minutes is probably due to the systematic error mentioned above.

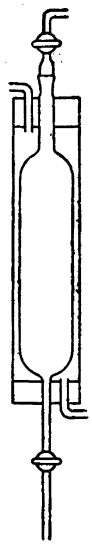

Fig. 1. Shape of experimental arrangement.

The results from all the experiments in strongly acid solution are in Table 2. In the tables the concentrations of chlorine are corrected by multiplying them by the specific gravity ϱ of the solutions, because the concentrations determined by the experimental procedure are not moles per litre but moles per 1 000 g of the solution. As the specific gravity of the solutions at 25° C did not exceed 1.04, the corrections are small.

The values of k_1 are in column 5, Table 2. They are a little different in the two series of experiments, but it has not been possible to trace the difference. It is obvious that k_1 is independent of the concentration of hydrogen ion *i.e.*

Table 1. The reaction between chlorine and formic acid at 25° C in solution 0.098 M as to hydrogen ion and 0.98 M as to chloride ion. In column 5 is given $f(x) = r_1 \ln \frac{a-x}{a} + r_2 \ln \frac{b}{b-x}$; *the constant* k_1 *is determined graphically* $k_1 = 481$; $t_{calc.} = \frac{1}{481} f(x)$.

$r_1 = 5.717 \cdot 10^4$; $r_2 = 4.868 \cdot 10^4$.

1	2	3	4	5	6
t min. obs.	$a-x$	x	$b-x$	$f(x)10^{-4}$	t min. calc.
0	0.02030	0	0.00994		
3	0.01971	0.00059	0.00935	0.129	2.7
10	0.01847	0.00183	0.00811	0.450	9.4
20	0.01713	0.00317	0.00677	0.899	18.7
30	0.01605	0.00425	0.00569	1.372	28.5
40	0.01510	0.00520	0.00474	1.913	39.8
50	0.01439	0.00591	0.00403	2.427	50.5
60	0.01388	0.00642	0.00352	2.880	59.9
70	0.01340	0.00690	0.00304	3.392	70.5
80	0.01305	0.00725	0.00269	3.836	79.8
90	0.01271	0.00759	0.00235	4.343	90.3
100	0.01243	0.00787	0.00207	4.833	100.5
110	0.01222	0.00808	0.00186	5.256	109.3
120	0.01201	0.00829	0.00165	5.740	119.3
130	0.01181	0.00849	0.00145	6.273	130.4
140	0.01163	0.00867	0.00127	6.831	142.0
150	0.01148	0.00882	0.00112	7.368	153.0

the velocity is inversely proportional to the hydrogen ion concentration even in strongly acid solution. There is thus no evidence for the reaction

$$Cl_2 + HCOOH \rightarrow 2H^+ + 2Cl^- + CO_2 \qquad (2)$$

as asserted by Shilov and Slyadnev.

An experiment in 0.1 *M* sodium hydroxide and 0.98 *M* sodium chloride showed a very small consumption of chlorine, even after several days and nights, so we can regard the velocity as practically nil. From this experiment it is evident that the hypochlorite ion does not react with the formate ion.

There remains then the question about any possible reaction between hypochlorous acid and formic acid and formate ion, respectively. An attempt has been done to explain the kinetics in weakly acid and basic solution, but the results from these experiments will be dealt with in a following paper.

Table 2. The results from experiments in strongly acid solution. Column 5 shows that the constant k_1 is independent of the concentration of hydrogen ion i.e. the velocity is inversely proportional to the concentration of hydrogen ion.

1	2	3	4	5
Initial conc. total chlorine	Initial conc. H^+	Initial conc. Cl^-	$[Na^+]$	k_1
I 0.0182	0.490	0.98	0.490	500
II 0.0207				465
I 0.0228	0.196	0.98	0.784	485
II 0.0237				503
I 0.0150	0.098	0.98	0.882	500
II 0.0203				481
I 0.0240	0.049	0.98	0.931	460
II 0.0233				494

Initial conc. formic acid = 0.01 Mean: 486

SUMMARY

The oxidation of formic acid by chlorine has been investigated at 25° C in strongly acid solution and strongly basic solution. It is shown that the experimental results in strongly acid solution can be calculated from the expression $k_1 t = f(x) = r_1 \ln \frac{a-x}{a} + r_2 \ln \frac{b}{b-x}$ where a and b are the initial total concentrations of chlorine and formic acid respectively, and $r_1 = (1 + \frac{h+2a}{K_F}) \frac{1}{a-b}$, $r_2 = (1 + \frac{h+2b}{K_F}) \frac{1}{a-b}$ in which h represents the initial concentration of hydrogen ion.

From the experiments in strongly acid solution it is obvious, that the rate of reaction is inversely proportional to the hydrogen ion concentration, and in consequence of this circumstance the reaction takes place between Cl_2 and $HCOO^-$:

$$Cl_2 + HCOO^- \rightarrow H^+ + 2Cl^- + CO_2$$

in agreement with the results of Bognár for the reaction between bromine and formic acid, and the results Gróh reports in his paper, while there is no evidence for a reaction between Cl_2 and HCOOH as claimed by Shilov and Slyadnev.

An experiment in strongly basic solution shows that hypochlorite ion does not react with formate ion.

The velocity constant $k_1 = 486$ mole litre^{-1}min.$^{-1}$ at 25° C is in agreement with the value $k = 431$ stated by Gróh.

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A Spectrophotometric Method for the Determination of Sulphate and Organic Sulphur on the Micro- and Ultramicroscale

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Among the numerous methods proposed for the determination of sulphate, those based on its precipitation in the form of benzidine sulphate are becoming increasingly important [1–3]. The method presented below belongs to this category.

In earlier methods the isolated benzidine sulphate has been treated in various ways; it has been titrated with alkali [1] and with permanganate [4], or treated in different ways to permit colorimetric determination [5–7], and it has even been oxidized, the resulting carbon dioxide being then determined manometrically [8]. The present method is based on the fact that benzidine and its salts strongly absorb ultraviolet light. The complete procedure is as follows: the sulphate is precipitated as benzidine sulphate, is isolated, dissolved in hydrochloric acid, and diluted to a certain volume, and its absorption measured in a spectrophotometer. The salts of benzidine have a distinct absorption maximum [9] at 2 500 Å, so strong that the optimal range of operation with 1 cm cells is 1—15 mg of benzidine sulphate/l. The precipitate is crystalline and easily filtered, but is of such low density that it is not very suitable for centrifugation. Its solubility in water is 98 mg/l at room temperature [10] and the precipitation cannot, therefore, be made from a highly diluted solution. Strong acidity raises the solubility further [11], one l of 2.8 *N* hydrochloric acid dissolving 1.93 g of benzidine sulphate at 25°, but it is most advantageous to work with slightly acid solutions. Powerful oxidizing agents such as free halogens, nitrite, chromate, and hydrogen peroxide, and some ions giving slightly-soluble benzidine salts, such as cyanoferrate II and III, oxalate, and, to a certain extent, phosphate, have an interfering influence. Further information on the precipitation of benzidine sulphate is to be found in the literature [1–3].

The considerable solubility in water of the benzidine sulphate prevents the method from being, *mutatis mutandis*, directly applicable to ultramicro quantities. The solubility is best reduced by using alcoholic solutions (Table 1).

Table 1. The solubility of benzidine sulphate in the system ethanol + water.

% Ethanol	mg benzidine sulfate/l
0 (10)	98
50	10.1
95	1.16

Acetone is unsuitable because of its absorption of ultraviolet light. The benzidine hydrochloride reagent must be neutral, as even the small excess of mineral acid which is present in the normal reagent seems to dissolve some of the precipitate. The precipitation must be carried out in hot solution.

Some results obtained by applying the micro- as well as the ultramicro method to standard solutions are given in Fig. 1. A solution of 515.3 mg of carefully dried ammonium sulphate in 250 ml of water was used as standard. This solution contains 0.5 mg of sulphur/ml. For the ultramicro method this solution was diluted to 5 μg of sulphur/ml, and suitable quantities were pipetted and evaporated as described below.

I. THE MICRO METHOD

Apparatus: 1) A spectrophotometer for ultraviolet light; 2) A few small Büchner-funnels of about 1.5—2 cm diameter; 3) One or more 1 l measuring flasks; 4) Slow filter paper.

Reagents: 1) Benzidine hydrochloride: 6.7 g of benzidine + 20 ml of about 6 *N* hydrochloric acid are diluted to 1 l, allowed to stand until the benzidine is dissolved, and filtered if necessary. The solution gradually becomes unusable because of mold growth[12]; 2) 0.2 *N* acetic acid; 3) 50 % ethanol; 4) Hydrochloric acid, one part of conc. acid + three parts of water.

The sample to be analysed should be free from interfering substances, almost neutral, and 0.1—0.01 *N* with respect to sulphate. A suitable volume is 1—10 ml containing 0.1—1.5 mg of sulphur. The solution is acidified slightly with 0.25—2 ml of acetic acid and 0.5—2 ml of benzidine hydrochloride reagent is added. One ml of 0.1 *N* sulphate (= 1.6 mg of sulphur) requires about 2 ml of reagent. The precipitate is allowed to stand for 10—30 min. and is then filtered and eventually washed with a few ml of 50 % ethanol. The whole funnel is placed in 200 ml of warm hydrochloric acid. Any precipitate remaining in the precipitation vessel is likewise dissolved in this solution. After some cautious stirring, the precipitate is dissolved and the funnel and filter paper are removed and rinsed with water. The solution is poured into a measuring flask, diluted to 1 l with water, and measured spectrophotometrically at 2 500 Å. The solvent is used as blank solution, for hydrochloric acid absorbs ultraviolet light to a certain extent. The accuracy is 1—2 %.

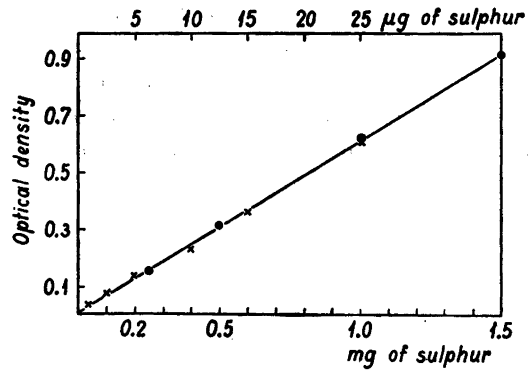

Fig. 1. Standard curve for the determination. Precipitate dissolved in hydrochloric acid. Wave length 2 500 Å; 1 cm cells.
● *Values obtained by the micromethod; dilution to 1 litre.*
× *Values obtained by the ultramicromethod; dilution to 25 ml.*

II. THE ULTRAMICRO METHOD

Apparatus: 1) The filtration is best carried out through fine glass wool *e.g.* Pyrex Brand in the apparatus shown in Fig. 2, and 2) 1—2 simple pipettes with fine tips for small drops of about 0.02 ml; 3) A measuring vessel, volume 25 ml.

Reagents: 1) Neutral benzidine hydrochloride solution: 500 mg of benzidine + 5.5 ml of 0.1 *N* hydrochloric acid + 100 ml of water are heated to boiling, cooled and filtered; 2) 0.2 *N* acetic acid; 3) 50 % ethanol saturated with benzidine sulphate; 4) Hydrochloric acid, one part of conc. acid + nine parts of water.

The sample to be analysed, containing 1—25 µg of sulphur in the form of a neutral sulphate solution, is evaporated, dissolved in 1—2 ml of 95 % ethanol, and acidified with one small drop of acetic acid. The sulphate is precipitated with benzidine hydrochloride, of which about one small drop is taken for each µg of sulphur. The latitude is some 3—12 drops/5 µg but smaller for larger quantities of sulphur. The precipitation is carried out in hot solution. After about 15—30 min. the solution is filtered, and the precipitate washed, at first with 50 % ethanol saturated with benzidine sulphate, and then with 95 % ethanol. The precipitate is dissolved in hydrochloric acid, transferred to the measuring vessel, diluted to 25 ml with hydrochloric acid, and measured spectrophotometrically. The accuracy is slightly lower than that of the micro method.

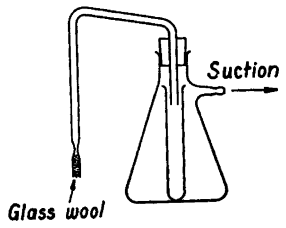

Fig. 2. Apparatus for filtration.

The method can also be used for the determination of sulphur in organic material, which must first be completely oxidized. The destruction is generally best carried out by the method of Carius, in a small sealed tube of about 0.5 cm diameter. The nitric acid is evaporated, and the remaining traces of free acid must be carefully neutralized, which is best done by means of ammonia.

The author wishes to express his gratitude to Professor T. Enkvist for his good advice throughout the work.

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Short Communications

On the System Manganese-Tellurium

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The compounds MnTe and $MnTe_2$ have earlier been prepared and investigated by X-ray methods. They are found to have structures characteristic of compounds of the transition elements Ti-Ni with the elements in the sixth group (O-Te), as MnTe has the NiAs structure [1] and $MnTe_2$ the pyrite structure [2,3]. In some of the related systems other phases of lower symmetry are also found [4], and we have prepared a number of alloys of manganese and tellurium in order to find out whether any such phases exist in this system.

Weighed quantities of manganese and tellurium (pure to about 99.9 and 99.7 % respectively) were kept at about 800° C for 30 hrs in evacuated silica tubes. The alloys were carefully crushed, then annealed at 500° C for two months and finally quenched in ice water. The following alloys were prepared and investigated: Mn (1), $MnTe_{0.5}$ (2), $MnTe_{0.8}$ (3), $MnTe_{0.9}$ (4), $MnTe_{1.0}$ (5), $MnTe_{1.1}$ (6), $MnTe_{1.2}$ (7), $MnTe_{1.3}$ (8), $MnTe_{1.4}$ (9), $MnTe_{1.6}$ (10), $MnTe_{1.8}$ (11), $MnTe_{2.0}$ (12), $MnTe_{2.1}$ (13), $MnTe_{3.0}$ (14), $MnTe_{3.5}$ (15), and Te (16). X-ray diagrams were taken of all the alloys, using FeK radiation (wave length Fe$K\alpha_1$ = 1.9360 Å) and a powder camera 114.6 mm in diameter. Lines from the following phases were observed on the diagrams:

Alloy	Phases observed
(1)	α-Mn
(2), (3)	α-Mn + MnTe
(4), (5)	MnTe
(6)–(10)	MnTe + $MnTe_2$
(11)	$MnTe_2$
(12)–(15)	$MnTe_2$ + Te
(16)	Te

No other phases were detected. The lattice constants were determined to be:

α-Mn:	cubic, a = 8.911 Å (± 0.002 Å)
MnTe:	hexagonal, a = 4.146 Å, c = 6.709 Å (± 0.005 Å)
$MnTe_2$:	cubic, a = 6.951 Å (± 0.002 Å)
Te:	hexagonal, a = 4.457 Å, c = 5.916 Å (± 0.006 Å)

These measurements are in good agreement with those reported in the literature (α-Mn: a = 8.912 Å[5]; MnTe: a = 4.132 Å, c = 6.711 Å[1]; $MnTe_2$: a = 6.954 Å[2]; Te: a = 4.454 Å, c = 5.922 Å[6]. The values refer to the same value of the X-ray wave length as in the present investigation).

The lattice constants of α-Mn, MnTe, $MnTe_2$ and Te are found not to vary with the composition of the alloys within the experimental error (± 0.002 Å). However, a slight variation cannot be excluded, thus the lattice constant of $MnTe_2$ in the alloys (6)–(11) appear to be slightly (about 0.001 Å) greater than in the alloys (12)–(15). At any rate, the homogeneity range of the phases, if any, must be very small. The phase boundaries, or the exact composition, of the phases MnTe and $MnTe_2$ cannot be deduced from the present me-

asurements. The tellurium content in $MnTe_2$ appears to be less than required by the formula $MnTe_{2.00}$ at the temperature used in these experiments as this alloy (12) is found to be a two-phase preparation.

It has been pointed out by Biltz and Klemm [7], that the Mn^{++} ion is extraordinary stable, and it is therefore only to be expected that MnTe and $MnTe_2$, in contradiction to analogous compounds of related metals, have no, or a very small, homogeneity range.

The author wishes to thank Prof. H. Haraldsen for helpful discussions and working facilities.

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Strepogenin as a Growth Factor for *Lactobacillus bifidus*

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In 1941 the presence in liver of a new growth factor, effective for certain hemolytic streptococci, was demonstrated by Woolley [1]. Later Wright and Skeggs [2] established that enzymatic digests of casein were potent sources of this factor, and Sprince and Woolley [3,4] showed that the factor could be found in enzymatic hydrolysates of a number of proteins, insulin probably being the most potent source. The last-mentioned authors named the factor "strepogenin", introduced the use of *Lactobacillus casei* for its microbiological assay, and cited evidence for its tripeptide nature. More recently Wright *et al.* [5] reported that strepogenin, under certain conditions, is essential for *Lactobacillus bulgaricus*. They proposed the use of this organism in the assay of strepogenin, while Kodicek and Mistry [6], on the other hand, improved the *L. casei*-method for the same purpose.

Investigating the nutritional requirements of a *Lactobacillus bifidus* strain, isolated from the faeces of a breast-fed infant, the present authors [7] were able to show that strepogenin was essential for its maximal growth. Further evidence of the strepogenin requirement of *L. bifidus* is given in this paper.

In all experiments on the strepogenin requirement of our strain, labelled TM 2, the synthetic basal medium of Hassinen *et al.*[8] was employed. This medium which, in addition to lactose, sodium acetate, and salts A (KH_2PO_4, K_2HPO_4) and salts B ($MgSO_4$, $FeSO_4$, NaCl, $MnSO_4$), contains only ammonium acetate, cysteine, Ca-pantothenate and biotin, is reported to meet the nutritional requirements of *L. bifidus*. Inocula of the test-organism were prepared in the usual way as cell suspensions in sterile saline. Incubation of the tubes was carried out at 37° for about 70 hours. The growth was measured titrimetrically. The different protein digests investigated were prepared by hydrolysing the proteins with trypsin at 37° for 24 hours. To test the effect of acid-hydrolysis these digests were boiled with equal parts of conc. HCl for 3 hours.

The growth-promoting effect of the tryptic digests of certain proteins is evident from Table 1. Untreated proteins are much less active, while the acid-hydrolysates are completely inactive. Trypsin alone has practically no activity. A mixture of amino acids composed so as to simulate the amino acid composition of casein has only a very slight activity.

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The growth-promoting effect of the tryptic digests of certain proteins is evident from Table 1. Untreated proteins are much less active, while the acid-hydrolysates are completely inactive. Trypsin alone has practically no activity. A mixture of amino acids composed so as to simulate the amino acid composition of casein has only a very slight activity.

Table 1. Effect of different protein preparations on the growth of L. bifidus (TM 2).

	Addition to the basal medium	Consumption of 0.1 *N* NaOH ml/10 ml medium
Exp. A.	1. None	1.9
	2. Casein, 0.6 mg/ml	3.8
	3. Egg albumin, 0.6 mg/ml	7.9
	4. Blood albumin, 0.6 mg/ml	9.9
	5. Pepsin, 0.6 mg/ml	6.9
	6. Gelatin, 0.6 mg/ml	3.1
	7. Tryptic digest of casein, 0.6 mg/ml	10.4
	8. » » » egg albumin, 0.2 mg/ml *	14.4
	9. » » » blood albumin, 0.6 mg/ml	13.2
	10. » » » pepsin, 0.6 mg/ml	11.7
	11. » » » gelatin, 0.6 mg/ml	8.9
Exp. B.	1. None	1.4
	2. Acid treated tryptic digest of casein	1.1
	3. » » » » » egg albumin	1.6
	4. » » » » » blood albumin	1.6
	5. » » » » » pepsin	1.7
	6. » » » » » gelatin	1.4
	7. Trypsin, at the level corresponding to that used in the tryptic digests	2.7
	8. Amino acid mixture, composed so as to simulate the amino acid composition of casein	3.3

* Because of the high activity of the egg albumin hydrolysate this preparation was tested at a lower level than the others.

Table 2. Effect of the tryptic digest of egg albumin on the growth of different L. bifidus strains.

Strain	Consumption of 0.1 *N* NaOH ml/10 ml medium	
	Basal medium	Basal medium + egg albumin digest (200 μg/ml)
TM 2	3.2	17.9
T 3	0.6	10.6
T 10	0.8	9.7
K 15	0.7	11.1

In addition to strain TM 2 we have investigated several other *L. bifidus* strains isolated from the faeces of different breast-fed infants. As can be seen from Table 2 the results obtained are in good agreement with those of strain TM 2. Thus it seems evident that at least under the cultural conditions employed by us strepogenin is an essential growth factor for *Lactobacillus bifidus*.

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On the Synthesis of Monobromocyanoacetamide

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Although dibromocyanoacetamide was prepared by Hesse [1] as early as 1896, the monobromoderivative was not prepared until 1922 by Gupta and Thorpe [2]. They prepared monobromocyanoacetamide by partial debromination of dibromocyanoacetamide by means of dimethylaniline:

$$CBr_2(CN)CONH_2 + C_6H_5-N(CH_3)_2 \rightarrow C_6H_4Br-N(CH_3)_2 + CHBr(CN)CONH_2$$

A solution of dibromocyanoacetamide and dimethylaniline in benzene was kept at 80° for five hours. In this way monobromocyanoacetamide and *p*-bromoaniline were obtained in a yield of 55 per cent of the theoretical amount.

Dibromocyanoacetamide is obtained in a simple way by bromination of cyanoacetamide in aqueous solution in the presence of sodium acetate at low temperature. The dibromo-product separates during the reaction. Gupta and Thorpe stated, that all attempts to prepare monobromocyanoacetamide by direct bromination were fruitless.

However, a more careful study of the bromination reaction indicates, that the following three competitive reactions take place:

$$CH_2(CN)CONH_2 + Br_2 \rightarrow CHBr(CN)CONH_2 + HBr \quad (1)$$

$$CHBr(CN)CONH_2 + Br_2 \rightarrow CBr_2(CN)CONH_2 + HBr \quad (2)$$

$$CH_2(CN)CONH_2 + CBr_2(CN)CONH_2 \rightarrow 2\ CHBr(CN)CONH_2 \quad (3)$$

At a low temperature the reactions (1) and (2) are very fast; reaction (3) is evidently slow. At a higher temperature, however, reaction (3) is also fast. On bromination of cyanoacetamide in the cold, the formation of the dibromoderivative is also favoured by the fact, that it is less soluble than the monobromoderivative and thus quickly separates from the solution. It is likely, that a bromination of cyanoacetamide at a high temperature would lead mainly to the monobromoderivative, when equimolar parts of amide and bromine are used.

The best method for preparing monobromocyanoacetamide is, however, to make use exclusively of reaction (3). Thus, on heating equimolar parts of cyanoacetamide and dibromocyanoacetamide in aqueous solution, a good yield of the monobromo-product is obtained.

Both mono- and dibromocyanoacetamide are quantitatively debrominated by an acidified potassium iodide solution:

$$CHBr(CN)CONH_2 + 2\ HJ \rightarrow CH_2(CN)CONH_2 + J_2 + HBr$$

$$CBr_2(CN)CONH_2 + 4\ HJ \rightarrow CH_2(CN)CONH_2 + 2\ J_2 + 2\ HBr$$

These reactions are useful for the analytical determination of bromocyanoacetamides, since the liberated iodine can be titrated with sodium thiosulfate.

Experimental. 6.3 g (0.075 mole) of cyanoacetamide and 18.2 g (0.075 mole) of dibromocyanoacetamide were dissolved in 50 ml of hot water, and the solution was boiled for about two minutes, treated with some decolorizing charcoal and filtered hot. On cooling, monobromocyanoacetamide separated as white needles. Yield 20 g (80 %). The product may be recrystallized from hot water or preferentially from alcohol. M.p. 121–122°.

0.1929 g: 23.30 ml 0.1018 *N* $Na_2S_2O_3$.
0.2009 g: 24.19 » 0.1013 » HCl (Kjeldahl)
$C_3H_3ON_2Br$ = 163.0. Calc. N 17.19, Br 49.03
Found » 17.09, » 49.12

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Space Group Data on Barium Pentathionate

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Crystals of barium pentathionate dihydrate [1] have been obtained in two modifications, one triclinic (I) by crystallization from water, and one orthorhombic (II) by crystallization from aqueous methanol. The dimensions of the unit cells, from oscillation and Weissenberg photographs taken with CuK_α radiation, $\lambda = 1.54$ Å, are:

I, $a = 5.00 \pm 0.02$ Å, $b = 10.36 \pm 0.04$ Å, $c = 11.53 \pm 0.04$ Å, $\alpha = 110°$, $\beta = 98°$, $\gamma = 90°$. Two molecules per unit cell; density, calc. 2.56, found 2.59. No absent reflections. The Wilson [2] ratio, $\varrho = \langle|F_{obs}|\rangle^2 / \langle F^2_{obs}\rangle$, is 0.59 for 274 $0kl$ reflections in the range $\sin\vartheta$ from 0.25 to 1. The predicted value being 0.637 for centrosymmetric crystals and 0.785 for noncentrosymmetric crystals, this indicates that the space group is $C_i^1-P\bar{1}$.

II, $a = 5.00 \pm 0.02$ Å, $b = 10.30 \pm 0.04$ Å, $c = 21.78 \pm 0.06$ Å. Four molecules per unit cell; density, calc. 2.55, found 2.53. Absent reflections, $0kl$ when $k + l$ is odd, $hk0$ when h is odd. The Wilson ratio, calculated for $0kl$ reflections as above, is 0.57, which is in favour of the centrosymmetric space group $D_{2h}^{16}-Pnma$.

The crystals, for both modifications, appear as prisms elongated along the a axis and flattened along the b axis. In both cases there is perfect cleavage along (001). The relative distribution of the intensities of the $00l$ reflections, with the c axis of I doubled, is very similar for the two crystals.

A Patterson synthesis based on the $0kl$ data of I gave the y and z parameters of the barium atom, the space group being chosen as $C_i^1-P\bar{1}$. Subsequent Fourier syntheses, starting with the strongest reflections and signs calculated from the barium contributions alone, revealed the positions of the five sulphur atoms, in agreement with the barium-sulphur vectors of the Patterson map. The pentathionate ion is unbranched, and there is an apparent mirror plane perpendicular to the b axis at $z = 0$, $y = 1/4$ and passing through the barium atom and the central sulphur atom of the pentathionate ion at $z = -0.11$ and $z = 0.34$, respectively.

A mirror plane as a molecular symmetry element is in accordance with the requirements of the space group $D_{2h}^{16}-Pnma$ for II. A Patterson synthesis based on the $0kl$ data of II was carried out. The positions and numbers of vector peaks on the resulting map show that the structure of the pentathionate ion in this crystal is the same as in I, and that the space group of II is $D_{2h}^{16}-Pnma$. The barium atom and the central sulphur atom of the pentathionate ion lie in the fourfold positions *(c)* of this space group [3].

The pentathionate ion in barium pentathionate thus possesses a mirror plane of symmetry. The configuration of the sulphur chain is similar to that of the S_8 ring of orthorhombic sulphur [4] with three adjacent sulphur atoms removed.

Work on the crystal structure of barium pentathionate (I and II) is being continued. Also, work is being done on strontium and barium selenopentathionate and telluropentathionate, and on solvates of the above salts with ethanol and acetone.

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On the Use of 2-Amino-1-phenyl-propane (Benzedrine) for Optical Resolution of Acids

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The optical resolution of a racemic acid is usually performed by fractional crystallisation of its salt with some suitable, optically active alkaloid. There are less than ten such alkaloids commercially available and often only some of them will give crystalline salts with the acid in question. As the resolving power of these common alkaloids is rather capricious, it is not always possible to separate both the antipodes or even to prepare one of them in an optically pure condition without an unreasonable number of recrystallisations. In such cases where the common alkaloids fail to give satisfactory results α-phenylethylamine is often found to be useful. However, the disadvantage of this amine is that its optically active forms are not easily available as the resolution is carried out by means of the expensive (−)-malic acid [1]. For that reason the author has investigated the homologous 2-amino-1-phenyl-propane as regards its fitness for optical resolutions. The racemic base, which is also known as benzedrine, is rather cheap and the (+)-form is commercially available. The resolution is easily carried out by means of tartaric acid; recrystallisation of the bitartrate yields the (+)-form and the neutral tartrate the (−)-form [2].

The results from a series of experiments on resolution of different acids have been

Table 1. Experiments on resolution of some acids by means of (+)-2-amino-1-phenyl-propane. If not otherwise stated the optical activity refers to ethanolic solution.

Acid	Solvent	First salt fraction Yield in %	$[\alpha]_D$ of the acid		Excess of active acid %
			found	lit.	
α-Phenoxypropionic	50 % Benzene--petr. ether	50	−4.9°	−39.3°	12
α-(2-Naphthoxy)propionic	85 % Ethanol	34	−79.7°	−93.3°	85
α-(2-Methyl-4-chlorophenoxy)propionic	20 % Ethanol	73	0.0°	+19°	0
α-(2,4-Dichlorophenoxy)-propionic	20 % Ethanol	oil	—		
α-(3,4-Dichlorophenoxy)-propionic	30 % Ethanol	66	−5.8°	−39.3°	15
α-(2,4,5-Trichlorophenoxy)propionic	35 % Ethanol	55	−20.1°	−49.7°	40
α-Phenoxy-*n*-butyric	10 % Ethanol	35	+3.6°	+51.2°	7
α-(1-Naphthoxy)-*n*-butyric	40 % Ethanol	oil	—		
α-(2-Naphthoxy)-*n*-butyric	96 % Ethanol	30	−64.1°	−90.8°	71
α-(2,4-Dichlorophenoxy)--*n*-butyric	30 % Ethanol	oil	—		
α-(2-Naphthylmethyl)-propionic	35 % Ethanol	47	+5.7°[a]	+32.0°[a]	18
Propylsulphidesuccinic (acid salt)	10 % Ethanol--acetone	54	−13.2°	−137.2°[b]	10
β-Hydroxy-β-phenyl-pivalic	96 % Ethanol	42	−2.6°[b]	−9.0°[b]	29
α-Bromopropionic	Acetone	41	−6.7°	−29.5°[c]	20−25

a) in acetone b) in acetic acid c) homogenous

summarised in Table 1. It is notable that crystalline salts were obtained in all cases except three. Also it may be pointed out that most of the acids used in these experiments have rather small tendencies to yield crystalline salts with the common alkaloids. The acids liberated from the first fraction of the (+)-amine salts were obtained in very different degrees of optical purity. In two cases no or very small activity could be detected while in three cases the acids were obtained in a fairly high degree of optical purity. In the remaining six cases the products contained from 10 to 30 % excess of active acid which in many cases is enough to render a resolution possible by repeated recrystallisations. In view of this the use of 2-amino-1-phenylpropane for optical resolution of acids seems to be of great value as a complement to the common alkaloids and as a substitute for α-phenylethylamine.

The author is much indebted to *AB Astra* for a supply of benzedrine, which was used for the preparation of the optically active amine.

1. *Org. Syntheses, Collective Vol.* **II**, New York 1947, p. 506.
2. Nabenhauer, F. P. *U. S. P.* 2 276 508, 2 276 509 (1942).

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Volume Changes Accompanying Enzymatic Reactions with Ribonuclease and Deoxyribonuclease

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Previous investigations by Chantrenne, Linderstrøm-Lang and Vandendriessche [1] and Vandendriessche [2] have shown that the enzymatic breakdown of ribose nucleic acid (RNA) by ribonuclease is accompanied by volume changes that cannot be explained solely in terms of a hydrolytic splitting of an ester bond between a phosphate group and a hydroxyl group in ribose. In fact, the splitting of such a bond would produce a decrease in volume of about 2 ml/eq. at pH 4.6, while actually a contraction of 14 ml/eq. is found. Moreover, at certain temperatures (*e.g.* 30° C) this contraction is preceded by a very rapid increase in volume (curve 1, Fig. 1).

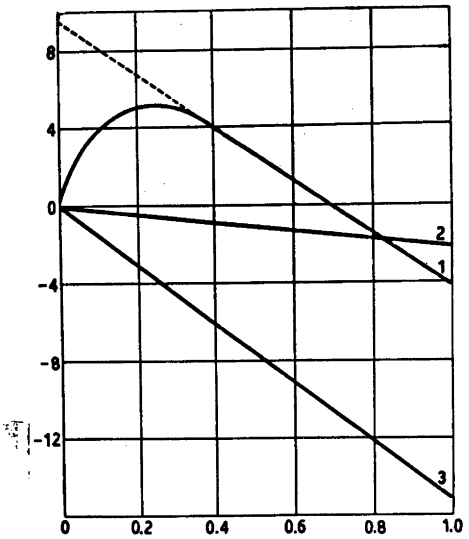

Fig. 1. Relation between volume changes and titration values (pH 4.6—30° C). Absc.: Base consumption in equivalents per mole (P_4 in case of nucleic acids). Ord.: Volume changes in ml per mole (id.).

1. *Yeast ribose nucleic acid and ribonuclease.*
2. *Thymus deoxyribose nucleic acid and deoxyribonuclease.*
3. *Ammonium uridine-2′—3′-phosphate and ribonuclease.*

Several explanations have been proposed for these phenomena, *viz.* that the dilatation is due to the breakdown of a superstructure, and that the contraction is caused by dipole formation. However, in the light of further experiments at other temperatures and pH-values (Vandendriessche [2]), none of the explanations offered were wholly satisfactory. It has now been found that the enzymatic break-

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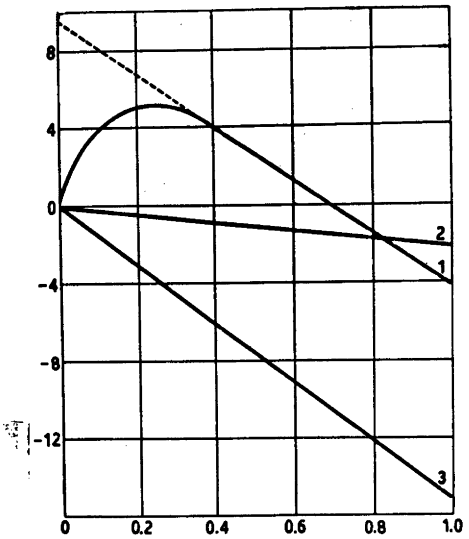

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down of deoxyribonucleic acid (DNA) at pH 4.6 and 30° C is accompanied by a contraction of only 2 ml/eq., and that no initial dilatation occurs (Fig. 1, curve 2). The experiments were made in the way described by Chantrenne, Linderstrøm-Lang and Vandendriessche [1] for the study of RNA-breakdown, *viz.* combined dilatometry and titration of liberated acid groups.

In view of these findings it is natural to correlate the observed difference in behavior between the RNA and the DNA with their main difference in structure, *viz.* the presence or absence of an OH group at position 2′. Since according to Brown and Todd [3] and Brown, Dekker and Todd [4] an intermediate 2′—3′ (cyclic) phosphate is formed in the breakdown of RNA, an investigation was made of the volume change accompanying the enzymatic degradation of ammonium uridine-2′—3′-phosphate. As shown in Fig. 1, curve 3, one titratable group is liberated in this substance by the action of ribonuclease and a contraction of 15 ml/eq. is observed. A similar investigation of cytidine-benzyl phosphate b, which according to Brown and Todd should give rise to cytidylic acid and benzyl alcohol with intermediary formation of cytidine-2′—3′ (cyclic) phosphate, led to results that may be qualitatively represented by curve 1, Fig. 1. Due to lack of material simultaneous estimations of volume change and release of acid groups could not be carried out. The curve for the relation between volume and time matched however closely those found for RNA showing a rapid initial increase in volume (*ca.* + 4 ml/mole at the maximum) followed by a slower fall. This diphosphate ester therefore behaves almost exactly as RNA, and it is therefore suggested that the initial increase in volume, observed in experiments with RNA and cytidine-benzyl-phosphate b is due to the formation of a 2′—3′-phosphate, while the contraction of about 15 ml/eq. equally characteristic of the degradation of RNA, uridine-2′—3′ phosphate and cytidine-benzyl-phosphate b is due to the opening of an ester bond in the cyclic phosphate. On this basis the experiments on DNA (Fig. 1, curve 2) are easily explained too since the absence of an OH group at position 2′ excludes the intermediary formation of cyclic phosphate so that the hydrolysis of the ester bond is uncomplicated and therefore is followed by the normal contraction of about 2 ml/eq.

From these results it would then appe ar that the formation of cyclic phosphate is accompanied by a dilatation of *ca.* 15—2 =13 ml/mole, an astonishingly high value for so small a molecular group. It is to be hoped that a theoretical investigation of this phenomenon may throw light upon the structural details of the cyclic phosphates.

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Note on the Stepwise Degradation of Peptides *via* Phenyl Thiohydantoins

PEHR EDMAN

Department of Physiological Chemistry, University of Lund, Lund, Sweden

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Anhydrous acetic acid saturated at room temperature with dry hydrogen chloride (approx. 2 *M*) brings about a rapid and complete cleavage of PTC-peptides. The fission in this medium of 0.01 *M* solutions of PTC-alanylglycine [1] and PTC-leucylglycine [1] at 37° C was followed by amino nitrogen determinations [5,6]. The reaction was found to be complete in less than 3 minutes.

Acetic acid with a small content of water dissolves large peptides and even many proteins. The effect on the reaction caused by the addition of water (from 5 % to 30 %) to the above mentioned medium (*i. e.* acetic acid-hydrogen chloride) was therefore studied. However, the presence of water did not appreciably increase the length of time required for complete cleavage.

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Molar Refractions and Parachors of Some Organic Phosphoryl Compounds

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A comparison has been made of the calculated and observed values of the molar refractions and the parachors of some organic phosphoryl compounds. At this institute molar refractions of some phosphoryl compounds have earlier been determined by Holmstedt [1].

Apparatus and Experiments. The density was determined by a pyknometer and the refractive index in a refractometer of Bellingham and Stanley's design. The surface tension was measured by means of a stalagmometer. The flow velocity of the liquid was regulated so that the number of drops per minute did not exceed fifteen. All determinations were performed at 20° C.

Calculations. The observed molar refraction was obtained from the Lorenz-Lorentz formula $MR = \frac{M(n^2-1)}{D(n^2+2)}$, and the parachor according to the formula $P = \frac{M\sqrt[4]{\gamma}}{D-d}$, where M = molecular weight, n = refractive index, γ = surface tension, D = density of the liquid and d = density of the vapour over the liquid (d is here extremely small compared with D and can therefore be ignored).

In Table 1 two series of calculated molar refractions are given. One is based on Jones, Davies and Dyke's [2] values for atomic refractions of phosphorus and oxygen, and the other on those of Kabachnik [3]. In both series Vogel's [4] values were used for the other atomic group refractions, with the exception of the values for chlor-

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Table 1. Molar refractions and parachors of some organic phosphoryl compounds

Compound	Density at 20° C	Molar refraction				Parachor		
		$n_D^{20°}$	$MR_{obs.}$	$MR_{calc.}$ a)	$MR_{calc.}$ b)	γ 20°	$P_{obs.}$	$P_{calc.}$
$(C_2H_5O)_2PO \cdot H$	1.072	1.4075	31.75	31.65	31.47	30.9	607.6	606.3c)
$(i \cdot C_3H_7O)_2PO \cdot H$	0.997	1.4080	41.11	41.00	40.82	26.4	755.5	757.3c)
$(C_2H_5O)_2PO \cdot F$	1.145	1.3719	31.00	31.36	—	25.9	307.6	312.8
$(i \cdot C_3H_7O)_2PO \cdot F$	1.058	1.3825	40.54	40.71	—	24.5	387.3	387.7
$(C_2H_5O)_2PO \cdot Cl$	1.210	1.4177	35.90	36.39	36.18	32.0	339.0	342.0
$(i \cdot C_3H_7O)_2PO \cdot Cl$	1.102	1.4175	45.85	45.74	45.53	28.3	419.9	417.0
$CH_3OPO \cdot Cl_2$	1.493	1.4344	26.01	26.31	25.93	34.9	242.5	244.9
$C_2H_5OPO \cdot Cl_2$	1.381	1.4347	30.78	30.96	30.58	32.8	282.3	283.1
$(CH_3)_2NPO \cdot (OC_2H_5)_2$	1.045	1.4180	43.70	43.57	43.90	30.3	406.6	409.8
$(CH_3)_2NPO \cdot OC_2H_5 \cdot Cl$	1.188	1.4404	38.06	38.13	38.29	34.9	351.1	350.7
$(CH_3)_2NPO \cdot F_2$	1.235	1.3470	22.32	22.63	—	25.1	233.9	233.5
$(CH_3)_2NPO \cdot Cl_2$	1.363	1.4637	32.77	32.69	32.68	36.1	291.3	291.7
$(C_2H_5)_2NPO \cdot Cl_2$	1.253	1.4641	41.83	41.99	41.98	35.7	370.7	371.4
$((CH_3)_2N)_2PO \cdot F$	1.115	1.4267	35.14	34.83	—	33.4	332.3	330.3

a) Jones, Davies and Dyke-Vogel-Holmstedt.
b) Kabachnik-Vogel.
c) Arbuzov and Vinogradova give 607.9 and 751.4.

ine in the Cl-P bond in the series according to Kabachnik [3] and the nitrogen in the N—P bond according to Jones, Davies and Dyke [2]. The new chlorine value was calculated by Kabachnik [3] and the new nitrogen value (1.71) by Holmstedt [1].

In calculations of the parachors, Gibling's [5–8] values with special structure corrections seem to be the most reliable. Other authors who have studied group parachors more recently are Samuel [9] and Vogel [4]. Arbuzov and Vinogradova [10], using Gibling's method, have shown that the molecules of phosphites are mainly in dimeric form, for which reason a correction ought to be made in the calculation of the parachor (see also Gibling [6]). In alkoxy groups corrections in respect of β-atoms were made except in the case of *iso*-propoxy compounds, where it was found necessary to correct only one of the two β-atoms.

Results. Close agreement was obtained between the calculated and the observed values for both the molar refractions and the parachors of the phosphoryl compounds studied, as shown in Table 1. Some of the phosphoryl halides, however, are easily hydrolysed by the humidity of the air, which complicated the determinations.

The author is indebted to the Head of this institute, Professor Gustaf Ljunggren, for the kind interest he has shown in this work.

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Note on Vitamin B_{12b}

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Vitamin B_{12b} was first isolated from *Streptomyces aureofaciens* fermentation broth by Pierce *et al.*[1]. The vitamin was believed to be identical with vitamin B_{12a} obtained by catalytic hydrogenation and subsequent exposure to air of vitamin B_{12}[2,3]. However, the opinion has been expressed that vitamin B_{12b}[1] and vitamin B_{12a}[2] should not be identical [4,5].

It can be shown in different ways that vitamin B_{12b} from *Streptomyces aureofaciens* (Lederle 7—9125) is not a homogeneous compound. Ionophoresis on paper in a acetate buffer containing 0.13 mg NaCN/ml has revealed eight different compounds active for *E. coli* 113—6 [6]. One of these is probably cyanocobalamin (obtained even when no cyanide was added to the buffer) as judged from chromatographic, ionophoretic and spectrophotometric examinations. One of the main compounds of the vitamin B_{12b} used, appears to be identical with the major compound of factor A[7]. It showed maxima at 2 780, 3 615 and 5 520 Å, which changed to maxima at 2 780, 3 685, 5 450 and 5 820 Å on addition of an excess of cyanide. Another factor obtained from "vitamin B_{12b}" appeared to be identical with the major component of pseudovitamin B_{12}[7,8]. A compound with chromatographic and ionophoretic properties identical with those of factor B[7] was also detected.

Besides the described factors, four others — all moving towards the anode — were detected. It appears likely that two of these are identical with the factors C_1 and C_2[9]. Vitamin B_{12s}[10] that also moves towards the anode seems to be one of the negatively charged factors for *E. coli* present in the "vitamin B_{12b}". The most predominant of these four factors which was not vitamin B_{12s} showed maxima at about 2 650, 3 600 and 5 500 Å (the preparation used was obtained from an ionophoretic experiment with borate buffer).

A yellow compound was also observed in the „vitamin B_{12b}" employed. Its spectrum was not similar to that of the vitamin B_{12} group of factors, and showed a plateau between 4 400 and 4 650 Å which changed to a maximum at 4 850 Å on addition of cyanide. This as well as its chromatographic behaviour in a mixture of *n*-butanol, acetic acid and water (80 : 15 : 29)[11] suggests that this yellow compound is similar to or identical with the yellow compound observed in a B_{12}-preparation by Schmid *et al.* [11]. The yellow compound obtained from "vitamin B_{12b}" showed activity for *E. coli* but this seems to be due to the presence of cyanocobalamin.

Vitamin B_{12} (Merck & Co., Inc.) also contains several vitamin B_{12}-factors but in this case cyanocobalamin is by far the main compound.

A growth factor for *Lb. lactis* Dorner and *Lb. leichmannii* occurring in Normocytin (Lederle), Reticulogen (Lilly & Co.) and in a *Streptomyces griseus* fermentation broth showing ionophoretic properties different from cyanocobalamin and hydroxycobalamin was reported in a previous paper [12]. A factor with a similar ionophoretic behaviour can also be obtained from "vitamin B_{12s}" provided no cyanide is added to the buffer. It appears to be the aquocomplex of the major component of factor A.

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Growth Factors for *E. coli* 113—3, other than the Vitamin B_{12}-Group or Methionine

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Methionine seems to be the only compound that has hitherto been reported as replacing the vitamin B_{12}-group as a growth factor for *E. coli* 113—3 under aerobical conditions [1-6]. We would like to report the occurrence of other substances stimulating the growth of *E. coli* 113—3 utilized in the agar cup plate method, in media [6,7] that are used for estimation of vitamin B_{12}.

A growth substance for *E. coli*, showing an R_F-value of 0.14 when chromatographed in water-saturated sec. butanol containing 3 % acetic acid and 25 mg/l KCN, was found in insects, *e.g.* ants, grasshoppers and millipedes, in normal human blood and (in higher concentrations) in blood from a patient suffering from leukemia, as well as in mushrooms. Ant eggs were found to be a good source of the factor. Ionophoresis on paper, of a concentrate of this substance obtained from ant eggs, revealed the presence of both acidic and basic groups. Autoclaving this concentrate in 1 *N* HCl or 1 *N* NaOH at 120° C for 1 hour did not decrease its microbiological activity. When hydrolysed in 6 *N* HCl at 100° C for 24 hours the factor disappeared and two new growth factors for *E. coli* with R_F 0.28 and 0.49 appeared. Acid hydrolyses of a sample of autolysed blood from the above mentioned patient with leukemia destroyed the factor having the R_F 0.14 and gave rise to three other factors, two with R_F-values 0.28 and 0.49 respectively, *i.e.* the same values as in the case of ant eggs, the third having R_F 0.67. Methionine has an R_F-value of 0.43 in this solvent. The type of growth caused by these four new factors for *E. coli* is similar to that obtained with methionine and is clearly different from that caused by the vitamin B_{12} group of factors. This indicates that they are active only in comparatively high concentrations. None of these new factors was found in casein hydrolysate nor could they be identified with any of twenty common amino acids. The factor with R_F 0.14 may be a peptide.

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Intermolecular Free Lengths in the Liquid State

IV. Dependence on Temperature

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In the previous works in this series the applicability of the concept of free length has been elucidated [1]. Thus, it has been shown that properties such as compressibility, surface tension and viscosity can easily be related to the free length between the surfaces of the molecules in a pure liquid or liquid mixture. The fact that the concept of free length has not formerly been used in full probably depends on the difficulty of calculating it exactly. The definition hitherto used by us is not ideal as an error is included in it the magnitude of which varies with the temperature.

Owing to the importance of the free length for the understanding of intermolecular processes a systematic investigation has been made here of the temperature dependence of the free length. This problem is principally the same as to find a formula describing the thermal expansion of a liquid system. The thermal expansion is an intermolecular process and is mainly independent of the absolute magnitude of the molecules which is only slightly temperature dependent. Therefore we may anticipate a simpler relation between the free length and temperature than between the density (molar volume) and temperature. We have in this work set up an empirical formula which has been found to describe accurately the temperature dependence of the free length. Using this the corresponding expression for the density has been obtained. For practical purposes values have been calculated for plotting graphs from which the zero point density and the critical temperature of a liquid system can easily be obtained from its densities at two temperatures.

RESULTS

When studying the temperature dependence of physical properties it is often convenient to compare the various substances in corresponding states. This is also the case when studying the intermolecular free length. The reduced free length is $L_r = L_T/L_C$ where L_T is the free length at the temperature T and L_c is the free length at the critical temperature T_c. Systematic attempts have been made in order to correlate the reduced free length with the reduced temperature T_r. Various possible relations have been proposed which were tested using the following three equations.

1. The free length between the surfaces of spherical molecules in hexagonal packing is

$$L_T = (2^{\frac{1}{2}}/N)^{\frac{1}{3}} (V_T^{\frac{1}{3}} - V_o^{\frac{1}{3}}) \quad (1)$$

where N is Avogadro's number, $V_T = M/\varrho_T$ and $V_o = M/\varrho_o$ are the molar volumes at T°K and 0°K.

2. The radius of the spherical molecules in hexagonal close packing is

$$r_o = 1/2(2^{\frac{1}{2}}/N)^{\frac{1}{3}} V_o^{\frac{1}{3}} \quad (2)$$

3. It is a known fact that for various substances there is generally a constant ratio between the densities at absolute zero and at the critical temperature $\varrho_o/\varrho_c = 3.75$ (Partington[2], p. 27). From this it follows that the free length at the critical temperature should be proportional to the molecular radius. Thus

$$L_c = cr_o \quad (3)$$

where c is constant for different substances.

The various relationships between the reduced free length and the reduced temperature, tested by these equations, were obtained from known formulae applying to the reduced state and to the thermal expansion (Partington [2]). The following equation is the one best fitting experimental data

$$1-L_r = (1-T_r)^p \quad (4)$$

where p is constant, L_r the reduced free length and T_r the reduced temperature. To make possible an experimental test of equation (4) the following relation between density and temperature was derived from (1), (2), (3) and (4)

$$\varrho_o = \varrho_T[1 + (c/2)-(c/2)(1-T_r)^p]^3 \quad (5)$$

The values for the constants c and p can be determined in the following way. From equations (1), (2), (3) and (4) a relation between the constants can be obtained

$$c = 2(1-f)/[f-1 + (1-T_r)^p] \quad (6)$$

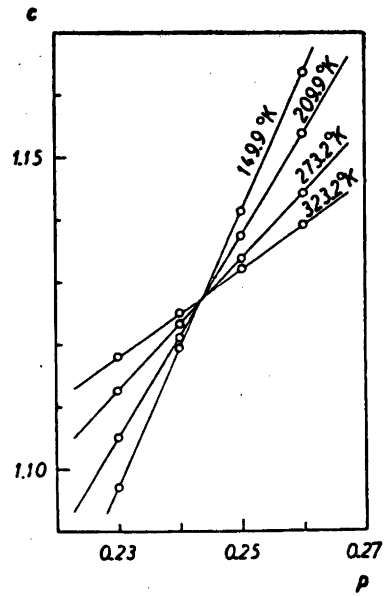

Fig. 1. Graphic determination of constants p and c for diethyl ether.

where $f = (\varrho_c/\varrho_T)^{\frac{1}{3}}$ if ϱ_c and ϱ_T are the densities at T_c°K and T°K. By plotting c as a function of p for a substance at different temperatures a number of curves are obtained whose point of intersection gives the desired values for the constants. In Fig. 1 this is shown for diethyl ether, and in Table 1 a few examples are given of p and c values obtained for various unassociated compounds. The density values and critical temperatures were taken from Timmermans [3]. The average value for the exponent is $p = 0.243$. This value is not far from $p = 1/4$ which value can be chosen without the accuracy of the

Table 1. Values obtained for the constants p and c.

Compound	p	c
Pentane	0.242	1.113
Hexane	0.240	1.117
Octane	0.236	1.150
Benzene	0.246	1.110
Difluorodichloromethane	0.250	1.128
Bromobenzene	0.244	1.124
Carbon tetrachloride	0.249	1.106
Ethyl acetate	0.240	1.155
Diethyl ether	0.244	1.128

proposed equations being reduced. The two corresponding values for the constants thus used in this work are

$$p = 1/4 \qquad c = 1.092 \tag{7}$$

An experimental test of the proposed formulae was thus possible. If equation (4) is valid constant values for the zero point densities ϱ_0 according to equation (5) should be obtained if the calculations are made at different temperatures. Such calculations were made for compounds other than those given in Table 1 and for which accurate density values were available between the melting point and critical temperature. The compounds 2-methylbutane, heptane, toluene, methylcyclohexane, carbon tetrachloride, chlorobenzene, methyl formate and ethyl mercaptan were investigated as well as octane which has c and p values differing most from those given in (7). The average deviation of the ϱ_0 values for all compounds, each studied at five different temperatures, was 0.28 per cent. The corresponding value for octane was 0.15 per cent, the smallness of which justifies the choice of the constants according to (7). Equation (5) describes the change of density with temperature more accurately than any of the corresponding formulae previously suggested (Partington [2], Sec. VIII B, C). The values obtained for the zero point densities are generally 4.3 per cent higher than those calculated from Sugden's formula [4]. But, as more constant values are obtained for each compound when calculated at widely different temperatures, our method of calculating the zero point densities is regarded as preferable.

The equations were also tested on liquid mixtures in the following way. The critical temperature and zero point density were calculated for each mixture by equation (5) from experimentally determined densities (Smyth *et al.*[5]) at two temperatures as described below. Densities then were calculated from (5) for other temperatures and were compared with those experimentally determined. Mixtures of the type heptane — ethylene chloride, heptane — ethyl acetate and heptane — butylbromide were investigated in the temperature range $-90°$ to $+ 70°$ C or $-90°$ to $+ 90°$ C at intervals of 20° C. The average deviation in the calculated density values for different temperatures and mixtures was 0.12 per cent. From that it can be concluded that non-associated liquid mixtures follow equation (4) and (5) with good accuracy.

DISCUSSION

It should be observed that the proposed equation (4) between the free length and the temperature is completely empirical. No satisfactory theoretical deduction could be obtained. However, in view of the accuracy with which the

Table 2. Values for plotting ϱ_0/ϱ_T as a function of T/T_c according to equation (5) and $L_T/V_T^{\frac{1}{3}}$ as a function of T/T_c according to equation (8). L is measured in Å, V_T in ml and the temperatures in degrees Kelvin.

T/T_c	ϱ_0/ϱ_T	$L_T/V_T^{\frac{1}{3}}$	T/T_c	ϱ_0/ϱ_T	$L_T/V_T^{\frac{1}{3}}$
0.300	1.1463	0.0591	0.575	1.3498	0.1267
0.325	1.1612	0.0646	0.600	1.3742	0.1336
0.350	1.1768	0.0702	0.625	1.4002	0.1411
0.375	1.1928	0.0759	0.650	1.4278	0.1488
0.400	1.2095	0.0816	0.675	1.4573	0.1568
0.425	1.2269	0.0876	0.700	1.4894	0.1652
0.450	1.2451	0.0938	0.725	1.5233	0.1740
0.475	1.2640	0.0999	0.750	1.5606	0.1832
0.500	1.2839	0.1062	0.775	1.6015	0.1931
0.525	1.3048	0.1128	0.800	1.6467	0.2036
0.550	1.3267	0.1195			

equation holds, it seems likely that it should be possible to give a theoretical deduction of this or a similar equation.

As the free length cannot be measured directly it was indirectly inferred, from the good agreement between experimental data and equation (5), that equation (4) is also valid with the same accuracy. The presumption for this is the validity of equations (1), (2) and (3). Of these (1) and (2) are simple stereometric formulae derived assuming spherical molecular shape and hexagonal packing. This is an oversimplification of the actual conditions. However, the influence of molecular shape on the free length has been studied previously and it was found that for low molecular weight substances the molecules can be regarded as spheres [1]. On the other hand form factors must be introduced when studying macromolecular compounds. The form of packing has even less influence on the equations (1) and (2). For cubical packing identical equations are obtained. Equation (3) is based on experimental data and the applicability of it is further illustrated by the good agreement in c values obtained for various compounds as shown in Table 4. For these reasons equation (4) can be regarded as valid.

For the practical use of the given equations it is convenient to make the calculations graphically. Values for drawing commonly used curves are given in Tables 2 and 3. If the zero point density ϱ_0 is required the ratio ϱ_0/ϱ_T is first graphically calculated from the reduced temperature. If the

Table 3. Values, calculated from equation (5), for plotting the critical temperature T_c °K as a function of the ratio $\varrho_{t_1}/\varrho_{t_2}$ of the densities at two temperatures t_1 and t_2 °C.

T_c (°K)	$\varrho_{t_1}/\varrho_{t_2}$						
	$t_1 = 0$ $t_2 = 15$ (°C)	$t_1 = 0$ $t_2 = 20$ (°C)	$t_1 = 0$ $t_2 = 30$ (°C)	$t_1 = 15$ $t_2 = 25$ (°C)	$t_1 = 15$ $t_2 = 30$ (°C)	$t_1 = 20$ $t_2 = 30$ (°C)	$t_1 = 20$ $t_2 = 60$ (°C)
400	1.03392	1.04614	1.07229	1.02422	1.03714	1.02498	1.11727
425	1.02920	1.03960	1.06152	1.02054	1.03137	1.02109	1.09571
450	1.02572	1.03481	1.05382	1.01811	1.02739	1.01835	1.08143
475	1.02301	1.03109	1.04793	1.01601	1.02434	1.01632	1.07114
500	1.02088	1.02819	1.04320	1.01444	1.02191	1.01465	1.06330
525	1.01907	1.02572	1.03944	1.01320	1.01999	1.01338	1.05704
550	1.01765	1.02376	1.03631	1.01211	1.01835	1.01226	1.05209
575	1.01640	1.02206	1.03367	1.01120	1.01698	1.01135	1.04803
600	1.01538	1.02043	1.03143	1.01048	1.01583	1.01057	1.04450
625	1.01438	1.01926	1.02935	1.00978	1.01477	1.00990	1.04155
650	1.01353	1.01811	1.02764	1.00921	1.01395	1.00936	1.03895
675	1.01285	1.01723	1.02617	1.00888	1.01320	1.00879	1.03658
700	1.01211	1.01632	1.02477	1.00827	1.01250	1.00833	1.03456
725	1.01156	1.01550	1.02355	1.00785	1.01184	1.00794	1.03275
750	1.01109	1.01480	1.02248	1.00746	1.01129	1.00755	1.03113
775	1.01057	1.01413	1.02145	1.00716	1.01078	1.00722	1.02969
800	1.01011	1.01353	1.02054	1.00683	1.01032	1.00692	1.02838
900	1.00863	1.01156	1.01750	1.00580	1.00876	1.00586	1.02404
1 000	1.00755	1.01008	1.01532	1.00508	1.00770	1.00514	1.02096

free length L_T is required at a certain temperature the ratio $L_T/V_T^{\frac{1}{3}}$ is first graphically obtained from the reduced temperature according to

$$L_T = V_T^{\frac{1}{3}}(2^{\frac{1}{3}}/N)^{\frac{1}{3}}[1-(1-T_r)^{\frac{1}{2}}]/[1 + (2/c)-(1-T_r)^{\frac{1}{2}}] \quad (8)$$

which equation has been obtained from (2), (4) and (5).

If the critical temperature is unknown for a liquid system, it can easily be obtained graphically from (5) if two densities ϱ_{t_1} and ϱ_{t_2} are known at two temperatures T_1 and T_2°K (t_1 and t_2° C). The ratio $\varrho_{t_1}/\varrho_{t_2}$ is plotted as a function of T_c for the two temperatures. Some values are given in Table 3 which have been calculated using −273.2° C as the value of the absolute zero. Some examples of calculated critical temperatures are given in Table 4. The average error for 18 non-associated compounds is 1.4 per cent. This method of calculating the critical temperature is of special interest for the study of associated liquids which will be treated in a subsequent work.

Table 4. Critical temperatures calculated from the ratio of densities at two temperatures $\varrho_{t_1}/\varrho_{t_2}$.

Compound	ϱ_{t_1} at (t_1 °C)	ϱ_{t_2} at (t_{t2} °C)	T_c (°K) Calc. from $\varrho_{t_1}/\varrho_{t_2}$	T_c (°K) Calc. mean value	T_c (°K) Expt. observed	Per cent deviation
Hexane	0.67704 (0°)	0.66380 (15°)	512			
	0.67704 (0°)	0.65055 (30°)	516			
	0.6593 (20°)	0.6502 (30°)	508	512	508.0	+ 0.7
*Cyclo*hexane	0.78310 (15°)	0.76928 (30°)	558			
	0.78310 (15°)	0.74060 (60°)	555			
	0.77853 (20°)	0.76914 (30°)	552	555	553.9	+ 0.2
Benzene	0.89996 (0°)	0.88420 (15°)	548			
	0.88420 (15°)	0.86844 (30°)	554			
	0.8790 (20°)	0.8357 (60°)	552	551	561.7	− 1.9
Chlorobenzene	1.12792 (0°)	1.09550 (30°)	623			
	1.1062 (20°)	1.0955 (30°)	638			
	1.1062 (20°)	1.0636 (60°)	640	633	632.4	0.0
Ethyl acetate	0.92453 (0°)	0.90657 (15°)	515			
	0.90657 (15°)	0.88851 (30°)	521			
	0.90665 (15°)	0.89446 (25°)	517	518	523.3	− 1.0
Diethyl ether	0.73622 (0°)	0.71925 (15°)	468			
	0.71925 (15°)	0.70205 (30°)	472			
	0.71930 (15°)	0.70768 (25°)	470	470	466.8	+ 0.7

Sometimes it is of interest to know the temperature derivative of the free length

$$dL_T/dT = p(L_c/T_c)(1-T_r)^{p-1} = p(L_c-L_T)/(T_c-T) \quad (9)$$

The equations given in this work for calculating the free length and the zero point densities should replace those previously used if good accuracy is required. The formulae obtained make possible an exact study of the temperature dependence of intermolecular properties by relating them to the free length. For instance, it has appeared that the fluidity for non-associated liquids is a strictly linear function of the free length within large temperature ranges.

SUMMARY

The reduced free length in a liquid system $L_r = L_T/L_c$ is related to the reduced temperature $T_r = T/T_c$ by the equation $1-L_r = (1-T_r)^p$ where L_T and L_c are the intermolecular free lengths at the temperature T and at the

critical temperature T_c respectively. The molecular radius is $r_0 = L_c/c$. As p and c vary only slightly with different compounds they can be put equal to 1/4 and 1.092, respectively. From this equation formulae were derived which make possible calculations of zero point densities and critical temperatures for a liquid system if the densities at two different temperatures are known. Values are given for plotting curves for graphical computations of such data. Knowledge of the temperature dependence of the free length is of value for the study of intermolecular properties and processes.

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Bioautographic Separation of Vitamin B_{12} and Various Forms of Folinic Acid Occurring in Some Brown and Red Seaweeds

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The growth promoting activity of extracts of six different species of seaweed for *Lactobacillus leichmannii* 313 (ATCC 7 830), *Lb. lactis* Dorner (10 697), *Lb. lactis* Dorner (8 000), *Leuconostoc citrovorum* (8 081), and *Streptococcus faecalis* (8 043) was reported in a previous paper [1].

The present paper deals with chromatographic separation and bioautographic identification of the vitamin B_{12} and folinic acid (citrovorum factor, CF) type of growth factors in extracts of these algae.

EXPERIMENTAL

Materials and methods. Samples of *Phaeophyceae, i.e. Sphacelaria arctica (Sphacelariales)*, *Laminaria saccharina (Laminariales)* and *Fucus vesiculosus (Fucales)*, and of *Rhodophyceae, i.e. Furcellaria fastigiata (Gigartinales)*, *Polysiphonia nigrescens (Ceramiales)*, and *Rhodomela subfusca (Ceramiales)*, were collected on the east and west coasts of Sweden in the autumn of 1951, as reported previously [1].

Fresh algal samples were dried at room temperature, then ground and extracted with boiling water for 30 min. under reflux to release the growth factors. Drops of the extracts and of a standard solution were placed on 16 × 48 cm sheets of Whatman No. 1 filter paper and dried. These large sheets made it possible to run a standard solution and 4–5 extracts simultaneously. Descending chromatograms with *n*-butanol, water and acetic acid (125 : 125 : 30) were run overnight and the solvent front was allowed to advance about 40–42 cm from the starting line. The chromatograms were then dried.

Lactobacillus lactis Dorner (ATCC 8 000) and *Leuconostoc citrovorum* (ATCC 8 081) served as test organisms. The general technique associated with the maintenance of the

lactobacilli, the substrates etc. was the same as employed when using the agar cup plate method [2]. Tomato juice, fumaric acid and sodium-ethyloxalacetate were omitted from the assay medium in the case of *L. citrovorum*. Sterilized portions of the agar medium (150 ml) were cooled to 45—50°, mixed with 6 ml of a suspension of twice washed cells of *Lb. lactis* Dorner (LLD) (density: galvanometer reading 40 in Coleman spectrophotometer at 5 000 Å) or *L. citrovorum* (density: galv. reading 70) and poured into rectangular dishes of the same size as the chromatographic sheets. After solidification the chromatograms were spread out on the surface of the agar plates. The sheets were left on the agar plates during the whole incubation period (16—18 hrs) and removed only before readings were made. The incubation temperature was 37°. The development of *L. citrovorum* plates was found to be satisfactory at this temperature, which is higher than that recommended for the growth of this organism.

A solution containing 0.5 μg/ml of vitamin B_{12} (Cobemin, Merck), 0.1 mg/ml of guanine desoxyriboside (or alternatively hypoxanthine desoxyriboside), and 0.1 mg/ml of thymidine was used as a standard in the tests with LLD. The standard mixture used for the tests with *L. citrovorum* contained 0.1 mg/ml thymidine and 0.3 μg/ml of synthetic citrovorum factor (Leucovorin, Lederle, lot No. 7—1 142—4 B). A calcium salt of a natural citrovorum factor (Ca CF)-isolated from horse liver by Keresztesy and Silverman [3], and an injectable liver concentrate (Reticulogen, Lilly) was also used for comparison.

Separation of the LLD active factors in algal extracts. Crystalline vitamin B_{12} separates — if not protected from light — into two fractions, a slower fraction considered to be vitamin B_{12b} (hydroxocobalamin), and a faster moving fraction which is vitamin B_{12} (cyanocobalamin) [4,5]. The conversion of cyanocobalamin to hydroxocobalamin by light has been observed by several authors (*cf.*[6]).

As shown in Fig. 1, the extracts of three of the six algae tested, *viz. Laminaria saccharina, Polysiphonia nigrescens, Rhodomela subfusca*, seem to contain vitamin B_{12b} but none of them vitamin B_{12}. With the agar cup plate method we have already shown the existence of LLD active factors in these algae [1]. However LLD responds not only to vitamin B_{12} and B_{12b} but also to desoxybriosides if present in relatively high concentrations. Besides vitamin B_{12b} several desoxyribosides have been detected in these algal extracts. Thymidine was clearly present in all seaweeds investigated except *Fucus vesiculosus*. Cytosine-, guanine-, and hypoxanthine-desoxyridosides have very similar R_F-values in butanol-acetic acid as shown by Smith and Cuthbertson [4]. By employing the method described by Carter [7] for the separation of desoxyribosides, using Na_2HPO_4 in amyl alcohol as solvent, it was found that the slowest moving desoxyriboside present in *Sphacelaria arctica, Laminaria saccharina* and *Furcellaria fastigiata* was in all three cases hypoxanthine desoxyriboside.

There was no opportunity of testing a pure sample of adenine desoxyriboside, but one of the samples of thymidine employed as a standard contained some adenine desoxyriboside as impurity. The latter substance has a characteristic R_F-value between those of hypoxanthine desoxyriboside and thymidine and it was found that *Sphacelaria arctica* and *Furcellaria fastigiata* contained adenine desoxyriboside in quantities sufficient for detection. Other desoxyribosides possibly present in the seaweed extracts investigated, but in very low concentrations — less than 10 μg/ml of extract — as well as vitamin B_{12} if present only in concentrations less than 0.01 μg/ml, could not be detected in the extracts because such low levels were outside the range of the estimations.

Growth factors for Leuconostoc citrovorum. The organism *Leuconostoc citrovorum* 8 081, used originally in the research of the anti pernicious anemia (APA) principle [8], gives response with the natural and the synthetic citrovorum factor (CF, folinic acid) and with thymidine if present in high concentrations but not with the other desoxyribosides or with vitamin B_{12} [9,10]. The "folinic acid" or "citrovorum factor" has been shown to be N_5-formyl-5,6,7,8-tetrahydro folic acid [11,12].

Extracts of the algae have been chromatographed and the chromatograms developed bioautographically with *L. citrovorum* according to Winsten and Eigen [13] (Table 1). All the chromatograms except that of *Fucus vesiculosus* showed a thymidine spot with the same R_F-value as a spot developed on chromatograms with *Lb. lactis* Dorner (*cf.* Fig. 1). In at least four of the six seaweeds a citrovorum factor with the same R_F-value as that of the synthetic

Table 1. Distribution of growth factors, active towards Leuconostoc citrovorum 8 081, in red and brown seaweed extracts when chromatographed in a n-butanol, water, acetic acid mixture.

Spots with the same R_F-value as that of:	Rhodophyceae			Phaeophyceae		
	Sphacelaria arctica	*Laminaria saccharina*	*Fucus vesiculosus*	*Furcellaria fastigiata*	*Polysiphonia nigrescens*	*Rhodomela subfusca*
Leucovorin and Ca-CF	+	+	+	+*	+	nil
Thymidine	+	+	nil	+	+	+

* And other slowly moving factors (see text).

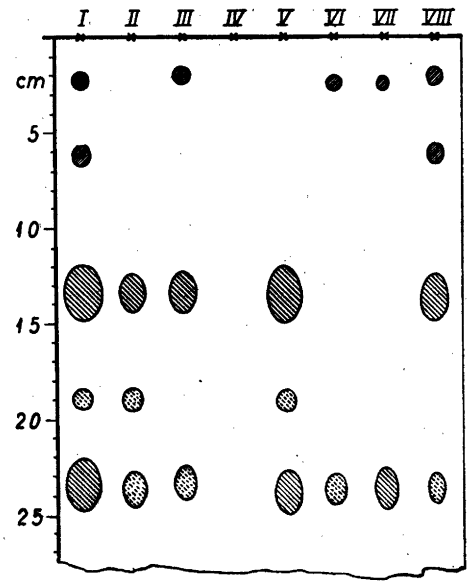

Fig. 1. Factors detected with Lactobacillus lactis Dorner 8 000. Columns I—VIII, spots representing zones of growth caused by:

I Standard: vit. B_{12b}, vit. B_{12}, desoxyribosides of hypoxanthine, adenine and thymine.

II Sphacelaria arctica: desoxyribosides of hypoxanthine, adenine and thymine.

III Laminaria saccharina: vit. B_{12b}, desoxyribosides of hypoxanthine and thymine.

IV Fucus vesiculosus: no factors for LLD.

V Furcellaria fastigiata: desoxyribosides of hypoxanthine, adenine and thymine.

VI Polysiphonia nigrescens: vit. B_{12b}, and thymine—desoxyriboside.

VII Rhodomela subfusca: vit. B_{12b}, and thymine—desoxyriboside.

VIII Reticulogen (Lilly): vit. B_{12b}, vit. B_{12}, desoxyribosides of hypoxanthine and thymine.

CF (tetrahydroformylfolic acid, Leucovorin) [12], was found. In our experiments the calcium salt of the natural CF (Ca CF) isolated from horse liver [14, 15] showed the same R_F-value as Leucovorin which is in agreement with the results reported by Sauberlich [16]. Dietrich *et al.* also found that autolyzed liver from several animal species gave spots similar to that of synthetic CF [17].

In addition to the CF detected in the algae, three further more slowly moving citrovorum factors were found in *Furcellaria fastigiata* as seen in Fig. 2, column II. The citrovorum factors of this alga were investigated more closely

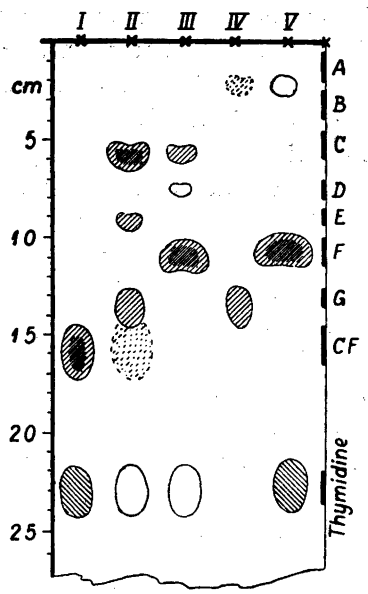

Fig. 2. Factors detected with Leuconostoc citrovorum 8 081. Solid lines indicate regular, dotted lines occasional growth. Shadowing was used when growth was more intensive. Columns I—V, spots representing zones of growth caused by;

I Standard: Leucovorin (Lederle) and Ca-citrovorum factor (in the same spot), and thymidine.

II Furcellaria fastigiata.

III Furcellaria fastigiata, enzyme treated.

IV Horse liver.

V Horse liver, enzyme treated.

as described below and were compared with liver and yeast which have been reported to possess CF of multiple nature [13].

Because synthetic di- and triglutamates of folinic acid are active towards *L. citrovorum* (*cf.* Shive *et al.*[11]) in a similar way, as di- and triglutamates of folic acid are towards *Lb. casei* and *S. faecalis* the existence of these conjugates of CF in nature was assumed. Winsten and Eigen [13] reported in their early work two slowly moving growth factors for *L. citrovorum* in liver which could be converted by an enzyme in rat stomach homogenate to a faster moving factor with high growth promoting activity. Furthermore Hill and Scott [18] have shown that dried brewers' yeast contains the CF largely in bound form, from which it can be released by a hog kidney extract known to contain folic acid conjugase. From the results obtained they conclude "that folic acid conjugase and the CF-liberating enzyme of hog kidney are strikingly

similar if not identical". For these reasons the hog kidney enzyme was used for the comparison of the citrovorum factors in *Furcellaria*, liver and yeast.

Chromatograms were made of portions of a hot water extract of *Furcellaria*, of fresh horse liver homogenated in a Waring blendor (1 g of fresh liver per 10 ml of water) and of a solution of yeast extract (Difco) steamed for 30 min. in an autoclave (1 g per 10 ml water). In order to release the citrovorum factors from their microbiologically inactive bound form or from their conjugates, similar to those of folic acid (*cf.* [16—19]), other portions of the same extracts were mixed with equal volumes of a suspension containing 1 g hog kidney homogenate and 0.14 mole cysteine HCl per liter, according to Hill and Scott [18, 19]. The suspension was adjusted to pH 4.5. The extracts were digested under toluene at 40° C for at least 16 hours. After evaporation of the toluene they were chromatographed and the chromatograms developed with *L. citrovorum*. The kidney extract did not contain significant amounts of factors stimulating the growth of this lactobacillus. Fig. 2 shows the results obtained with *Furcellaria* and liver.

Liver does not seem to contain a factor identical with the slowest moving factor in *Furcellaria*, neither before nor after enzyme treatment, although liver gives a spot representing a still lower R_F-value. Both *Furcellaria* and liver have a factor — on the horizontal zone *G* — with R_F-value slightly lower than that of the synthetic CF and Ca CF of horse liver (zone *CF*). The main effect following enzymatic digestion appears to be that in the case of both *Furcellaria* and liver a heavy spot due to a factor in the zone *F* appeared. The slow moving factor, zone *C*, in *Furcellaria* decreased on the same treatment.

Enzymatic digestion of yeast extract (Difco), released four factors with R_F-values lower than that of tetrahydroformylfolic acid. Yeast extract showed features different from those of liver and *Furcellaria* before and after digestion but it was not further investigated whether any of the factors of yeast extract were identical with the CF type of factors of extract of horse liver and the alga *Furcellaria*. Doctor and Couch [20] found, however, that the microbiologically inactive precursors of the CF in liver and in yeast are similar at least in the respect of their movement in different solvent systems. It is of interest to note that in our experiments the CF-conjugase of hog kidney did not liberate CF itself but instead only more slowly moving factors. The experiments clearly show that several naturally occurring factors active towards *L. citrovorum* exist, some or all of which may be conjugates of folinic acid (CF).

SUMMARY

Paper chromatograms of hot water extracts of three brown and three red seaweeds were developed bioautographically using *Lactobacillus lactis* Dorner, and *Leuconostoc citrovorum*.

1. Vitamin B_{12b} seems to be present in the following three algae: *Laminaria saccharina*, *Rhodomela subfusca* and *Polysiphonia nigrescens*.

2. The desoxyribosides of adenine, hypoxanthine and thymine seem to be present in different concentrations in the different algae.

3. In five algae, *Sphacelaria arctica*, *Laminaria saccharina*, *Fucus vesiculosus*, *Furcellaria fastigiata* and *Polysiphonia nigrescens*, a citrovorum factor similar to N_5-formyl-5,6,7,8-tetrahydrofolic acid was found. *Furcelleria fastigiata*, the alga richest in growth factors, contained three slow-moving citrovorum factors other than formyltetrahydrofolic acid. A comparison of the citrovorum factors of *Furcellaria fastigiata* and of horse liver before and after treatment with hog kidney homogenate, which contains CF-conjugase, shows that *Furcellaria* seems to contain two slow moving factors which are not present in horse liver. The same treatment released several slow moving citrovorum factors in a yeast extract (Bacto).

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Studies on Ribonuclease and Desoxyribonuclease Activities in Homogenates from Human Placenta

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The biological importance of the nucleic acids has stimulated the interest in the enzymes related to these compounds. Numerous investigations on the mechanism of action and the kinetics of these enzymes have been carried out. The subject has been reviewed recently [1,2]. However, attempts to establish a correlation between the developmental stage of a tissue and the activities of these enzymes, have not led to any positive results.

In connection with an investigation on the quantitative changes of nucleic acids during the growth of the human placenta [3,4,19], it was considered desirable also to carry out some analyses on the ribonuclease (PNase) and desoxyribonuclease (DNase) activities of this tissue at different stages of development. The object of the present study is to find out whether there exists a correlation between the enzyme activities and the developmental stage of the tissue.

MATERIALS AND METHODS

Tissue preparation. Placentas from different stages of pregnancy were obtained at abortions provoked on social-medical indications according to Swedish law, and at parturitions. All the patients were in good health. The age of the placentas was estimated from the over-all (crown-heel) length of the foetus, applying Haase's rule (quoted from Wylie and Amidon [5]).

Immediately after removal the placentas were freed from the amnion membrane and the decidua. The remaining foetal placenta was cut into small pieces, washed in glass-distilled water and frozen in a beaker immersed in a dry ice-alcohol mixture. The preparation was immediately brought to the laboratory and homogenized in glass-distilled water for four minutes in a Bühler homogenizator. The tissue concentration was about 10 per cent. Precautions were taken to keep the temperature in the homogenate close at 0° C. This homogenate was used for the quantitative assay of the enzyme activities, and the determinations were begun within an hour after removal of the placentas.

Substrates. Commercial yeast nucleic acid was used as the original substance of the substrate for the PNase determinations. All the preparations were purified in the same way. The crude preparation was dissolved in a 10 % sodium chloride solution by adding some concentrated NH_3 to pH 6—6.5. The concentration of the nucleic acid was about 3 to 4 per cent. The solution was dialyzed against running tap water (about + 10° C) for 20 days and against frequently changed distilled water (+ 4° C) for two days. The nucleic acid was then precipitated by adding three volumes of absolute alcohol and a few ml of a 10 % sodium acetate solution. The precipitate was washed a few times with absolute alcohol and dried with ethyl ether. The physico-chemical data of the preparation used in this study are summarized in Table 1. The extinction values are calculated by the method of Chargaff and Zamenhof [6].

In the determinations of DNase activity desoxyribonucleic acids (D.N.A.) from calf thymus was used as substrate. It was prepared by Hammarsten's [7] method. The only modification was that at the last precipitations with absolute alcohol a few ml of a 10 % (w/v) sodium acetate solution were added instead of a sodium chloride solution. The physico-chemical data of the preparation used in this study are summarized in Table 1.

For convenience the substrates are referred to as D.N.A. and yeast nucleic acid, but it is understood that it is always the neutralized salt of the nucleic acids to which reference is made.

General procedure for the determination of enzyme activities. The PNase and DNase activities were measured by estimating the release of acid-soluble substances containing pentose and desoxypentose sugar. The colorimetric determinations were carried out as described earlier [8,9]. By running correspondent determinations of acid-soluble substances containing phosphorus, the correlation between phosphorus and sugar was evaluated. By this means the enzyme activities can be expressed in terms of the number of micrograms of acid-soluble phosphorus. The main reason why the colorimetric sugar determinations were preferred throughout the study is that the tissue and substrate blanks, in the DNase determinations being zero, are considerably lower than the blanks when using the phosphorus determination procedure, owing to the relatively greater amount of acid-soluble tissue phosphorus. The sensitivity of the colorimetric methods and their convenience also contributed to this choice.

Determination procedure for the PNase activity. The homogenate, containing between 2 mg and 3.5 mg of total nitrogen per ml, was diluted 25 times with glass-distilled water. Duplicate determinations were made with two amounts of tissue. 0.5 ml of a 1/15 *M* phosphate buffer (pH 7.40—7.45) and 0.5 ml of a 1 % purified yeast nucleic acid solution

Table 1. Physico-chemical data of the substrates used.

Substance	N/P	*E* max	*E* min	ε(P)max	ε(P)min	ε(P) 280	pH
D.N.A.	1.69	260	231	8 045	3 198	5 097	6.0
P.N.A.	1.56	258	228	8 199	2 980	4 217	6.2

N = nitrogen; P = phosphorus; *E* max, *E* min = wave length in mμ for maximal and minimal extinction; ε (P) = atomic extinction coefficient with respect to phosphorus.

were used. The total volume of the reaction mixture was 2 ml and the final * pH 7.55—7.60. The determinations were carried out in a Warburg apparatus with a constant temperature water bath at 37° C under continuous shaking of the reaction vessels. The yeast nucleic acid was placed in the side-arm and added after temperature equilibrium had been attained. The incubation time was 60 minutes. The reaction was stopped by adding 2 ml of an ice-cold solution of 0.25 % (w/v) uranyl acetate in 10 % (w/v) trichloroacetic acid (T.C.A.) [10]. The tubes were allowed to stand at + 4° C for one hour and were then centrifuged. An aliquot of the centrifugate was used for the colorimetric sugar determinations. Appropriate tissue and substrate blanks were carried out for all analyses. The activity is expressed as the increase in acid-soluble phosphorus during 60 minutes per mg of total tissue nitrogen or per μ g of tissue D.N.A. phosphorus (D.N.A.P.); see Discussion.

Determination procedure for the DNase activity. The determinations were carried out with five amounts of tissue, using from 0.1 ml to 1.0 ml of homogenates, containing 2 mg to 3.5 mg of total nitrogen per ml. 0.5 ml of a 0.2 *N* acetate buffer (pH 4.95—5.00) and 1 ml of a 1 % (w/v) D.N.A. solution were used. The volume was made up to 3.5 ml and the final pH was 5.0 to 5.1. The determinations were carried out in the Warburg apparatus for 180 minutes at 37° C. The reaction was stopped by adding 1.5 ml of an ice-cold 20 % (w/v) T.C.A. solution. After standing at + 4° C for about 15 minutes the tubes were centrifuged. An aliquot of the centrifugate was used for the colorimetric sugar determinations, which were made immediately. Tissue and substrate blanks were zero. The activity is expressed as the increase in acid-soluble phosphorus during 180 minutes and is given for a point on the concentration-activity curve, which corresponds to an amount of tissue, containing 1 mg of total nitrogen or 20 μg of D.N.A.P.

Other determination procedures. Tissue D.N.A.P., after having been extracted by the method of Schneider [11], was determined as described earlier [9]. Every determination, including the extraction procedure, was run in duplicate.

The duplicate nitrogen determinations were done by the micro-Kjeldahl technique [12]. Phosphorus was determined according to Teorell's [13] modification of Fiske and Subba-Row's [14] method. Manipulations and centrifugations (M.S.E. refrigerated centrifuge) were carried out as close to 0° C as possible. All the solutions were prepared daily.

The colorimetric determinations were done in a Beckman quartz spectrophotometer, using a 1 cm cell. The pH values were electrometrically controlled with a Radiometer pH-meter 3.

EXPERIMENTAL

Prior to the adoption of determination procedures for the PNase and DNase activities in placental homogenates, the influence of a variety of factors on these activities had to be studied.

1. Determination of PNase activity

Influence of pH. The effect of hydrogen ion concentration is demonstrated in Fig. 1. The optimum is situated near pH 7.6. The inhibitory effect of the borate buffer should be noted [15].

* By final pH is meant the pH value of the whole reaction mixture at the beginning of the reaction. In order to avoid losses it was measured in a duplicate mixture.

Table 2. Relationship between homogenate concentration and PNase activity.

Amount of homogenate in mg total nitrogen	Activity per μg D.N.A.P.
0.028	28.6
0.045	30.2
0.056	29.4
0.090	29.4
0.112	29.3
0.180	31.6
0.280	30.0
0.450	23.2
0.560	19.3

Activity expressed as increase in μg of acid-soluble phosphorus during 60 minutes. D.N.A.P. = desoxypentose nucleic acid.

Tissue concentration. To establish the relationship between homogenate concentration and enzymic activity the experiment summarized in Table 2 was undertaken. A rather wide tissue concentration range is determinable and it covers safely the homogenate concentrations used.

Incubation time. The relationship between activity and incubation time is dependent on the concentration of the homogenate used. Under the present experimental conditions the linearity between enzymic activity and time is sustained for more than 60 minutes. In order to check that every experiment was conducted within the appropriate time and tissue concentration all analyses were carried out in duplicate with two amounts of tissue.

Stability of the PNase activity in the homogenate. No decrease in PNase activity was observed after 12 hours. After 24 hours the activity had decreased by about 15 per cent.

Digestibility of the substrate. The digestibility of different batches of yeast nucleic acid, purified in the same way, varied considerably. The same preparation was therefore used throughout this study.

Substrate concentration. The effect of the substrate concentration on the PNase activity is demonstrated in Fig. 2. Five mg of yeast nucleic acid were considered adequate for the PNase determinations. An increase in substrate concentration brought about a considerable increase in blank values.

2. Determination of DNase activity

Influence of pH. Fig. 3 shows a rather sharp activity peak at pH 5.0 for a 12 week old placenta. For a full term placenta maximum activity is situated near pH 5.5. This displacement of the pH maximum was found to be correlated to the age of the placenta. Before the sixteenth to eighteenth week of pregnancy maximum was invariably situated in the range of pH 5.0. At the middle of pregnancy the activities at pH 5.0 and pH 5.5 were about the same. In full term placentas maximum was situated near pH 5.5.

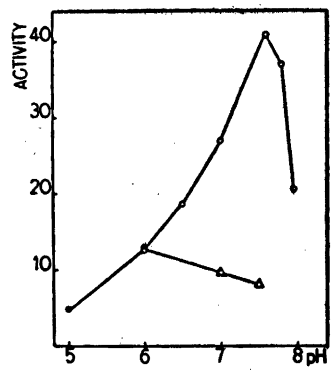

Fig. 1. Effect of pH on the PNase activity in placental homogenate. Activity expressed as increase in acid-soluble phosphorus per μg of tissue D.N.A.P. per 60 minutes.

●——● *0.2 N acetate buffer.*
○——○ *1/15 M phosphate buffer.*
△——△ *0.2 M boric acid, 0.05 M sodium tetraborate.*

Influence of magnesium. It was noted that magnesium inhibited the placental DNase activity at pH 5.0. Fig. 4 shows the effect of inhibition at different concentrations of magnesium.

Tissue concentration. Fig. 5 shows some DNase activity curves from homogenates of placentas of varying ages, where the activity has been plotted against the amount of homogenate expressed as μg of tissue D.N.A.P. In the concentration range chosen, all the curves show a lag phase in the rate of formation of acid-soluble enzymic hydrolysis products, but from a certain point all the curves are linear.

Because of the configuration of the homogenate concentration curves and for the sake of comparison between different placentas, the activities were calculated for a tissue nitrogen or tissue D.N.A.P. value common to all the placentas.

Incubation time. Fig. 6 demonstrates the time-activity curve for placental DNase activity. This curve also exhibits a lag phase. Because of this fact and of the relatively weak DNase activity in full term placentas an incubation time of 180 minutes was used.

Stability of the DNase activity in the homogenate. A fairly rapid inactivation of the DNase activity was observed at pH 5.0. The activity eight hours after the homogenization procedure was 65 per cent of that immediately after homogenization. In contrast to this finding the activity at pH 5.5 showed an increase during the same period, amounting to 47 per cent. After 22 hours, however, the activity at this pH had also decreased by 20

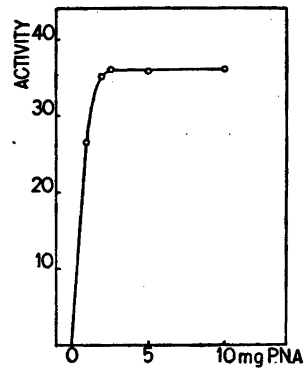

Fig. 2. Effect of substrate concentration on the PNase activity in placental homogenate. P.N.A. = yeast nucleic acid.

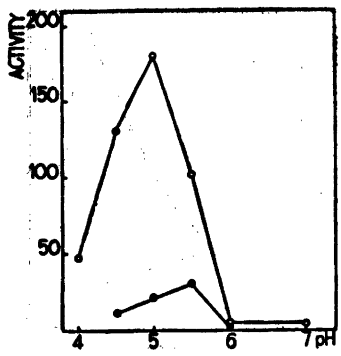

Fig. 3. Effect of pH on the DNase activity in placental homogenate. Activity expressed as increase in acid-soluble phosphorus per 20 μg of tissue D.N.A.P. per 180 minutes.
0.2 N acetate buffer pH 4.0—6.0
1/15 M phosphate buffer pH 6.0—7.0
12 week old placenta.
full term placenta.

per cent compared with the initial value. It should be noted that the activity at pH 4.5 showed principally the same pattern as that at pH 5.0 and that the pH 6.0 value principally resembled that at pH 5.5. No corrections for the decrease in activity at pH 5.0 have been made.

Substrate concentration. The influence of the substrate concentration is demonstrated in Fig. 7. 10 mg of D.N.A. were considered adequate for the DNase activity determinations.

3. Inhibitor experiments

Some experiments were carried out to demonstrate the presence of an inhibitor in homogenates from human placenta against crystalline DNase. The reactions were run at pH 7.0, using a 0.1 *N* veronal-acetate buffer, and at pH 5.0, using a 0.1 *N* acetate buffer. Crystalline DNase, obtained from Worthington Chemical Corp., was used in a concentration of 30 μg per ml at pH 5.0 and 3 μg per ml at pH 7.0. The enzyme was stabilized by 0.25 per cent gelatin [16]. The DNase was activated by magnesium, which in the reaction mixture had a concentration of 0.01 molarity. Besides 0.25 ml of a 0.140 *M* magnesium sulphate solution and 0.5 ml of the DNase solutions, the same volumes were used as those quoted for the placental DNase activity determinations. The incubation time was 30 minutes.

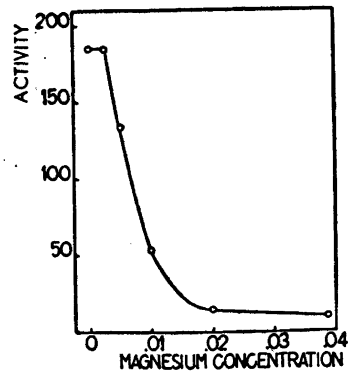

Fig. 4. Effect of magnesium on the DNase activity in placental homogenate. Magnesium concentration expressed as final molarity.

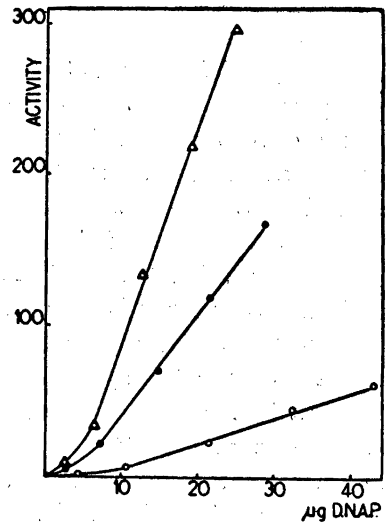

Fig. 5. Relationship between tissue concentration and DNase activity in placental homogenate. The amount of tissue expressed as µg of D.N.A.P. Activity expressed as increase in acid-soluble phosphorus per 180 minutes.

△——△ *11 week old placenta.*

●——● *20 week old placenta.*

○——○ *40 week old placenta.*

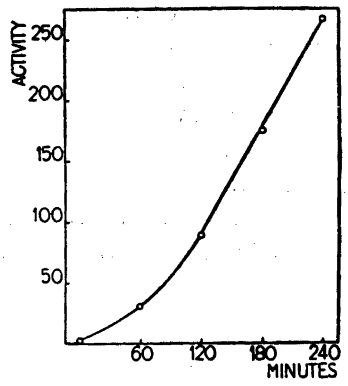

Fig. 6. Rate of formation of acid-soluble phosphorus by incubation of D.N.A. with placental homogenate. Activity expressed as increase in acid-soluble phosphorus per 20 µg of tissue D.N.A.P.

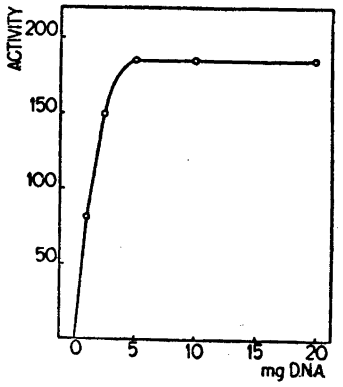

Fig. 7. Effect of substrate concentration on the DNase activity in placental homogenate. Activity expressed as in Fig. 3. D.N.A. = desoxypentose nucleic acid.

RESULTS

PNase activity. Table 3 summarizes the figures for the PNase activity in homogenates from placentas of different ages. When the activity is calculated per mg of total tissue nitrogen, the values show some variability between different placentas, but seem to be approximately constant throughout development. This approximate constancy is also observed when the enzymic activity is calculated per μg of tissue D.N.A.P.

DNase activity. Table 4 is a condensation of the analytical results concerning DNase activity in homogenates from placentas at different phases of development. The activity, expressed per 20 μg of tissue D.N.A.P., shows a marked decrease during the aging of the placenta. The activity of a 10 week old placenta is about ten times that of a full term placenta. If the activity is expressed per 1 mg of total tissue nitrogen there is also an indication of such a decrease, but the correlation to the age of the placenta is not as well established as when the tissue D.N.A.P. is used as a basis of reference.

Inhibition of crystalline DNase. The inhibitor experiments demonstrated the presence of an inhibitor against crystalline DNase. Small amounts of homogenate, corresponding to about 0.2 mg of total tissue nitrogen, caused an inhibition of 70 per cent. The inhibitor exerted its effect at pH 7.0. At pH 5.0 the reverse was observed, the crystalline DNase being activated by placental homogenate with about 100 per cent. Under these conditions of the experiment the DNase which was incubated at pH 5.0 in the presence of placental homogenate, had an activity that was about 20 per cent of that of the pure crystalline DNase activity at pH 7.0. The problem will be further studied.

Table 3. PNase activity during development of placenta.

Age in weeks	PNase activity/mg N	PNase activity/μg D.N.A.P.
10	323.9	36.5
11	419.0	41.4
13	354.8	27.3
14	342.7	25.5
17	364.8	25.3
20	493.4	37.8
40	501.3	39.7
40	478.0	33.1

Activity expressed as increase in μg of acid-soluble phosphorus during 60 minutes. N = total nitrogen; D.N.A.P. = desoxypentose nucleic acid; Every value the mean of duplicates.

Table 4. DNase activity during development of placenta.

Age in weeks	DNase activity/1 mg N	DNase activity/20 μg D.N.A.P.
10	68	217
11	90	226
13	73	133
13	100	183
14	64	110
14	82	195
17	111	131
20	69	108
20	42	113
40	10	22
40	11	23
40	12	20

Activity expressed as increase in μg of acid-soluble phosphorus during 180 minutes. N = total nitrogen; D.N.A.P. = desoxypentose nucleic acid phosphorus; The activity values calculated from the regression lines, see text.

STATISTICAL ANALYSIS

The error of the method has been calculated from differences of individual pairs of values according to the following formula (the error dependent on the level of the substance measured)

$$k = \sqrt{\frac{\sum w^2}{2n}}$$

where k = the relative error (coefficient of variation)

$$w = \frac{x-y}{\frac{1}{2}(x+y)}$$ (x and y duplicate determinations)

n = number of pairs.

For the calculation of the error of the method for the DNase determination procedure the curved part of the line (Fig. 5) has been omitted throughout the material by leaving out the first point of observation. To each placenta a straight line has been fitted to the remaining points by the method of least squares [17,18], each point being given the same weight. The residual variance has been determined by standard methods. With the method used for the

Table 5. Accuracy of determination methods used revealed by statistical analysis.

Substance	n	Relative error in single determination
Total nitrogen	28	± 1.4
D.N.A.P.	30	± 1.7
PNase activity	16	± 5.9
DNase activity	36	± 7.1

D.N.A.P. = *desoxypentose nucleic acid.* n = *number of determinations.*

determination of DNase activity the error in a y-determination (y = activity) should be proportional to the y-value. This assumption has been confirmed by plotting the mean deviation (square root of the residual variance) against $\bar{y}$ (mean y-value) for each line. Under these conditions the mean deviation from a straight line related to the mean y-value gives an estimate (Q) of the relative error. The mean relative error (coefficient of variation = $\bar{Q}$) has been determined according to the formula

$$\bar{Q} = \sqrt{\frac{\sum Q^2}{n}}$$

where n = is the number of lines.

The results of the statistical analysis of the material are summarized in Table 5.

DISCUSSION

From the experiments described here it is evident that a correlation can be demonstrated between the developmental stage of the placenta and the DNase activity (calculated with the tissue D.N.A.P. as a basis of reference) in the corresponding homogenate. No such correlation was established for the PNase activity.

It has been demonstrated that the average D.N.A.P. content per nucleus in the placenta is constant during different stages of development [19], as calculated from the D.N.A.P. contents and cell counts on suspensions of isolated nuclei. The calculation of the enzymic activities per μg of D.N.A.P. thus gives a relative expression for the average per tissue unit. The D.N.A.P. content of a tissue sample gives an estimation of the total number of units in it and seems to be a better expression for the active mass than is, for instance, total nitrogen and wet or dry weight.

The homogenates used in this investigation are suspended in distilled water. Examination by phase contrast microscopy reveals almost complete disintegration of whole cells and nuclei. The mitochondria, however, are not disrupted by the mechanical homogenization procedure, but it is considered that these particles disintegrate in distilled water [20], which may be the case especially after previous freezing and thawing. Studies on the cellular distribution of DNase and PNase activities have revealed that the mitochondrial fraction is endowed with enzymic activity. The extent of this activity varies considerably in different tissues and according to the methods used [21–23]. In calf thymus cells [24] the mitochondrial DNase activity is 12 per cent of that of the whole homogenate and the mitochondrial PNase activity 23 per cent, in mouse liver [25] the corresponding figures are 73 per cent and 58 per cent.

It should be emphasized that the complete disintegration of the structural formations of the cell, which certainly play an important rôle in connection with intracellular enzymic activity, means that what is measured is not the average *in vivo* intracellular enzymic activity but the average maximal activity under the conditions chosen.

The DNase activity of a young human placenta has its pH optimum in the range of 5.0 and is inhibited by magnesium (see foregoing). This is in sharp contrast to the observations on the enzyme, which is prepared from pancreatic tissue and available in crystalline form. This enzyme exerts its maximal activity between pH 6 and 7 and requires magnesium for activation [16,26–29]. A broad pH spectrum for the optimal DNase activities from different organs has been demonstrated [30]. The inhibitory effect of magnesium has also been demonstrated for spleen [31] and calf thymus [24] DNase activity. The optima for these enzymic activities are also situated near pH 5.

The initial, non-linear, increase in the rate of formation of acid-soluble enzymic degradation products (Fig. 6) is in agreement with observations by Kunitz [29] on crystalline pancreas DNase. The fact that the initial lag phase is more marked when increasing the substrate concentration, is thought to be due to the higher viscosity of the more concentrated D.N.A. solutions [29].

The observed decrease in DNase activity during the development of the placenta can be brought about by a decrease in the enzyme concentration of the tissue, a decrease of an activator of the enzyme, an increase of an inhibitor against the enzyme, or by a combination of all these factors. Zamenhof and Chargaff [32,33] reported the existence of a specific DNase inhibitor which was found in yeast cells. Inhibition of crystalline DNase from beef pancreas by a great variety of both normal and neoplastic tissues has been demonstrated [34–37].

The present investigation has shown that a strong inhibition of crystalline pancreas DNase is brought about by homogenates from human placenta at pH

7.0 but that the activity is increased at pH 5.0, both findings being in agreement with the results obtained by Henstell and Freedman [38] on extracts of whole blood or bone marrow. These authors hold that the degree of inhibition of crystalline DNase is correlated to the maturity of the cells and discuss the possibility of this inhibitor to control cell maturation and development. It is questionable, however, if any conclusions regarding the regulation of tissue DNase activity may be drawn from experiments on the inhibition of crystalline pancreas DNase by tissue homogenates or extracts.

Up to the present comparatively little work has been done on the relationship between PNase and DNase activities and the developmental stage of a tissue. An approach to the problem has been made by Greenstein and Jenrette [39], Greenstein [40-42], Greenstein and Stewart [41] and Greenstein and Thompson [43]. In a series of investigations on these enzymic activities in normal and neoplastic tissues these workers examined the PNase and DNase activities in adult resting and regenerating rat liver and in hepatomas and foetal livers of the same species. These authors, however, were not able to correlate the activities to the physiological state of these different tissues. When comparing the DNase activities of induced tumors and the normal tissue of origin no consistent results were obtained.

With respect to the constancy of the PNase activity during different stages of cellular development the present investigation confirms the results obtained by Greenstein and co-workers [39-43]. It may be emphasized, however, that all these investigations, including the present one, have been carried out with yeast nucleic acid as a substrate. The possibility that experiments with organ-specific, highly polymerized P.N.A. might give other results cannot be ruled out.

The DNase activity measurements in the investigations by Greenstein and co-workers [39-43] were done by the viscosimetric technique. This fact, however, does not necessarily exclude a comparison between these investigations and the present one. Laskowski [44] has presented evidence indicating that probably the same enzymic activity is measured, when estimating the decrease in viscosity as when determining the amount of acid-soluble enzymic degradation products of the D.N.A. All the determinations by Greenstein and co-workers, however, were run at pH 7. Moreover, the measurements were made on a water extract which was allowed to stand at + 5° C for 18 hours. A considerable inactivation of the enzyme during this time cannot be ruled out (see under Experimental).

Lately, Allfrey and Mirsky [23] have brought some evidence of a correlation between the DNase activity of a tissue and its D.N.A. turnover, measured as the incorporation of N^{15}. The enzymic determinations were run near pH 5. From these experiments on a diversity of adult animal tissues these authors

put forward the hypothesis that there exists "a possible connection between the DNase concentration of a tissue and its capacity for proliferation or regeneration". However, comparative studies made by these workers on enzymic activities of foetal and adult tissues or young, adult and regenerating rat livers have not brought any direct support of this hypothesis.

In a recent investigation [45], using the same technique as that presented in this paper, a correlation between the growing state, expressed by the mitosis frequency, and the DNase activity was established in normal and regenerating bone marrow from hens. The results from these studies are thus in principal agreement with those obtained for placental tissue. In the homogenates from bone marrow activation of the DNase activity in the range of pH 5.0 by magnesium was observed, but a considerable activity was present also without addition of magnesium. A full account will be given in a report to be published.

SUMMARY

1. Methods based on the determination of acid-soluble nucleic acid degradation products containing pentose and desoxypentose have been worked out for the determination of PNase and DNase activities in human placental homogenates.

2. The DNase activity in a young placenta has its pH optimum in the range of pH 5.0 and is inactivated by magnesium at this pH.

3. This DNase activity has been measured in homogenates of placentas of different ages. A correlation between enzymic activity (calculated with tissue D.N.A.P. as a basis of reference) and the developmental stage has been established. The activity of a 10 week old placenta is ten times that of a full term placenta.

4. The PNase activity has its pH optimum near pH 7.6.

5. This PNase activity is approximately constant throughout development.

To Professor E. Hammarsten and Dr. Bo Thorell I want to acknowledge my indebtedness for their constructive criticism and helpful suggestions I wish also to express my deep gratitude to Professor K. Myrbäck for valuable discussion.

I am also indebted to Dr. Nils Blomqvist for useful suggestions on statistical problems. To Mrs. M. Thorell, who made the drawings, I want to express my deep thanks.

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Alkalimetric Determination of Phosphoric Acid and Phosphates

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Various methods have been worked out for determination of phosphoric acid and phosphates on the basis of the following principle: With a suitable indicator present, the sample is neutralized to the first equivalence-point of the phosphoric acid (pH = *ca.* 4.5 corresponding to $H_2PO_4^-$). Then a suitable excess of a salt is added, the cation of which — by the methods hitherto worked out at a somewhat higher pH — reacts with the phosphate and quantitatively forms a precipitate of the tertiary phosphate (or a *welldefined* basic phosphate).

The precipitation may be:

$$H_2PO_4^- + 3\ Ag^+ \rightarrow Ag_3PO_4 + 2\ H^+$$

The liberated — or gradually liberated — hydrogen ions are titrated with a strong base.

A calcium salt (or a barium salt) can be used as mentioned by Kolthoff and Stenger [1], who also give the most important literary references. Titration is carried out by addition of a calcium salt until pH = *ca.* 9 (phenolphthalein).

Also a silver salt can be used. This method has been thoroughly investigated by Brunisholz [2], who, too, refers to earlier literature. A further specified excess of silver nitrate is used and titration is carried out until pH = *ca.* 6 (chlorophenol red).

In the present paper a method is proposed, according to which precipitation of phosphate is carried out by means of cerous nitrate:

$$H_2PO_4^- + Ce^{+++} \rightarrow CePO_4 + 2\ H^+$$

As was shown by the experiments, the precipitation of cerous phosphate at the first equivalence-point of the phosphoric acid was so complete that the

liberated hydrogen ions could be titrated to the pH of the first equivalence-point. This procedure offers the following advantages:

1) The experimental and theoretical advantage that the same indicator can be used when fixing the pH as well as when carrying out the titration itself.

2) The theoretical advantage that the possibility of the presence of acids consuming the base between the pH of the first equivalence-point and the end-point of the titration is eliminated. (The actual metal ions added (cerous ions) precipitate only when $pH > 7$).

On the other hand, like the above-mentioned methods, the present method will be inhibited or rendered impracticable by the presence of acids consuming the base in the proximity to the first equivalence-point and thus preventing an accurate fixation of pH being made. Futhermore, the method will be inhibited by the presence of substances which — like dihydrogen phosphate ions — react with cerous ions under liberation of hydrogen ions.

As to the choice of indicator when the sample is to be neutralized to the first equivalence-point, we have followed Pierre *et al.*[3] These authors recommend a mixed indicator consisting of 0.02 % methyl orange and 0.1 % bromocresol green. They state that the pH-range of methyl orange is 3.1 —4.4 and that of bromocresol green 4.0—5.6, while the mixed indicator in question at $pH = 4.3$ has a characteristical change of colour from greenish yellow to light green. However, it is not clear at which ion strength these values off pH are valid.

During our titrations a white porcelain plate was used as underlay, and in the experiments a daylight bulb was used when necessary as source of illumination. In preparing the mixed indicator the method of Pierre *et al.* was followed:

> "The most satisfactory method of bringing both indicators into solution is to weigh 0.1 gram of bromocresol green and 0.02 gram of methyl orange into an agate mortar, and to grind these with a pestle as small amounts of sodium hydroxide solution are added, using a total of 2 cc 0.25 *N* sodium hydroxide or its equivalent. The solution is transferred to a beaker and then to a 100-cc volumetric flask and made up to volume with water. Ten drops of the indicator mixture are used per 150 cc of the solution titrated. While small variations from this amount give equally satisfactory results, it seems best to keep the ratio of indicator to solution to near one drop to 15 cc of solution titrated."

For the alkalimetric titration 0.1 *N* sodium hydroxide (made of 50 % sodium hydroxide diluted with boiled and cooled water) was used. This sodium hydroxide was standardized against hydrazine sulfate. For the fixation of the first equivalence-point 0.1 *N* hydrochloric acid was used. The cerous salt solution was prepared from the purest $Ce(NO_3)_3,6H_2O$.

Table 1. The solution of phosphate used contains 1.140 % PO_4 (gravimetrically determined). After fixation at pH = ca. 4.3 a cerous salt is added, and the hydrogen ions liberated are titrated alkalimetrically to the same pH.

Series of expts.	Number of determinations	% PO_4 found	Greatest deviation from mean value (absolute)	Deviation from gravimetric determination (relative)	Remarks
I	6	1.152	0.003	+ 1.1 %	No addition of NaCl.
II	7	1.151	0.002	+ 1.0	As I, but heating and cooling down before titration.
III	6	1.147	0.002	+ 0.6	Addition of 1 g NaCl
IV	5	1.141	0.001	+ 0.1	» » 2 » »
V	5	1.139	0.002	− 0.1	» » 3 » »
VI	5	1.138	0.002	− 0.2	» » 4 » »
VII	3	1.136	0.001	− 0.4	» » 5 » »
VIII	9	1.140	0.001	0	» »2.5 » » + $CaCl_2$ equivalent to the amount of phosphate.
IX	9	1.140	0.002	0	As VIII, but twice as much $CaCl_2$.

As sample was used a *ca.* 0.125 *M* solution of Na_2HPO_4 *p.a.* The phosphate content of the solution was determined gravimetrically as magnesium pyrophosphate; result: 1.140 % PO_4. In the following experiments 10.182 ml of this solution of Na_2HPO_4 were used.

METHOD

The indicator (and in some of the experiments also sodium chloride) is added to the sample. Fixation at the first equivalence-point is carried out by means of 0.1 *N* hydrochloric acid. A small excess of the cerous nitrate solution is added, and titration is made with a standard 0.1 *N* sodium hydroxide until the indicator assumes the same colour as at the first equivalence-point. Total volume: 75—100 ml.

RESULTS

The results are shown in Table 1. In No. I series of experiments the addition of cerous nitrate caused immediate precipitation of cerous phosphate. In series No. II heating until boiling was carried out after the precipitation of

cerous phosphate, by which procedure the precipitate disappeared and reappeared only when, during the titration, about 0.5 ml 0.1 *N* sodium hydroxide was still left to be added. Such a variation in procedure may be used to denote that the end-point of the titration is being approached, otherwise it does not seem to be of any advantage.

In Nos. I and II series of experiments the fixation is evidently made at a pH-value in the solution which is below the first equivalence-point. The first equivalence-point here is roughly estimated to be at pH = 4.5 (*cf.* below), while titration is carried out until pH = *ca.* 4.3. Accordingly, more hydrogen ions are bound in the phosphate than corresponding to the first equivalence-point, and the additionof cerous ions consequently liberates too many hydrogen ions, which makes the results too high.

In the following series of experiments the ionic strength in the solution is increased by addition of sodium chloride, and so the pH for the equivalence-point is decreased. On the basis of series Nos. III—VII it is seen that in connection with the concentrations used here an addition of *ca.* 2.5 g sodium chloride will bring about such an ionic strength that the first equivalence-point will become identical with the pH-value at which the mixed indicator has its characteristical change of colour.

In series Nos. VIII and IX this ionic strength has been roughly adhered to, and the experiments show that addition of reasonable amounts of a calcium salt does not handicap the method. If calcium ions are present, however, titration must be carried out at a somewhat slower rate in order to leave time for a possible precipitate of calcium phosphate to be converted into cerous phosphate.

In some experiments in a later series the precipitated cerous phosphate was filtered off, and then washed, dried and weighed. Calculated under the assumption that the precipitate is pure $CePO_4$, this precipitate should weigh 0.2905 g; three experiments gave the following results: 0.2910 g; 0.2915 g; 0.2925 g. Consequently, the cerous phosphate precipitation should be practicable also as a gravimetric method.

Theory: An addition of 2.5 sodium chloride evidently gives a suitable ionic strength, which is in agreement with what is to be expected theoretically. Bjerrum and Unmack [4] state the following expressions for the first two dissociation constants in solutions of sodium chloride (at 18° C):

$$pk_{H_3PO_4} = 2.178 - 0.35 \sqrt[3]{c} + 0.18\ c$$

and

$$pk_{H_2PO_4^-} = 7.277 - 1.13 \sqrt[3]{c} + 0.185\ c$$

where c is the concentration of sodium ions and where the formulae are valid for $0.1 > c > 0.003$. Bjerrum and Unmack also state:

$$pk_{H_3PO_4} = 2.120 \quad \text{and} \quad pk_{H_2PO_4^-} = 7.227$$

when the ionic strength is nil.

In the titrations carried out, where the volume during the fixation at the first equivalence-point before the titrations was *ca.* 75 ml, c is approximately equal to 0.03 without addition of sodium chloride. From the above formulae we get:

$$pk_{H_3PO_4} = 2.074 \quad \text{and} \quad pk_{H_2PO_4^-} = 6.932$$

Further, the approximate value of c is 0.6 after addition of 2.5 g sodium chloride. Certainly this value is far above the exact validity range of the above-mentioned formulae, but if these are still used, we get:

$$pk_{H_3PO_4} = 1.991 \quad \text{and} \quad pk_{H_2PO_4^-} = 6.435$$

If the first equivalence-point is calculated by means of the approximated formula:

$$pH = \tfrac{1}{2} (pk_{H_3PO_4} + pk_{H_2PO_4^-})$$

we get:

$$pH = 4.50 \quad \text{for} \quad c = 0.03$$

and

$$pH = 4.21 \quad \text{for} \quad c = 0.6$$

Also the interval of transition of the indicator is somewhat altered, and even if no exact statement can be made with regard to the mixed indicator, reference can be made to papers dealing with methyl orange by Güntelberg and Schiödt [5] and with bromocresol green by Chase and Kilpatrick [6]. In these papers the classical dissociation constants for the two indicators have been determined in solutions of potassium chloride, and conditions here cannot be essentially different from conditions in solutions of sodium cloride. The experimental results from the two papers cited have been used in Table 2.

Table 2. The classical dissociation constant indicated by pk for methyl orange (according to Güntelberg and Schiödt [5]) and for bromocresol green (according to Chase and Kilpatrick [6]) in dependence with the concentration of the electrolyte (mainly KCl).

Electrolyte moles per liter	Methyl orange pk (18°)	Bromocresol green pk (25°)
0.0	3.49	
0.1	3.37	4.51
0.2	3.36	
0.5	3.43	4.39
1.0	3.58	4.44
2.0	3.85	4.53

The values of p*k* for both indicators, initially decreasing and later on increasing, explain, when compared with the decreasing (finally only slightly decreasing) values of pH for the first equivalence-point, the results of Nos. III—VII series of experiments.

SUMMARY

An alkalimetric method for determination of phosphoric acid and phosphates has been worked out. Principle: The solution is neutralized to the first equivalence-point of phosphoric acid ($H_2PO_4^-$). By addition of cerous ions cerous phosphate is precipitated and two hydrogen ions per group of phosphate are liberated. This amount of acid is titrated with a strong base to the same pH as before.

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Argentometric Determination of Bromide with Nitroferroin as Redox Indicator

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The use of starch-iodine as redox indicator in connection with the argentometric determination of iodide has been known for many years (see *e.g.* Kolthoff and Stenger [1]). The principle of the method is as follows (see also Charlot and Bézier [2]):

The iodide solution is treated with starch and a very little free iodine (or a very small amount of the iodide present is oxidized to iodine). The solution is titrated with silver nitrate. The end-point of the titration is indicated by the disappearance of the blue colour of the starch-iodine complex. The oxidation potential of the liquid is:

$$E = E_0 + 0.03 \cdot \log \frac{[J_2]}{[J^-]^2}$$

During the titration, the starch-iodine complex acts as redox indicator with transition interval from blue to colourless when $E = ca.$ 0.90 volt is exceeded (corresponding to the absence in the solution of iodide ions necessary for the formation of the blue starch-iodine complex). When the blue colour has just disappeared, it is still possible to show the presence of free iodine in the solution; but if the titration is continued beyond the end-point, the free iodine may react as follows:

$$J_2 + 3\ H_2O \rightleftharpoons JO_3^- + 5\ J^- + 6\ H^+$$

which is equivalent to the equilibrium being forced to the right.

Crawford and Bishop [3] have used *o*-dianisidine as redox indicator instead of starch-iodine. Kolthoff [4] has investigated the titration potentiometrically.

In the present paper the same principle has been made the basis of a determination of bromide. As the normal potential, $2\ Br^-/Br_2$ (= 1.06 volt),

is somewhat greater than the normal potential, $2\,J^-/J_2$ (= 0.53 volt), the redox indicator to be used must have a transition interval which is somewhat larger than that of starch-iodine. For the bromide/bromine system the following equation is valid:

$$E = 1.06 + 0.03 \cdot \log \frac{[Br_2]}{[Br^-]^2}$$

At the equivalence-point for the titration of Br^- with Ag^+ (as the solubility product for silver bromide is $10^{-12.2}$) we get:

$$E = 1.06 + 0.03 \cdot \log[Br_2] + 0.37 = 1.43 + 0.03 \cdot \log\,[Br_2]$$

Therefore, the transition interval of the redox indicator should be about 1.30—1.35 volt (somewhat dependent on $[Br_2]$). Nitro-ferroin meets this requirement, as it can be used in the range at about 1.30 volt. The transition interval is, however, somewhat dependent on pH (a little smaller values for E in strongly acidic liquids — *cf.* Smith and Richter [5]).

In the following experiments solutions of potassium bromide and silver nitrate, which were made by weighing chemicals *p.a.*, were used. The potassium bromide used was 0.09988 *M*; the silver nitrate used was 0.09989 *M*.

EXPERIMENTAL

Method: To 20 ml water are added 5 ml 4 *M* nitric acid, 2 drops of 0.025 *M* nitro-ferroin and 2 drops (= 0.10 ml) of saturated bromine water. A few drops of 0.1 *M* potassium bromide are added, and then 0.1 *M* silver nitrate is added dropwise, until a transition takes place from red to nearly colourless (a faint violet). In order to ensure a correct fixation of the transition another drop of 0.1 *M* potassium bromide is added and then the small amount of 0.1 *M* silver nitrate which is now required for transition.

The sample in which Br^- is to be determined, is added. Titration is now carried out with standard 0.1 *M* silver nitrate. The flask is held in slight rotation, and titration is continued until the red precipitate changes colour from red to yellow. A white porcelain plate is used as underlay. The transition from red to yellow is not denoted by a significant change of colour, and it is difficult to observe it in artificial light. However, the transition seems to be very sharp.

RESULTS

In Table 1 are given the results from a series of experiments, which show that the method yields results that are *ca.* 1 ‰ too high. In the experiments the same solutions as were used in the determination itself, were used for the fixation of the primary transition of the indicator; in principle, however, it is not necessary to know the exact concentration of the solutions that are used for the fixation of this transition of the indicator.

Table 1. Determination of Br^- using nitro-ferroin as indicator. Calculated: $M_{KBr} = 0.09988$. Found as mean value (last column but one): $M_{KBr} = 0.09997$. Found as result of control determination No. 2 of the same solutions (last column): $M_{KBr} = 0.10012$.

Expt. No.	Fixation of Indicator ml KBr	ml $AgNO_3$	Determination ml KBr	ml $AgNO_3$	Found M_{KBr}	Control No. 2
1	0.60	0.66	20.16	20.18	0.09999	0.10009
2	0.56	0.63	19.96	20.01	0.10014	0.10033
3	0.30	0.40	20.23	20.26	0.10004	0.10026
4	0.37	0.45	20.02	20.02	0.09989	0.09999
5	0.35	0.45	20.16	20.17	0.09994	0.10003
6	0.41	0.50	20.61	20.61	0.09989	0.10013
7	0.48	0.55	20.23	20.24	0.09994	0.10008
8	0.31	0.39	19.96	19.96	0.09989	0.10004
9	0.47	0.58	20.05	20.06	0.09994	0.09994
10	0.33	0.41	20.37	20.39	0.09999	0.10018

When the titrations were finished the following control of the results was carried out: A few drops (0.20—0.30 ml) of the standard 0.1 *M* potassium bromide were added and the solution was titrated with silver nitrate. This procedure was repeated, and the results from this control No. 2 are given in the last column of Table 1. It appears that on the whole the results are a further 1 ‰ too high. When control No. 3 was carried out in the same way, the transition was not usually sharp, presumably because the dissolved bromine gradually evaporates from the solution or possibly is oxidized. Consequently, it was not possible to show the presence of free bromine in the solution after the titration had been finished.

An attempt at following the titration potentiometrically was made. Approaching the equivalence-point the potential became constant rather quickly, but just before the equivalence-point the potential (E = *ca.* 1.2 volt) became oscillatory and unstable, probably because of the evaporation of the free bromine due to the constant stirring.

During the determinations no iodide nor chloride must be present, which limits considerably the possibilities of the method in question. The method is mainly theoretically interesting.

By an argentometric method worked out by Uzel [6] for determination of bromide α-naphtoflavone is used as indicator. Although Uzel gives a different explanation of the mechanism, his method is presumably related to the method stated above.

SUMMARY

A new argentometric method for determination of bromide has been worked out. In this precipitation titration, nitro-ferroin is used as redox indicator, a small amount of free bromine having been added beforehand.

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Cuprous Compounds of Acetylene

III. Identification of Dissolved Addition Compounds in Chloride Solutions

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In a previous paper [1] of this series the subject was the equilibria between acetylene and its cuprous addition complexes in aqueous solutions of cuprous chloride and hydrochloric acid. Variations of the hydrochloric acid concentration cause considerable changes in the activity coefficients of the ions present, since HCl is the predominating electrolyte in these systems. It is therefore of interest to investigate a system, where the ionic strength can be kept constant by use of an indifferent electrolyte. This electrolyte must be a strong acid of high concentration, in order to repress the formation of acetylidic compounds. We have used *perchloric acid*, which is supposedly indifferent at the formation of cuprous complexes, and whose mixtures with hydrochloric acid show a fairly constant HCl activity coefficient at a given ionic strength [2].

The majority of the measurements in the present paper concerns the system

$$x \text{ m } HCl,\ (4-x) \text{ m } HClO_4,\ CuCl(s),\ C_2H_2.$$

The mixture of the two acids thus remains of constant total concentration, but in the system are also present inorganic cuprous chloride complexes, acetylene and acetylene-copper compounds. However, it will be demonstrated, that even in the complete system the ionic strength, with a fair approximation, can be regarded as constant $\mu = 4$ m, provided x is smaller than 1.

Solutions of this composition remain colourless on absorption of acetylene, and the concentration of the yellow acetylidic complex ion can be neglected in comparison with that of the addition complexes. There is no interfering irreversible acetylene reaction and no noticeable oxidation of cuprous copper.

Table 1. Solubility of cuprous chloride in 4 molal mixtures of hydrochloric and perchloric acid, 25° C.

HCl_t	S
0.1000	0.00468
0.1500	0.00708
0.199	0.00977
0.415	0.0230
0.500	0.0293
0.600	0.0368
0.800	0.0531
1.000	0.0729
1.500	0.1318
2.00	0.210
3.00	0.427
4.00	0.735

Symbols

P_A	Acetylene pressure in atm,
$[C_2H_2]_t$	Total concentration of dissolved acetylene,
$[C_2H_2]_{Cu}$	Concentration of copper-bound acetylene,
a	Absorption coefficient, accounting for the "physical" solubility of acetylene; in moles/1 000 g $H_2O \times$ atm,
HCl_t	Formal concentration of HCl in perchloric acid mixtures, where HCl, $HClO_4$ and H_2O are regarded as components,
$[Cl^-]$	Concentration of "free" chloride ion,
$CuCl_t$	Formal concentration of dissolved cuprous chloride,
S	Solubility of cuprous chloride (acetylene absent).
	All concentrations are given in m, *e.g.* moles/1 000 g of water.

The inorganic system

The solubility of cuprous chloride in 4 molal mixtures of hydrochloric and perchloric acid has been determined analytically (see "Apparatus and Procedure") and the results are found in Table 1. The measurements are reproducible within $\pm$ 1 %, and the analysis is so performed that the relative accuracy is approximately the same throughout the whole series. In diagrams 1 a and b the quotient S/HCl_t has been plotted against HCl_t, yielding a curve which approaches a straight line at lower HCl-concentrations. Within the interval

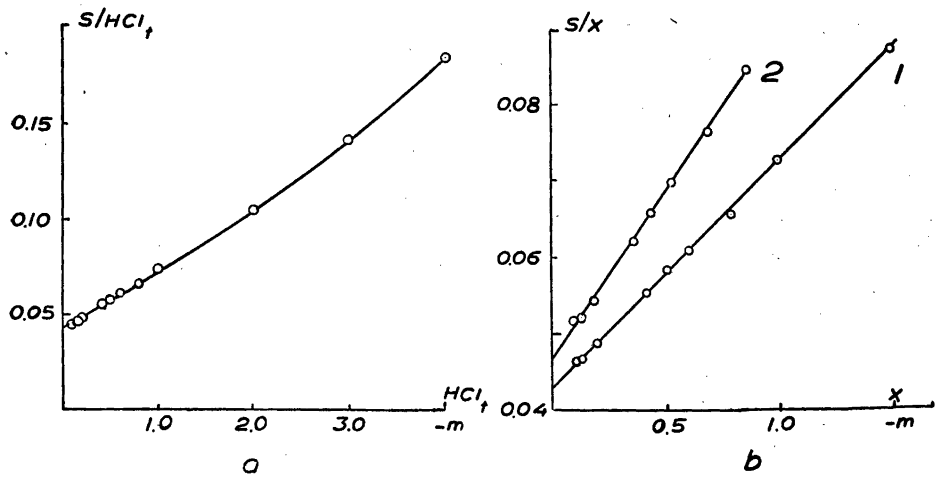


Fig 1. Relation between S and HCl_t.

Curve 1: $X = HCl_t$
Curve 2: $X = HCl_t - 2\ S$

$0.1 < HCl_t < 1.5$ there is no significant deviation from a linear relation, and thus:

$$S = a_1 \cdot HCl_t + a_2 \cdot HCl_t^2 \quad (0.1 < HCl_t < 1.5) \tag{1}$$

where the graphically determined constants are $a_1 = 0.0435$ and $a_2 = 0.0294$.

If the activity coefficients are assumed constant within the interval $0.1 < HCl_t < 1.0$, the solubilities can be referred to the two equilibria:

$$CuCl(s) + Cl^- = CuCl_2^- \qquad \beta_{11} = 0.0447 \tag{2a}$$

$$CuCl(s) + 2Cl^- = CuCl_3^{2-} \qquad \beta_{12} = 0.0405 \tag{2b}$$

which imply the following relation between the variables measured:

$$S = \beta_{11} \cdot [Cl^-] + \beta_{12} \cdot [Cl^-]^2 \tag{3}$$

$$HCl_t = [Cl^-] \cdot (1 + \beta_{11} + 2\ \beta_{12} \cdot [Cl^-]) \tag{4}$$

Elimination of $[Cl^-]$ yields:

$$S = \frac{\beta_{11}}{1-\beta_{11}} \cdot (HCl_t - 2\ S) + \frac{\beta_{12}}{(1-\beta_{11})^2} \cdot (HCl_t - 2\ S)^2 \tag{5}$$

Obviously (1) and (5) are not identical functions, but within the interval mentioned no larger difference than 0.7 % is found between S-values computed from the two functions, using for the four constants the numerical values

stated above. A direct graphical check of (5) against the experimental values is included in diagram 1 b, where the quotient $S/(HCl_t - 2\,S)$ is plotted against $(HCl_t - 2\,S)$.

The ionic strength of the system changes on the formation of $CuCl_3^{2-}$ (and other, higher charged, complexes) but because of the low solubility the change remains unimportant. If only the equilibria (2) are considered, the relative change in the ionic strength amounts to $0.0075 \times HCl_t^2$. Even if other complexes are present to some extent, the ionic strength can be regarded as satisfactorily constant if $HCl_t = 1.0$ is put as the upper limit for the interval.

These solubility determinations in systems with constant ionic strength definitely support the generally accepted, but poorly verified, opinion [3], derived from Bodländer's classical work (1902), that the species $CuCl_2^-$ and $CuCl_3^{2-}$ predominate in aqueous solutions of cuprous chloride and a dissociating chloride. However, it is not to be excluded that complexes of a higher chloride content occur in amounts of some importance; the fairly arbitrary limitation of the HCl_t-interval is a condition for the interpretation according to (2). Regarding the copper content of the complexes no information of value can be extracted from these equilibrium experiments, where the cuprous chloride activity is kept constant. (Potentiometric measurements, of the same kind previously [1] described, show that, in the present system also, the main part of the dissolved copper is to be found as mononuclear complexes.) A detailed discussion of the inorganic complexes falls however outside the scope of this paper.

The physical solubility of acetylene

Table 2 records measurements of the acetylene absorption in 4 molal aqueous solutions of hydrochloric acid, perchloric acid, and mixtures of the two (CuCl absent). No deviation could be found from a proportionality between the partial pressure and the molal concentration of dissolved acetylene.

Perchloric acid has a definite salting-in effect: in 4 molal acid the absorption coefficient α is 0.0509 moles/1 000 g $H_2O \times$ atm, for pure water α is 0.0424. Other electrolytes tried cause a salting-out of acetylene.

For a 4 molal mixture of hydrochloric and perchloric acid the absorption coefficient within the interval $0 < HCl_t < 1$ can be expressed by the empirical formula:

$$\alpha = 0.0509 - 3.5 \cdot 10^{-3} \cdot HCl_t \qquad (6)$$

In the following it has been assumed that this absorption coefficient can be applied also to systems which contain cuprous chloride. The inorganic cuprous chloride complexes and the acetylene compounds together do not

Table 2. Solubility of acetylene in 4 molal mixtures of hydrochloric and perchloric acid, 25° C.

HCl_t	P_A	$[C_2H_2]_t$	a	HCl_t	P_A	$[C_2H_2]_t$	a
0.0000	0.1478	0.00724	0.049	0.494	0.1498	0.00739	0.0493
	0.2616	0.01308	0.0500		0.2998	0.01472	0.0491
	0.3141	0.01589	0.0506		0.3882	0.01920	0.0495
	0.4522	0.02289	0.0506		0.4950	0.02439	0.0493
	0.5769	0.02929	0.0508		0.6371	0.03140	0.0493
	0.6240	0.03164	0.0507		0.7573	0.03724	0.0492
	0.7177	0.03646	0.0508		0.8758	0.04301	0.0491
	0.8690	0.04406	0.0507				
	0.9285	0.04718	0.0508				
0.1115	0.1447	0.00738	0.0510	0.793	—	—	0.0481 (means)
	0.2339	0.01193	0.0510				
	0.3611	0.01837	0.0509				
	0.4745	0.02412	0.0508	1.000	0.0604	0.00279	0.046
	0.5814	0.02953	0.0508		0.1399	0.00653	0.0467
	0.7187	0.03651	0.0508		0.2683	0.01260	0.0470
	0.8581	0.04355	0.0508		0.3890	0.01827	0.0470
	0.9438	0.04787	0.0507		0.5433	0.02549	0.0469
	1.016	0.05158	0.0508		0.6764	0.03185	0.0471
					0.7645	0.03588	0.0469
0.2905	—	—	0.0497 (means)		0.8603	0.04029	0.0468
					0.9913	0.04648	0.0469
				2.00	—	—	0.0437 (means)

a = absorption coefficient in moles/1 000 g H_2O × atm.

reach a concentration higher than 0.15 m, and the change in the ionic strength will presumably not surpass 1 %. The concentration of complex bound acetylene is computed from the total absorption according to:

$$[C_2H_2]_{Cu} = [C_2H_2]_t - a \cdot P_A \qquad (7)$$

Acetylene absorption in the presence of cuprous chloride

The absorption experiments primarily aim at a determination of the empirical relation between the concentration of complex bound acetylene, $[C_2H_2]_{Cu}$, and the two variables P_A and HCl_t in a 4 m mixture of hydrochloric and perchloric acid, saturated with CuCl. The HCl concentration is varied

Table 3. Acetylene absorption in 4 molal mixtures of hydrochloric and perchloric acid saturated with cuprous chloride. 25° C.

HCl_t	P_A	$[C_2H_2]_t$	$[C_2H_2]_t/P_A$	HCl_t	P_A	$[C_2H_2]_t$	$[C_2H_2]_t/P_A$
0.1167	0.1254	0.00992	0.0791	0.199	0.1416	0.01180	0.0833
	0.2174	0.01725	0.0793		0.2037	0.01681	0.0825
	0.2885	0.02290	0.0794		0.3276	0.02692	0.0822
	0.3934	0.03109	0.0790		0.4118	0.03393	0.0824
	0.4882	0.03866	0.0792		0.5218	0.04270	0.0818
	0.5691	0.04510	0.0792		0.6187	0.05071	0.0820
	0.6889	0.05461	0.0793		0.7043	0.05788	0.0822
	0.7565	0.05994	0.0792		0.8106	0.06659	0.0821
	0.8449	0.06707	0.0794				
	0.9440	0.07492	0.0794				
0.415	0.1720	0.01771	0.1030	0.494	0.0639	0.00704	0.110
	0.2381	0.02452	0.1030		0.1634	0.01762	0.1072
	0.3082	0.03169	0.1028		0.2225	0.02373	0.1067
	0.3382	0.03494	0.1033		0.3072	0.03299	0.1074
	0.4207	0.04343	0.1031		0.4010	0.04311	0.1075
	0.4710	0.04882	0.1037		0.4533	0.04878	0.1076
	0.5255	0.05434	0.1034		0.5431	0.05831	0.1074
	0.5831	0.06023	0.1033		0.6179	0.06645	0.1075
	0.6185	0.06449	0.1042		0.6787	0.07296	0.1076
	0.7062	0.07361	0.1042		0.7510	0.08074	0.1075
					0.8174	0.08785	0.1075
0.617	0.0866	0.01025	0.118	0.793	0.0606	0.00800	0.132
	0.1353	0.01622	0.1199		0.1317	0.01751	0.1330
	0.2044	0.02463	0.1205		0.2004	0.02677	0.1331
	0.2461	0.02945	0.1197		0.2741	0.03659	0.1335
	0.2877	0.03462	0.1203		0.3509	0.04681	0.1334
	0.3479	0.04201	0.1208		0.4037	0.05388	0.1335
	0.3728	0.04507	0.1209		0.4965	0.06605	0.1330
	0.5167	0.06242	0.1208		0.6019	0.08007	0.1330
	0.6045	0.07246	0.1199		0.6696	0.08898	0.1329
	0.6553	0.07879	0.1202		0.7369	0.09786	0.1328
	0.7438	0.08941	0.1202		0.8034	0.1067	0.1328
	0.8013	0.09582	0.1196		0.8706	0.1154	0.1326
	0.8827	0.1067	0.1209		0.9289	0.1231	0.1325
	0.9433	0.1131	0.1199				
	0.9529	0.1144	0.1200				

Continued

Table 3 continued

HCl_t	P_A	$[C_2H_2]_t$	$[C_2H_2]_t/P_A$	HCl_t	P_A	$[C_2H_2]_t$	$[C_2H_2]_t/P_A$
0.962	0.0348	0.00509	0.146	0.962	0.1145	0.01661	0.1451
	0.0839	0.01217	0.1450		0.1709	0.02437	0.1426
	0.1234	0.01786	0.1447		0.2485	0.03601	0.1449
	0.1519	0.02198	0.1447		0.3091	0.04506	0.1458
	0.2171	0.03163	0.1457		0.3350	0.04878	0.1456
	0.2717	0.03993	0.1469		0.4101	0.05992	0.1461
	0.3000	0.04410	0.1470		0.4614	0.06758	0.1465
	0.3637	0.05334	0.1466		0.5025	0.07348	0.1463
	0.4130	0.06054	0.1466		0.5565	0.08174	0.1469
	0.4521	0.06638	0.1468		0.6263	0.09182	0.1466
	0.5202	0.07655	0.1472		0.6684	0.09815	0.1468
	0.5727	0.08373	0.1462		0.7128	0.1046	0.1467
	0.7008	0.1028	0.1467		0.8304	0.1217	0.1466
	0.7469	0.1100	0.1472		0.8490	0.1243	0.1463
	0.8081	0.1188	0.1470		0.9856	0.1446	0.1467
1.000	0.1159	0.01791	0.155	1.000	0.0973	0.01476	0.1514
	0.1917	0.02885	0.1520		0.1628	0.02465	0.1514
	0.3103	0.04710	0.1518		0.2518	0.03828	0.1520
	0.3846	0.05817	0.1512		0.3270	0.04957	0.1516
	0.4937	0.07404	0.1500		0.4113	0.06191	0.1505
	0.5698	0.08575	0.1505		0.4833	0.07267	0.1504
	0.6711	0.1006	0.1499		0.5580	0.08470	0.1518
	0.7572	0.1135	0.1499		0.6049	0.09086	0.1502
	0.8764	0.1309	0.1494		0.6483	0.09758	0.1505
	0.9497	0.1416	0.1493		0.7070	0.1066	0.1508

between 0 and 1 m and the acetylene pressure between 0.1 and 1.0 atm approximately. Unfortunately the measurements cannot be extended to higher pressures, since a solid addition compound forms [1].

There is a surface adsorption of acetylene on solid CuCl, which can cause noticeable errors in these systems, where the concentration of dissolved acetylene is low, in spite of the fact that the adsorbed amounts are quite small. The effect is most apparent at low pressures and depends of course on the relative amount of solid phase, and its particle size. The surface adsorption can be demonstrated, and also arithmetically eliminated, by a comparison between two or several experiments, differing only in the quantity ratio solid phase/solution. But the adsorption error will become negligible at 1 atm and normally also insignificant at 0.1 atm, provided a rather coarse-crystalline substance, in moderate excess, is used.

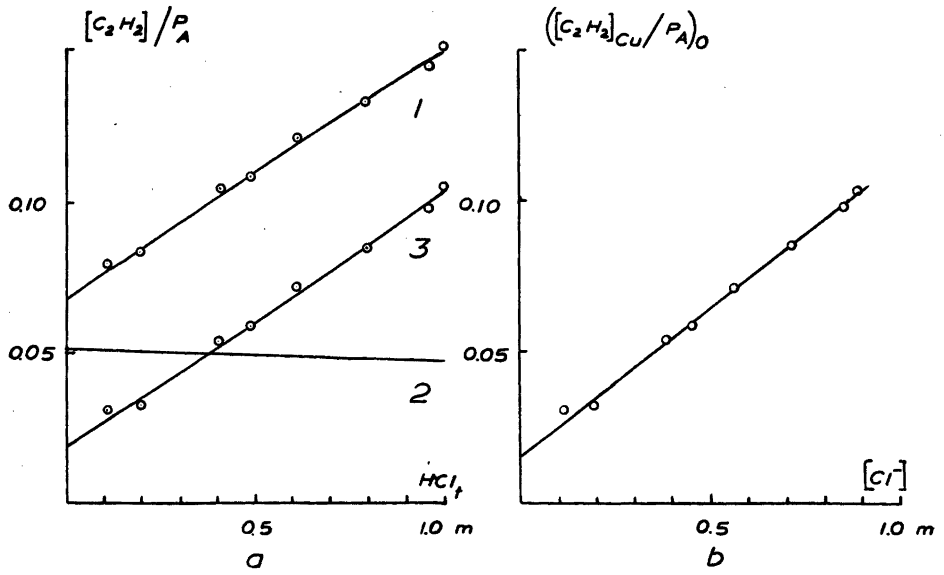


Fig. 2. The absorption quotient as a function of HCl_t and of $[Cl^-]$.

a 1: In the presence of solid CuCl; $[C_2H_2]_t/P_A$
2: CuCl absent - a according to (6)
3: $[C_2H_2]_{Cu}/P_A$, computed as $[C_2H_2]_t/P_A - a$
b : $[Cl^-]$ computed according to (4)

Each separate experiment of Table 3 ($0.1 < HCl_t < 1.0$) will give a relation between $[C_2H_2]_t$ and P_A at a constant HCl_t. This relation is a proportional one in all cases. The tendency of the quotient $[C_2H_2]_t P_A$ to diminish with increasing P_A, observed in some experiments (*e.g.* one of the experiments at $HCl_t = 1.000$), can be explained completely by the surface adsorption. Otherwise the variations in the quotient values are within the experimental error. Supposing that a is independent of P_A the same thing holds for the quotient $[C_2H_2]_{Cu}/P_A$, computed according to (7). For these systems thus holds the same rule as for solutions containing only hydrochloric acid, saturated with CuCl, *i.e.*:

$$[C_2H_2]_{Cu}/P_A \text{ constant at } \begin{cases} \text{CuCl (s)} \\ HCl_t \text{ constant} \end{cases} \qquad (8)$$

The quotients $[C_2H_2]_t/P_A$ and $[C_2H_2]_{Cu}/P_A$ thus are functions of HCl_t only, under the conditions stated. Curve 1 in Fig. 2 *a* shows that the relation between $[C_2H_2]_t/P_A$ and HCl_t is linear (with a constant term). The quotient values are taken from Table 3, and are mean values of all the readings which

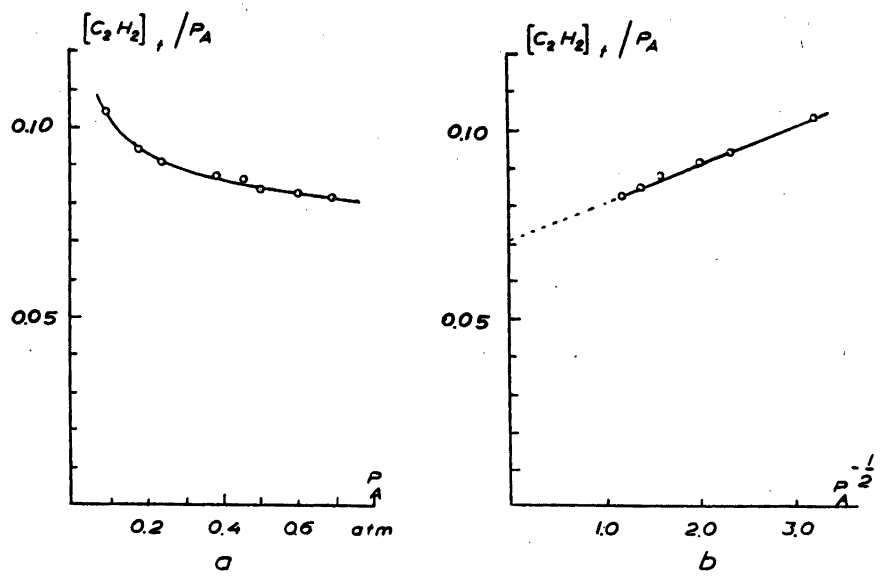


Fig. 3. Relation between acetylene absorption and partial pressure in 4-molal perchloric acid saturated with CuCl.

correspond to pressures over 0.1 atm. The absorption coefficient α which accounts for the "physical" solubility, is given by curve 2, representing the empirical function (6). Curve 3 corresponds to the difference 1—2 and thus gives $[C_2H_2]_{Cu}/P_A$ as a function of HCl_t.

The absorption measurements in systems with solid CuCl, within the interval $0.1 < HCl_t < 1.0$, can thus be summarized in the following empirical formula:

$$[C_2H_2]_{Cu} = P_A \cdot (A + B \cdot HCl_t) \qquad \begin{cases} A = 0.016 \\ B = 0.086 \end{cases} \tag{9}$$

For the quantity $([C_2H_2]_{Cu}/P_A)_0$, previously [1] defined as the limit value of the quotient when P_A tends to zero at a constant HCl_t, an analogous formula holds:

$$([C_2H_2)_{Cu}/P_A)_0 = A + B \cdot HCl_t \tag{10}$$

Measurements of the acetylene absorption in 4 m perchloric acid (with solid CuCl), *i.e.* at $HCl_t = 0$, gives quite different results. As is obvious from Table 4 the absorption is larger than to be expected from (9). The absorption is not proportional to the pressure; the quotient $[C_2H_2]_t/P_A$ decreases by about 20 % when the pressure is raised from 0.1 to 0.7 atm, *cf.* Fig. 3 a.

Table 4. Acetylene absorption in 4 molal perchloric acid saturated with cuprous chloride, 25° C.

P_A	$[C_2H_2]_t$	$[C_2H_2]_t/P_A$
0.0967	0.01004	0.1038
0.1844	0.01743	0.0945
0.2439	0.02232	0.0915
0.3902	0.03412	0.0874
0.4598	0.03970	0.0863
0.5052	0.04232	0.0838
0.6087	0.05047	0.0829
0.6977	0.05717	0.0819
0.7244	(0.06417)	(0.0886)

To perform a more complete investigation of the acetylene absorption within the interval $0 < HCl_t < 0.1$ does not seem very inviting. The concentration of complexbound acetylene cannot be computed with satisfactory accuracy, since the physical solubility predominates. A solid acetylidic compound (violet complex) precipitates even at low pressures in most experiments. (Thanks to supersaturation it was possible to raise the pressure, in the experiment of Table 4, unto 0.72 atm before the violet complex began to precipitate.)

Table 5 shows the results of an absorption experiment in a *homogenous system*, where the CuCl concentration lies close to the solubility, S, for the mixture of acids employed. In this case $[C_2H_2]_{Cu}$ is not proportional to P_A but to the parameter

$$\pi_A \equiv P_A \ (1-[C_2H_2]_{Cu}/CuCl_t) \qquad (11)$$

as in the previous experiments [1] with homogenous solutions of cuprous chloride in hydrochloric acid only. The accuracy of the $[C_2H_2]_{Cu}$ value decreases with rising pressure, since physically dissolved acetylene will become a greater portion of the total absorption, at the highest pressure corresponding to 61 %. If HCl_t and $CuCl_t$ fall below the extreme values, chosen for the experiment of Table 5, it would be difficult to determine $[C_2H_2]_{Cu}$ with reasonable accuracy. Thus a complete investigation of the acetylene absorption in homogenous solutions is out of the question.

Previously [1] it has been shown that, in *saturated* solutions of CuCl in hydrochloric acid, $CuCl_t$ increases equimolarily with $[C_2H_2]_{Cu}$ when the acetylene pressure is raised. The high concentration of inorganic CuCl-complexes,

Table 5. Acetylene absorption in homogenous solution. 25° C.

System: 0.962 m HCl; 3.038 m $HClO_4$; 0.0713 m CuCl.

P_A	$[C_2H_2]_t$	$[C_2H_2]_t/P_A$	$[C_2H_2]_{Cu}/P_A$	$[C_2H_2]_{Cu}/\pi_A$
0.0431	0.00589	0.1367	0.0891	0.0941
0.0911	0.01225	0.1345	0.0869	0.0979
0.1636	0.02074	0.1268	0.0792	0.0970
0.1981	0.02481	0.1252	0.0776	0.0989
0.2715	0.03220	0.1186	0.0710	0.0974
0.3623	0.04084	0.1127	0.0651	0.0974
0.4373	0.04748	0.1086	0.0610	0.0976
0.5351	0.05537	0.1035	0.0559	0.0964
0.6175	0.06187	0.1002	0.0526	0.0967
0.7110	0.06869	0.0966	0.0490	0.0958
0.7783	0.07340	0.0943	0.0467	0.0952
0.8729	0.08004	0.0917	0.0441	0.0960
0.9307	0.08386	0.0901	0.0425	0.0956
1.0066	0.08879	0.0882	0.0406	0.0954
1.149	0.09782	0.0851	0.0375	0.0945
1.308	0.1074	0.0821	0.0345	0.0939
1.512	0.1197	0.0792	0.0316	0.0959
1.654	0.1281	0.0775	0.0299	0.0978

however, causes the relative change in $CuCl_t$ to become quite small. But in the mixtures of hydrochloric and perchloric acid studied here, $CuCl_t$ attains values much higher than the initial one, S. A direct analytical examination of the relation between $CuCl_t$ and $[C_2H_2]_{Cu}$ has therefore been carried out.

The system 0.5 m HCl; 3.5 m $HClO_4$; CuCl (s) has been chosen for such a measurement. A nitrogen-acetylene mixture of known composition is passed through the solution until equilibrium is attained; a sample is taken for analysis after the solid phase has been allowed to sediment. Table 6 shows that the difference $CuCl_t - S$ closely coincides with $[C_2H_2]_{Cu}$.

Apparatus and procedure

The performance of the acetylene absorption experiments has been described in a previous paper [1] of this series.

Determination of CuCl solubility

Long-necked Kjeldahl-flasks, provided with a stopper and in the middle of the neck a side tube with a stopcock, are charged with fine-crystalline CuCl and after a careful evacuation the acid-mixture, freed from air by stripping with nitrogen, is added through the side tube. In those cases where the solubility is slight ($HCl_t < 0.5$ m) the

Table 6. Solubility of cuprous chloride in the presence of acetylene, 25° C.

System: 0.500 m HCl; 3.500 m $HClO_4$; CuCl (s).

P_A	Analysis after	$CuCl_t$	$[C_2H_2]_{Cu}$	$CuCl_t - S$	$[Cu]_{C_2H_2}$
0.000	45 min.	0.0286			
	75 »	0.0289	—		—
0.778	35 »	0.074_3			
	75 »	0.074_6			
	100 »	0.075_0	0.047	0.046	0.048
0.974	25 »	0.086_0			
	55 »	0.086_5	0.058	0.057	0.060

$CuCl_t$ according to analysis
$[C_2H_2]_{Cu}$ from the empirical function (9)
S from the empirical function (1)
$[Cu]_{C_2H_2}$ calculated as $CuCl_t - [CuCl]_{00}$, with $[CuCl]_{00}$ from (2) and (16).

substance is washed twice; the liquid being decanted and sucked out through the side tube. The flask is placed in a 25° C thermostat with a shaking device.

For sampling a swift stream of nitrogen is passed through the side tube venting through the neck and an immersion filter (Pyrex F) is introduced, enabling a suitable quantity to be sucked into an evacuated weighing bottle. The quantity is so adapted that each time about 1 millimole (minimum 0.5 millimole) of dissolved CuCl is taken for analysis, and by addition of perchloric acid the composition of the solutions is approximately standardized. Hydrochloric acid, and the main part of the water and the perchloric acid are distilled off, Cu^I simultaneously being oxidized to Cu^{II} quantitatively [4]. After cooling water is added and the solution is boiled free of chlorine and chlorine oxides. The copper content is determined iodometrically in an acetate buffer with 0.02 *N* thiosulphate after addition of thiocyanate [5]. This obviously is a method for the determination of the *total* copper content, but by checking the colour of the sample and comparison with solutions where a known amount of Cu^{II} has been added, it is easy to state that the contribution from Cu^{II} is negligible.

There is no significant difference between samples taken after 2½ and 5 hours respectively, nor on comparison with checks where the equilibrium is reached from a supersaturated solution (prepared by preheating to 30°). Experiments with and without metallic copper give coinciding values. The results in Table 2 are means from two or more analyses.

Nitrogen-acetylene mixtures of known composition for the determination of the *CuCl-solubility at a given acetylene pressure* (Table 6) are prepared by a previously described [6] technique, where constant gas flow is adjusted with the aid of "critical orifices".

The gas is introduced into the cylindrical reaction vessel either through its fritted disc bottom or through a tube above the surface of the liquid. The gas escapes through an open vertical tube, used for sampling of liquid. The velocity in the tube is great enough to prevent back diffusion of air. The samples are taken without filtering to avoid precipitation of acetylene-bound CuCl which might result from a pressure drop. Before sampling complete sedimentation is awaited and checked in side-light. The analysis is then performed as described above.

Conclusions from the equilibrium experiments

1. Interval $0.1 < HCl_t < 1.0$

To find a relation between $[C_2H_2]_{Cu}$ and the concentration of free chloride ion, $[Cl^-]$, it is necessary to compute $[Cl^-]$ from a given HCl_t by subtracting the chloride content of both inorganic complexes and acetylene compounds. But the limit value $([C_2H_2]_{Cu}/P_A)_0$ can be referred to a system, where the acetylene compounds have no influence on the chloride ion balance and where $[Cl^-]$ can be computed according to (4). If (4) is inserted in (10) a quadratic relation between $([C_2H_2]_{Cu}/P_A)_0$ and $[Cl^-]$ results, but the contribution from the second order term is so small that it cannot be determined with any degree of certainty. Fig. 2 demonstrates that the limit quotient can be given, with acceptable accuracy, as a linear function of $[Cl^-]$ also:

$$[C_2H_2]_{Cu}/P_A)_0 = 0.015 + 0.097 \cdot [Cl^-] \qquad (12)$$

If we assume that the acetylene absorption can be referred to equilibria of the type

$$n\ C_2H_2 + p\ CuCl(s) + q\ Cl^- = (C_2H_2)_n(CuCl)_p(Cl^-)_q \qquad (13)$$

and that the ions involved have constant activity coefficients, then it follows from (12) that there must exist one complex with $q = 0$, whose concentration is independent of $[Cl^-]$, and one complex with $q = 1$, whose concentration rises proportionally with $[Cl^-]$. Other experimental data indicate that the complexes mainly occurring have $n = p = 1$.

The predominating species thus are $C_2H_2 \cdot CuCl$ and $C_2H_2 \cdot CuCl_2^-$. If then we assume that *only* these two compounds and the inorganic CuCl-complexes occur, the following relations should hold for systems with solid CuCl:

$$[C_2H_2 \cdot CuCl] = k_2 \cdot P_A \qquad k_2 = 0.015 \qquad (14\ a)$$

$$[C_2H_2 \cdot CuCl_2^-] = k_3 \cdot P_A \cdot [Cl^-] \qquad k_3 = 0.097 \qquad (14\ b)$$

and

$$[C_2H_2]_{Cu} = [Cu]_{C_2H_2} = P_A(k_2 + k_3 \cdot [Cl^-]) \qquad (15)$$

$$HCl_t = [Cl^-] \cdot (1 + \beta_{11} + 2\ \beta_{12}[Cl^-] + k_3 \cdot P_A) \qquad (16)$$

It now remains to try if (15) and (16) fit the experimental results with acceptable accuracy, or if there are deviations, indicating the presence of noticeable amounts of other complexes.

a) The experiments show the quotient $[C_2H_2]_{Cu}/P_A$ to be independent of P_A. But according to (15) and (16) the quotient should diminish when P_A increases, as a consequence of the consumption of chloride ion by the formation of $C_2H_2 \cdot CuCl_2^-$. At a low HCl_t the computed change is small, but at $HCl_t = 1.0$ it would amount to 6 % within the interval $0.1 < P_A < 1.0$ atm, corresponding to 4 % of the directly measured quotient $[C_2H_2]_t/P_A$; the experiments show this quotient to be certainly constant within $\pm$ 0.5 %. This would indicate that complexes with $n > 1$, *i.e.* containing more than one acetylene molecule, are present to such an extent that the expected effect is compensated for. But the experiments cannot be said to prove definitely the existence of such complexes; the difference is not great, and minor variations in activity coefficients and/or acetylene's physical solubility are possible.

b) At a finite, constant P_A formulae (15) and (16) give $[Cl^-]$ values (and k-constants) which deviate slightly from the corresponding values at $P_A = 0$. But in each separate case a linear relation between $(C_2H_2]_{Cu}$ and $[Cl^-]$ is found to express satisfactorily the experimental results.

c) The difference $CuCl_t - S$ in Table 6 is only an approximate measure of the concentration of acetylene-bound copper, since the chloride ion consumption due to the formation of $C_2H_2 \cdot CuCl_2^-$ should cause the concentration of inorganic copper complexes to deviate slightly from S in the presence of acetylene. The values for $[Cu]_{C_2H_2}$ in the last column of Table 6 have been calculated according to

$$[Cu]_{C_2H_2} = CuCl_t - [CuCl]_{00}$$

where $CuCl_t$ is the analytically determined total concentration, and $[CuCl]_{00}$ is the concentration of copper bound in inorganic complexes, computed according to (2) and (16). As demonstrated by the table these $[Cu]_{C_2H_2}$ values exceed by some per cent the empirical $[C_2H_2]_{Cu}$ values, but considering the moderate accuracy of the solubility determinations the difference cannot be regarded as significant.

2. $HCl_t = 0$

The acetylene absorption in 4 molal perchloric acid (with solid CuCl) will find its simplest explanation by the assumption of a cation complex whose concentration is determined by the equilibrium:

$$C_2H_2 + CuCl(s) = C_2H_2 \cdot Cu^+ + Cl^- \qquad (17)$$

Cation complex and chloride ion thus form in equimolar amounts, and if the inorganic CuCl-complexes are neglected, we find:

$$[C_2H_2 \cdot Cu^+] = k_1^{\frac{1}{2}} \cdot P_A^{\frac{1}{2}} \tag{18}$$

Physical solubility and the uncharged complex $C_2H_2 \cdot CuCl$ further contribute to the total absorption, whereas $C_2H_2 \cdot CuCl_2^-$ can be disregarded. For the total absorption we then get:

$$[C_2H_2]_t/P_A = \alpha + k_2 + k_1^{\frac{1}{2}} \cdot P_A^{-\frac{1}{2}} \tag{19}$$

A linear relation consequently is to be expected between the absorption quotient and $P_A^{-\frac{1}{2}}$, and Fig. 3 b shows that this is the case. Graphically the following values are obtained:

$$\begin{cases} k_1 = (0.011)^2 = 1.2 \cdot 10^{-4} \\ \alpha + k_2 = 0.069 \end{cases}$$

The constant k_1 thus determined will have a very low accuracy. But the experiment can be regarded as an acceptable proof of the existence of the cation compound.

The main conclusions from the equilibrium experiments will thus be: in 4 m mixtures of hydrochloric and perchloric acid acetylene and CuCl form the following three addition complexes

$$C_2H_2 \cdot Cu^+ \qquad C_2H_2 \cdot CuCl \qquad C_2H_2 \cdot CuCl_2^-$$

and their concentration in the presence of solid CuCl can be computed from the equilibrium formulae:

$$\begin{cases} [C_2H_2 \cdot Cu^+] = k_1 \cdot P_A \cdot [Cl^-]^{-1} & k_1 = 1.2 \cdot 10^{-4} \\ [C_2H_2 \cdot CuCl] = k_2 \cdot P_A & k_2 = 1.5 \cdot 10^{-2} \\ [C_2H_2 \cdot CuCl_2^-] = k_3 \cdot P_A \cdot [Cl^-] & k_3 = 9.7 \cdot 10^{-2} \end{cases} \tag{20}$$

DISCUSSION

The cuprous addition compounds of *acetylene* are probably closely related to the corresponding olefine compounds. The dissolved cuprous addition compounds of *ethylene* in chloride solutions have been the subject of a series of papers [7] by Green, d'Angelo and Rote, and Park, all under the direction of Gilliland. The complexes formed seem to contain one atom of copper and one molecule of ethylene, as is demonstrated by experiments showing a linear relation between on the one hand the ethylene absorption and on the other

hand the activity (fugacity) of ethylene, and the concentration of "free" cuprous chloride (at a constant concentration of dissociating chloride). No definite conclusion has been reached as to the chloride content of the complexes; Green however finds it probable that the predominating complex has the composition $C_2H_4CuCl_2^-$. Similar results have been obtained for propylene.

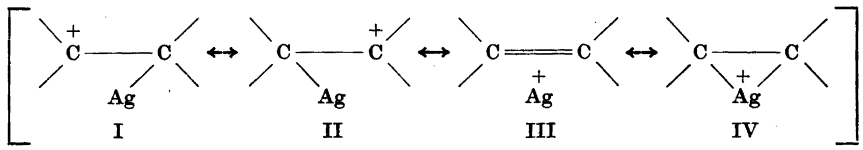
The dissolved cuprous complexes of ethylene are somewhat less stable than those of acetylene. Some preliminary measurements have shown that the constant k_c, *cf.* definition (13)[1], has the value 0.20 atm^{-1} for ethylene in 4 m HCl at 25° C, whereas the corresponding value for acetylene is 0.48 atm^{-1}.

Andrews, Keefer [8] and co-workers have studied the complex formation between allyl alcohol (and several other unsaturated alcohols and acids) and monovalent copper in chloride solutions. Here also the rule seems to hold that only 1 : 1 complexes exist. The main part of their equilibrium experiments aim at a determination of the chloride content of the complexes, and the types $A \cdot Cu^+$ and $A \cdot CuCl$ are found to exist. But the experiments have not been extended to systems of higher chloride concentrations, where possibly anion complexes of the type $A \cdot CuCl_2^-$ exist in this case also.

Winstein and Lucas [9] have shown that 1 : 1 complexes are typical of dissolved olefine complexes of silver ion also. Only for the diolefines can a tendency be observed to add two silver ions to each olefine molecule; compounds with two olefine molecules and one silver ion appear only when the olefine has an oxygen-containing group. It seems that divalent mercury also forms dissolved 1 : 1 complexes with olefines [10]. (Unfortunately one can hardly decide whether acetylene can form dissolved addition complexes with silver ion, since the formation of silver carbide, or complex compounds thereof, predominates entirely; silver carbide is stable [11] even in a strongly acid solution at a low silver ion activity.)

The question as to the nature of the metal-olefine coördination bond has been the subject of a lively discussion. On general chemical grounds a bond of the coördinate link type has been assumed, although the coördinated group has no "lone pair" of electrons. In the earlier literature [12] various suggestions are found for the solution of this difficulty. The fact that the complex formation does in no particular way favour either polymerization or isomerization of cis-trans olefines, forms a strong objection against those suggestions which involve an opening of the double bond and the formation of one carbon atom having only a sextet of electrons. Recently electronic interpretations have resulted in two ideas which, even though hypothetical, might be regarded as acceptable descriptions of the characteristics of this type of compound.

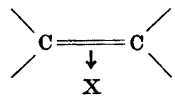
a) According to Winstein and Lucas [9] the Ag^+ complexes of the olefines are resonance hybrids of the following four forms:



As the C-C-Ag-angle can be considerably greater than 60° the strain of the three-membered ring need not be great. The strain energy has been estimated at 3—4 kcal, and the resonance energy at 10 kcal. The four forms can contribute in varying degrees in different metal-olefine compounds. Originally IV was omitted for the silver complexes, but later on a considerable contribution of this form was assumed in order to explain the stability of the cis-trans isomers. The donor property of the metal atom towards either of the two carbon atoms in form IV has not been discussed.

The same structure has been suggested by Andrews and Keefer [8] *et al.* for the olefine compounds of monovalent copper, excluding however form IV.

b) Werner [13] finds that the structure of the metal-olefine compounds might best be described by that special type of bond which was independently brought forth by Walsh [14] and by Dewar [15]; in both cases designed for compounds of quite different type. Dewars' idea has been developed from the observation that the a π-orbital of ethylene closely resembles the p-orbital of a separate atom with a lone pair of electrons. There seems to be no reason why the π-orbital should not be able to coalesce with a vacant bonding orbital of a third atom, resulting in a special kind of molecular orbital covering all three nuclei. Or otherwise expressed: a bond of the coördinate link type is not confined to lone-pair electrons in the donor atom; bonding electrons, under certain conditions, may also be donated to an acceptor atom. For compounds with this type of bond Dewar has suggested the name π-complex and the designation:



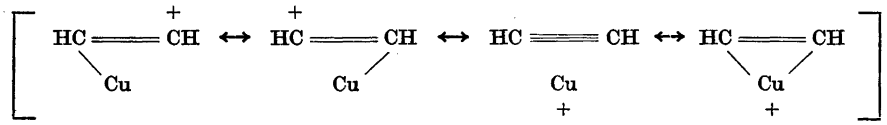
Obviously these two structure theories cannot be regarded as principally opposed; rather as two different vocabularies of resembling content. From a purely chemical view-point two similarities are obvious; both theories aim at the description of a *symmetrical* addition of the metal atom (disregarding such an obliquity as might result from an unsymmetrical structure of the olefine),

and both express that the addition will involve the multiple bond without *dissolving it entirely.*

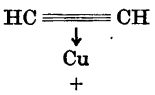
This last statement agrees well with the results from Raman measurements on dissolved olefine-silver complexes, performed by Taufen, Murray and Cleveland [16] in connection with Winstein's and Lucas' investigations. The strong double bond frequency has not disappeared in the silver complexes, but shows a general lowering of approximately 65 cm^{-1}, however still remaining in the double bond region. Other frequencies of the original compound appear essentially unaltered in the complex; no new lines corresponding to a metal-carbon linkage could be discerned.

The theory on the π-complexes has already played a certain role in the recent discussions on the reactivity of the olefines, intermolecular rearrangements etc., as is obvious *e.g.* from Dewar's own monograph [17]. As a rule a π-complex structure has been assumed only for hypothetical reaction intermediaries. The metal-olefines are practically the only kind of stable compounds which might be regarded as π-complexes.

It may be assumed that the cuprous addition compounds of acetylene have a similar structure, which might be formulated as a resonance hybrid of the four forms:



or as a π-complex:



(Further, the copper atom may add one or more atoms of normal donor properties, as *e.g.* chloride ion.)

The two π-bonds of acetylene are perpendicular to one another and thus electronically independent. The spatial spread, which according to Walsh primarily determines the power of donation, is no smaller than for ethylene.

Complex compounds, containing copper or silver carbide and an inorganic salt of either metal can, however, hardly be explained as π-complexes. The ability to add a large number of metal atoms in aqueous solution seems to be a characteristic property of these carbidic compounds, not only of silver carbide [18] and copper carbide [19], but also of monoalkylacetylides (according to unpublished results obtained in this laboratory). Already this "polyvalency" indicates that other bond forces are involved.

SUMMARY

Investigations of the solubilities of acetylene and cuprous chloride in mixtures of hydrochloric and perchloric acids of constant ionic strength show that the following dissolved species occur:

inorganic complexes $CuCl_2^-$, $CuCl_3^{2-}$,

acetylene addition compounds $C_2H_2 \cdot Cu^+$, $C_2H_2 \cdot CuCl$, $C_2H_2 \cdot CuCl_2^-$.

Equilibrium constants for the five compounds have been determined.

The bond between acetylene and copper in these addition compounds is presumably similar to the coördination bond in cuprous complexes of olefines.

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Kinetics of the Exchange of Cadmium between Amalgam and Cadmium Salt Solutions at Equilibrium Potential. I. The Rate Law in Perchlorate Solutions

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When a cadmium amalgam is brought in contact with a cadmium salt solution, the reversible reaction

$$Cd^{2+} + 2e = Cd \qquad (1)$$

sets in, resulting in a rapid attainment of the equilibrium potential difference between the two phases, and then the rates of the forward and the back processes of eq. (1) are equal. In the total electrode reaction are comprehended the electron exchange itself and furthermore the transport of Cd^{2+} in the solution and of Cd in the amalgam up to the interface. Then in the electron exchange we have also included the loosening and coordinating of ligands, *e.g.* water molecules or anions. The rate of the exchange of cadmium is controlled by the slowest of these partial processes under the experimental conditions.

The rate of the electron exchange itself can be investigated according to a method developed by Randles [1] and applied by him and Somerton [2]. The method involves measurements of the impedance of a dropping amalgam electrode. Measurements of a similar kind have been reported by Gerischer [3]. For cadmium the electron exchange is so rapid that from the impedance measurements only a lower limit for the rate constant can be obtained.

The aim of the present investigation is to study the exchange of cadmium at equilibrium potential by the use of a radioactive tracer Cd^{115}. By this method it is the overall reaction rate that is investigated, and thus a determination of the factors influencing the exchange rate will give us a possibility to decide which of the partial processes mentioned above is rate controlling for the whole reaction under the experimental conditions.

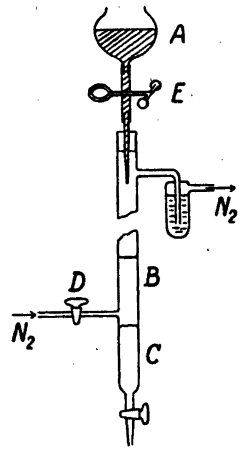

Fig. 1. The apparatus used at the exchange experiments.

Previously Gróh [4] used a radioactive lead isotope to determine the exchange rate on a dilute lead amalgam. From the measurements the author concluded that it was the rate of the electron exchange that was determined, but as the concentrations of lead in the two phases were not varied the rate law was not obtained, and thus it is hardly justified to draw such a conclusion.

THE EXPERIMENTAL METHOD

The apparatus used in the present investigation was similar to that of Gróh, and is drawn schematically in Fig. 1. The funnel A contained a *freshly prepared*, liquid, and inactive cadmium amalgam of known concentration (0.5—2 per cent by weight). The thickness of the amalgam layer in the funnel was kept approximately constant at all the measurements. The lowest part C of the burette was filled with carbon tetrachloride, and at B it contained the radioactive solution, supplied through the gas inlet tube. The solution had the following composition:

$$\begin{cases} (C_a + C_i) \text{ mC } Cd(ClO_4)_2 & (C_a + C_i \approx C_i) \\ 20 \text{ mC } HClO_4 \\ 900 \text{ mC } NaClO_4 \end{cases}$$

The burette and the solution were first freed from oxygen by a stream of nitrogen, and then the stop-cock D was closed and the thickness of the solution layer read off on a scale.

When the clip E was opened the amalgam squirted out, forming droplets, which passed the radioactive solution with great velocity. The nitrogen gas and the perchloric acid were intended to keep the surface of the droplets free from oxide. To get a well defined time of contact with the solution it was necessary to prevent adherent solution from following the amalgam into the carbon tetrachloride phase. This was effected by a small

addition (2 per cent by volume) of alcohol to the solution. After 0.5—1 ml of amalgam had passed the layer, the clip E was closed. All the exchange experiments were performed at a temperature of about 20° C.

The amalgam collected at the bottom of the burette was shaken with dilute (1 C) nitric acid, which dissolved the cadmium and a small amount of mercury. The mercury, present as mercurous ions, was removed quantitatively by addition of sodium chloride, after which the cadmium was precipitated as hydroxide. This was dissolved in a minimum of sulfuric acid, and the solution was electro-analysed. From the weight of the cadmium load on the catode the weight m of the corresponding amount of amalgam was calculated. m was not necessarily equal to the amount of amalgam collected, as the cadmium separation may not have been complete. The cadmium was then redissolved in concentrated nitric acid, the solution was diluted to the volume v (= 15 ml), and on 10 ml the beta radioactivity was measured with a G-M tube for liquids (type M.6, 20th Century Electronics). The number of counts per minute (cpm) obtained was taken as a measure of the concentration C'_a of radioactive cadmium in this solution. Immediately afterwards the solution in the burette — suitably diluted — was measured with the same G—M tube, giving us the concentration C_a of radioactive cadmium in this solution. Thus the quotient C'_a/C_a was independent of the tube used.

If instead of the procedure described all the amalgam collected had been weighed and dissolved in nitric acid, corrections due to radiation absorption by the mercury dissolved would have been necessary to give correct values of C'_a/C_a. But this way would not have been expedient.

From Table 1 it is evident that C'_a was always extremely small in comparison with C_a. This means that C_a did not decrease perceptibly during an exchange experiment. The average concentration $\bar{q}_a$ of radioactive cadmium in the amalgam collected — calculated per unit weight — was obtained from the equation:

$$\bar{q}_a = \frac{v}{m} C'_a \qquad (2)$$

CHEMICALS

Cadmium amalgam. Calculated amounts of fine-grained cadmium and mercury of analytical grade were mixed and a little perchloric acid was added to remove oxide from the surface of the cadmium grains. Then the cadmium dissolved rapidly in the mercury and the amalgam formed was freed from acid by washing with hot water. It was dried with a piece of filtering-paper and used at once.

Cadmium perchlorate. A weighed amount of cadmium containing some Cd^{115} (from A.E.R.E., Harwell, England) was dissolved in nitric acid, and the nitrate formed was converted into perchlorate by heating with perchloric acid. Then the whole dose was taken for the preparation of a stock solution of known concentration.

The perchloric acid used was of analytical grade, and the sodium perchlorate was purified by repeated crystallizations.

DEDUCTION OF THE RATE LAW FROM THE MEASUREMENTS

In addition to the quantities $\bar{q}_a$ and C_a, introduced above, the following will be used:

q_a, q_i = the concentrations of active and inactive cadmium respectively in the amalgam phase close to the interface.

C_i = the concentration of inactive cadmium in the solution phase.

The taking up of radioactive cadmium in the amalgam phase depends upon the process

$$\overset{*}{Cd}^{2+} + Cd \rightarrow \overset{*}{Cd} + Cd^{2+} \quad (\overset{*}{Cd} = Cd^{115})$$

which we will denote $(\overset{*}{Cd}, Cd)$. Its rate, *i.e.* the number of moles passing the interface in each direction per unit time and unit area, is denoted h_1. Furthermore, we have the following three processes with their own rates:

Process	Rate
(Cd, Cd)	h_2
$(Cd, \overset{*}{Cd})$	h_3
$(\overset{*}{Cd}, \overset{*}{Cd})$	h_4

The rate h of the total exchange reaction is given by the relation

$$h = \sum_{j=1}^{4} h_j \tag{3}$$

If m_0 is the weight of an amalgam droplet, s_0 the area of its surface, and t the time of contact between the phases, we have the following equation for the increase of the concentration of radioactive cadmium in the amalgam phase per unit time:

$$m_0 \frac{d\bar{q}_a}{dt} = s_0 (h_1 - h_3) \tag{4}$$

For $\frac{d\bar{q}_a}{dt}$ we have:

$$\frac{d\bar{q}_a}{dt} = \frac{d\bar{q}_a}{dx} \cdot \frac{dx}{dt}$$

where x is the thickness of the solution layer. Generally t cannot be exactly proportional to x, but from the following it will be evident that the only condition necessary for the deduction is that $\lim \frac{dt}{dx}$ for $x \rightarrow 0$ had the same value in the different measurement series, and no doubt this condition was fulfilled, as the concentration of sodium perchlorate was the same in all solutions, and also other experimental details were kept unchanged. However, the variation in $\frac{dt}{dx}$ with x must surely be small, and for the sake of shortness

Table 1. Measurements of the exchange of radioactive cadmium between perchlorate solutions and amalgams.

q_i per cent 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	1180	2.6	14.1	0.643	2.8
1.00	2.23	1180	4.9	40.6	1.013	5.1
1.00	2.23	1180	8.3	69.4	1.002	8.8
1.00	2.23	1180	10.9	85.5	0.890	12.2
1.00	4.47	2160	2.6	41.8	1.100	2.7
1.00	4.47	2160	5.4	67.1	0.853	5.5
1.00	4.47	2160	8.7	147	1.211	8.4
1.00	4.47	2160	12.5	197	1.170	11.7
1.00	13.4	6030	2.9	108	0.957	2.8
1.00	13.4	6030	5.4	267	1.368	4.9
1.00	13.4	6030	9.1	281	0.877	7.9
1.00	13.4	6030	12.1	339	0.892	9.5
1.00	4.38	2890	2.2	34	0.904	2.0
1.00	4.38	2890	3.3	74	1.176	3.3
1.00	4.38	2890	6.0	60	0.904	3.4
1.00	4.38	2890	9.5	94	1.268	3.8
2.00	2.23	734	2.9	14.2	0.982	3.0
2.00	2.23	734	4.8	29.8	1.372	4.5
2.00	2.23	734	9.0	44.7	1.134	8.1
2.00	2.23	734	12.3	62.9	1.222	10.5
0.50	4.47	1340	2.5	26.0	1.140	2.6
0.50	4.47	1340	5.0	78.1	1.794	4.9
0.50	4.47	1340	9.0	113.5	1.530	8.3
0.50	4.47	1340	13.0	110.0	1.076	11.5

in our equations we prefer to treat the derivate as a constant. Thus the expression $\frac{s_0}{m_0} \cdot \frac{dt}{dx}$ is a constant that we may include in the rate constant, if no absolute value of the latter is searched for. Then the differential equation for the radioactive exchange can be written:

$$\frac{d\bar{q}_a}{dx} = h_1 - h_3 \qquad (5)$$

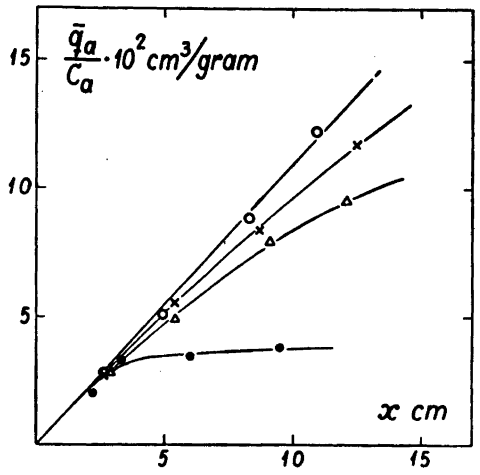

Fig. 2. $\frac{\bar{q}_a}{C_a}$ *as a function of* x *for* $q_i = 1.00$ *per cent and for different values of* C_i. *1.* $C_i =$ *2.23 mC (○); 2.* $C_i = 4.47$ *mC (×); 3.* $C_i = 13.4$ *mC (△); 4.* $C_i = 4.38$ *mC (●) and the amalgam aged.*

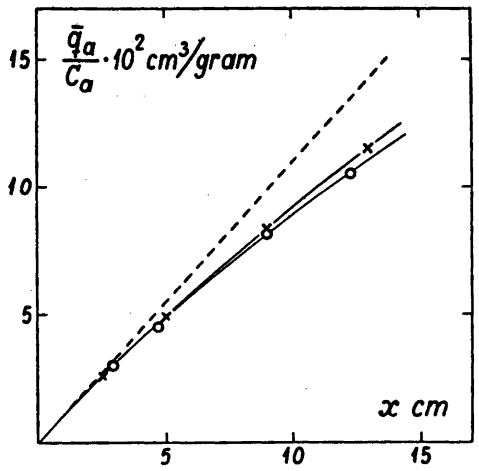

Fig. 3. $\frac{\bar{q}_a}{C_a}$ *as a function of* x *for different values of* q_i *and* C_i. *1.* $q_i = 2.00$ *per cent,* $C_i = 2.23$ *mC (○); 2.* $q_i = 0.50$ *per cent,* $C_i = 4.47$ *mC (×). The slope of the dashed line indicates the value of* k_0.

Since $q_a = 0$ for $x = 0$, eq. (5) gives us the useful relation:

$$\lim_{x \to 0} \frac{\mathrm{d}}{\mathrm{d}x}\left(\frac{\bar{q}_a}{C_a}\right) = \left(\frac{h_1}{C_a}\right)_{x=0} \tag{6}$$

The values obtained from the exchange measurements are collected in Table 1. The exchange experiments within one and the same measurement series were performed at a constant value of C_a. During the subsequent determination of $\bar{q}_a$, the values of C'_a and C_a decreased somewhat because of the rather short half-life (43 d.) of Cd^{115}. This circumstance has no influence on the quotients C'_a/C_a and $\bar{q}_a/C_a$, but in order to have all the values in a measurement series referred to the time of the exchange experiment, C'_a/C_a was multiplied by the initial value of C_a, recorded in the third column of Table 1, giving us the corresponding values of C'_a in the fifth column.

All the measurement series of Table 1 except the fourth one were carried out with freshly prepared amalgams. The first three series corresponded to a constant value of q_i, whereas C_i and C_a were varied. Then, in the fifth and sixth series other concentrations q_i were used, and furthermore, on account of the decay of Cd^{115}, the quotient C_i/C_a varied considerably from the first to the sixth series.

From Figs. 2 and 3 it is clear that when $\bar{q}_a/C_a$ is plotted against x, approximately straight lines or very slightly curved lines are obtained. Thus it is very easy to determine with great accuracy the value of $\frac{\mathrm{d}}{\mathrm{d}x}\left(\frac{\bar{q}_a}{C_a}\right)$ for $x = 0$. Now from the graphs we can make the very interesting discovery that at $x = 0$ (*i.e.* $q_a = 0$) *the derivate has the same value* k_0 ($= 1.10 \cdot 10^{-2}$ cm$^2 \cdot$ g^{-1}) *for all the curves, independent of* q_i, C_i, and C_a. Then, according to eq. (6) we have:

$$(h_1)_{x=0} = k_0 \cdot C_a \tag{7}$$

As Cd and $\overset{*}{\mathrm{Cd}}$ have identical chemical properties, the rate $(h_2)_{x=0}$ of the (Cd, Cd) process must necessarily follow the quite analogous equation: $(h_2)_{x=0} = k_0 \cdot C_i$. Before the amalgam has become radioactive, the (Cd, $\overset{*}{\mathrm{Cd}}$) and ($\overset{*}{\mathrm{Cd}}$, $\overset{*}{\mathrm{Cd}}$) processes cannot take place, and thus we have: $(h_3)_{x=0} = (h_4)_{x=0} = 0$. Then, if we apply eq. (3), the expression for the rate of the total cadmium exchange is obtained:

$$h = k_0 (C_a + C_i) \tag{8}$$

It should be emphasized that *as this rate is independent of the time of contact, eq. (8) is valid for all x-values,* though it has been derived for $x = 0$. The factor k_0 we will call the rate constant. Thus it has been proved that under the experimental conditions of this investigation the rate of the cadmium exchange is proportional to the total cadmium concentration in the water solution and independent of the cadmium concentration in the amalgam.

On the basis of eq. (8) it is easy to derive the general expressions for the partial rates h_j. The fraction $C_a/(C_a + C_i)$ of all cadmium entering the amalgam is radioactive, and the fraction $q_i/(q_a + q_i)$ of all cadmium leaving the amalgam is inactive. Thus for h_1 we get:

$$h_1 = h \frac{C_a q_i}{(C_a + C_i)(q_a + q_i)} = k_0 \frac{C_a q_i}{q_a + q_i} \tag{9a}$$

and for h_3 the corresponding expression:

$$h_3 = k_0 \frac{C_i q_a}{q_a + q_i} \tag{9b}$$

Then the differential equation (5) for the radioactive exchange takes the final form:

$$\frac{\mathrm{d}\bar{q}_a}{\mathrm{d}x} = k_0 \frac{C_a q_i - C_i q_a}{q_a + q_i} \tag{10}$$

Eq. (10) fulfils the requirement that an equilibrium between the phases prevails (*i.e.* $\frac{d\bar{q}_a}{dx} = 0$) for $C_a/C_i = q_a/q_i$. Generally q_a can be neglected in comparison with q_i in the denominator of the right member and C_i, q_i, and C_a be considered as constants (see Table 1).

In addition to eq. (10) a relation between $\bar{q}_a$ and q_a could be obtained, if the differential equation for the diffusion in the amalgam phase is solved for the proper boundary conditions. But this matter will not be treated in the present paper.

DETERMINATION OF THE RATE CONTROLLING PROCESS

According to the theory of Randles [5], the rate of the electron exchange at a redox electrode in equilibrium with the solution is approximately proportional to $\sqrt{C_O\, C_R}$, where C_O and C_R are the concentrations of the oxidized and the reduced forms of the reactants. As the theory is quite the same for amalgam electrodes, the electron exchange at the cadmium amalgam would follow the rate law $h \approx$ const. $\sqrt{C \cdot q}$, where C and q are the total cadmium concentrations in the solution and the amalgam respectively. A comparison between this law and eq. (8) above, where $C_a + C_i = C$, shows clearly that in our experiments the electron exchange is not the rate controlling process.

The diffusion of cadmium in the amalgam up to the interface cannot be so either, for then h would have been a function of q and independent of C.

Finally it can be shown that the diffusion of cadmium ions in the solution up to the amalgam must follow a law of the same form as eq. (8). At $x = 0$ ($q_a = 0$) no radioactive ions leave the amalgam, and the arriving ones are discharged at once in the rapid electron exchange process. Thus, the concentration of $\overset{*}{\mathrm{Cd}}^{2+}$ close to the surface can be put ≈ 0. Outside the Nernst diffusion layer of the thickness δ, surrounding the droplet, the concentration has the constant value C_a, owing to the intense stirring at the experiments. Then, for the average concentration gradient C_a/δ, the rate $(h_1)_{x=0}$ of the net transport of $\overset{*}{\mathrm{Cd}}^{2+}$ per unit area of the interface is given by the diffusion equation:

$$(h_1)_{x=0} = \frac{D}{\delta} \cdot C_a \tag{11}$$

where D is the diffusion coefficient. In the same way as above, the general expression for h can be derived from eq. (11):

$$h = \frac{D}{\delta}\,(C_a + C_i) \tag{12}$$

From eq. (8) and (12) we get $k_0 = D/\delta$. If D is expressed in the usual dimension ($cm^2 \cdot sec^{-1}$), the constant $\frac{s_0}{m_0} \lim_{x \to 0} \frac{dt}{dx}$ must not be included in k_0.

Thus it has been fully proved that within the concentration ranges of C and q used in this investigation and at equilibrium potential, the diffusion of the cadmium ions is the rate controlling process for the exchange.

ON THE "AGEING" OF THE CADMIUM AMALGAM

At the exchange experiments described above all measurements were carried out within an hour after the preparation of the amalgam. Then the reproducibility in the values was very good. For the fourth series in Table 1 an amalgam was used that was about a week old. It had been kept in vacuum, and just before use it was treated with acid in the same way as a fresh amalgam to be freed from oxide. In Fig. 2 it is seen that the corresponding curve 4 at the lower x-values has a slope of about the same magnitude as the other curves, indicating that eq. (8) and (12) were still valid. At increasing x the $\bar{q}_a/C_a$-values very soon became approximately constant, but the reproducibility was very bad.

The course of the curve indicates that after a short time of contact an approximate equilibrium in the radioactive exchange was attained between a surface layer of the amalgam and the solution, as if the diffusion rate were lower in an aged amalgam than in a freshly prepared one. Concerning the electro-chemical properties it was found that a fresh and an aged amalgam gave exactly the same potentials, and when q was doubled, the cadmium activity increased in the proportion 1 : 1.85, indicating a monophase amalgam within this q-range.

Any explanation of the ageing process must take these facts into account. However, to give a plausible explanation further investigations of different kinds are of course necessary.

SUMMARY

The exchange of cadmium between liquid amalgams and cadmium perchlorate solutions at equilibrium potential is investigated by the use of a radioactive cadmium isotope.

A procedure is described, by which the taking up of radioactive cadmium by the inactive amalgam during short times of contact can be determined.

From these measurements the differential equation for the radioactive exchange is derived, and furthermore it is found that within the concentration

ranges used the rate of the total exchange is proportional to the cadmium concentration in the solution and independent of the concentration in the amalgam phase.

It is proved from the rate law obtained that the diffusion of cadmium ions through the Nernst diffusion layer is the rate controlling factor for the exchange under the experimental conditions given.

The amalgam used must be freshly prepared, otherwise the exchange experiments do not function well.

I am much indebted to Professor S. Bodforss for his kind interest in this investigation. My thanks are also due to Mr. L. Å. Appelqvist for his valuable help at the measurements.

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Preparation of Aromatic Carboxylic Acids by Side-Chain Oxidation with Dilute Nitric Acid at High Temperature and Pressure

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It is well known that aliphatic side-chains in aromatic compounds may be oxidized to carboxylic groups using nitric acid. Up to now this was generally performed by refluxing the organic compound with comparatively concentrated nitric acid. Very long reaction times were necessary using these older methods, the yields were low and large amounts of by-products were formed [1]. It has been claimed in an old German patent [2] that toluene can be oxidized to benzoic acid at 130—150° C under pressure but no figures were presented concerning pressure and yield. Later a series of alkylbenzenes have been oxidized in a similar way but the yields were low [3]. Recently it was claimed that terephthalic acid could be obtained in good yields when *p*-dialkyl benzenes were oxidized with nitric acid at 14 kg/cm^2. The reaction product was somewhat contaminated with toluic acid [4].

As nitric acid is an inexpensive oxidizing agent, research work was started in Bofors about ten years ago in order to find new ways of performing oxidations with nitric acid and to make them adaptable to full scale industrial manufacture. It was shown that a high temperature and a pressure as high as 30—50 kg/cm^2 gave fast and complete conversions and high yields of comparatively pure products [5]. In this way a considerable number of aromatic carboxylic acids have been prepared with good yield, *e.g.* benzoic acid, chloro- and nitrobenzoic acids, phthalic acids etc. This paper deals with some results, obtained by the oxidation of xylenes, cymenes, toluic aldehyde, ditolylsulfone and some chlorosubstituted aromatic hydrocarbons.

EXPERIMENTAL

For the experiments there was used a 2 500 ml autoclave made of stainless steel and fitted with stirrer, thermometer, pressure gauge, facilities for venting and two pressure chambers. The usual procedure was to charge the aromatic compound and the nitric acid, and then heat with stirring until the exothermic reaction started, generally around 170° C. The heating was then interrupted but nevertheless the pressure and temperature rose rapidly, thus indicating a violent oxidation reaction, and sometimes it was necessary to connect the autoclave with the pressure chambers in order to moderate the reaction. In certain experiments the pressure still rose, however, and then it was necessary to let out part of the gases formed. When the main part of the organic compound had been oxidized, which was when the pressure did not rise any more, the temperature was held at a certain level around 200° C for a certain time, and then the autoclave was emptied. The crude product was finally washed with water and dried.

This procedure was sometimes modified, so that only part of the components was charged at the beginning of the experiment and the remainder added later, when this first part had reacted. When this procedure was adapted, the pressure chambers were used for charging the autoclave using the autoclave's own pressure.

o-Xylene. Dilute nitric acid of 30 % concentration and *o*-xylene were charged in the autoclave. The nitric acid was used in a 30 % equivalent excess. The temperature was held at the level, indicated in Table 1, for 15 minutes, after the main reaction had been finished and the pressure also remained around the level indicated in the table during this period.

Table 1.

Temperature °C	Pressure kg/cm^2	Yield %	Eq. weight	Nitrogen (Dumas) %	HNO_3 in mother liquor %
168	39	69	87.0	0.5	9.7
183	43	57	83.9	0.4	11.2
187	49	69	84.6	0.3	6.7
189	49	70	83.9	0.4	8.6
191	50	71	83.8	0.3	6.5
194	52	69	83.2	0.3	7.9
172	39	74	87.7	0.7	9.1
172	42	80	88.4	0.6	9.7
183	50	66	84.7	0.3	9.6

As a further control to the purity, the crude products were analyzed by precipitating the K-salt of the phthalic acid in ethyl alcohol. This method, which is only approximate, indicated that the purity was between 94 and 97 % for all the samples.

The last three runs in the table were performed using the mother liquor from an earlier experiment under the same conditions.

As may be seen from the table, the best result was obtained when comparatively high temperatures and pressures were used.

The crude products in Table 1 crystallized from the mother liquors directly on cooling. The mother liquors from the filtration were evaporated and cooled, and then an additional quantity of crude product crystallized. This product had a comparatively high equivalent weight and a high nitrogen content. Consequently it was believed to contain a major part of mononitrotoluic acids, which are more soluble in water than is the phthalic acid. The weight of this crude product, crystallized from the mother liquor, was on an average about 20 % of the weight of the crude phthalic acid.

In a similar way *m-xylene* as well as mixtures of *m*-xylene and *p*-xylene have been oxidized. The results will be published later on.

p-Xylene. Using an equimolecular amount of nitric acid and charging all together the following results were obtained (Table 2).

Table 2.

Nitric acid conc. %	Temperature °C	Pressure kg/cm²	Yield %	Crude product Eq. weight	N(Dumas) %
15	169	40	84	90.6	0.7
15	172	37	79	91.2	0.7
25	165	37	86	93.1	0.5
25	174	38	85	91.0	0.5
25	196	40	83	86.3	0.5

In other series of experiments a certain surplus of nitric acid has proved to be preferable, in order to obtain a more complete oxidation. A rather high temperature, around 200° C, serves the same purpose. The crude product has been purified to pure terephthalic acid in a good yield. The purified as well as the crude products have been esterified with *n*-butyl alcohol to give pure terephthalic acid dibutyl ester in a good yield.

p-Cymene. The oxidation of *p*-cymene is still more violent than the oxidation of xylenes, and only small quantities could be oxidized, if the whole charge was put into the autoclave at the beginning of the experiment. If the *p*-cymene and the nitric acid were charged in portions, the capacity was, however, quite good. The experiments in Table 3 have been made using refined *p*-cymene with a specific gravity of 0.858. This quality, which was an ordinary refined *p*-cymene from a Swedish pulp mill, proved to have a purity of about 95 %, when carefully fractionated in a laboratory column (50 plates).

The values are representative of results obtained when *p*-cymene was oxidized with nitric acid using successive charging. It has, however, in other series of experiments, proved possible to obtain an 80–85 % yield using 15 % nitric acid.

In the same way a mixture of about 30 % *m*-cymene and about 70 % *p*-cymene was oxidized. The mixture was compared with a *p*-cymene of about 98 % purity. Using 30 % nitric acid in a 30 % excess at 200° C and 30 kg/cm², the mixture gave a 75 % yield, com-

Table 3.

Nitric acid conc. %	Nitric acid surplus %	Temp. °C	Pressure kg/cm²	Yield %	Crude reaction product Eq. weight	C %	H %	N %	Ash %
15	30	196	52	74	83.2	57.5	3.6	0.1	0.24
25	30	173	40	78	84.7	57.5	3.6	0.2	0.08
25	30	198	38	87	84.8	57.5	3.7	0.3	0.09
Calculated for terephthalic acid:					83.1	57.8	3.6		

pared with an 82 % yield from the pure *p*-cymene. The equivalent weights were about 85 for both products and the contents of nitroterephthalic acid, determined in a polarograph, were 2.5 and 1.8 % respectively.

p-Toluic aldehyde. *p*-Toluic aldehyde was made from toluene using the Friedel-Craft reaction and was identified by determination of density ($D_4^{20} = 1.024$) and refraction index ($n_D^{20} = 1.5453$). Aldehyde (40 g) and dilute nitric acid in 30 % excess, calculated for the oxidation to terephthalic acid, was charged, the autoclave was closed and the charge was heated while stirred. The heating was interrupted at about 180° C, when a vigorous reaction started, generating a rapidly rising pressure. The temperature rose to about 200° C and the pressure to 31—34 kg/cm². The pressure and temperature were held at that level for 15 minutes and the autoclave was then allowed to cool. The crude terephthalic acid was filtered, washed with water, dried and analyzed for equivalent weight and nitroterephthalic acid (polarographically). The result may be seen from Table 4.

Table 4.

Run No.	HNO_3 Conc. %	Temp. °C	Pressure kg/cm²	Crude terephthalic acid Yield	Eq. weight	Nitro-Compounds	Mother liquor HNO_3 %
1	15	200	31	79.5	83.1	1.6	6.2
2	15	206	31	83.5	83.6	0.9	5.9
3	30	203	33	84.2	84.5	2.9	10.7

The product from the experiments 2 and 3 were analyzed for C, H and N:

Run	C	H	N
2	57.7	3.6	0.2
3	57.8	3.6	0.3
Calculated:	57.8	3.6	

Another oxidation was made in which double the amount of aldehyde was charged and the corresponding amount of nitric acid added in two lots. When this first charge had reacted the rest of the nitric acid was charged under pressure. In that way a better capacity was obtained.

Ditolylsulfone. Ditolylsulfone, $CH_3 . C_6H_4 . SO_2 . C_6H_4 . CH_3$, is formed as a by-product of the sulfochlorination of toluene with chlorosulfonic acid. It is formed together with isomers, but the *p,p'*-compound is dominating.

For these experiments a starting material was used, which had been obtained as a by-product of the manufacture of toluene sulfonamides. It was leached several times with hot dilute sodium hydroxide to remove toluene sulfonamide and was then recrystallized from ethyl alcohol. After this treatment the material melted at 150—151° C and apparently consisted mainly of the *p,p'*-isomer.

The autoclave was charged with sulfone and nitric acid, the latter in 20 % excess over that required for the oxidation of two methyl groups to carboxylic groups. The autoclave was closed and the charge was heated while stirred. The heating was interrupted at about 180° C, when a vigorous reaction started, generating a rapidly rising pressure. The temperature rose to about 200° C and the pressure to 30—45 kg/cm². The temperature and pressure were held at that level for 15 minutes, and the autoclave was then allowed to cool. The crude product was filtered off, washed with water, dried and analyzed. The result may be seen from Table 5.

Table 5.

Sulfone g	HNO_3 conc. %	Temperature °C	Pressure kg/cm²	Crude product g	Yield %	Eq. weight	S %	N % Dumas
30	15	204	28	38	100	164.0	10.0	0.5
40	30	204	32	45	90	159.6	10.0	0.8
123	15	184	41.5	139	89	165.7	10.4	0.5
105	15	205	45	121	93	155.9	9.8	0.4

Calculated for $HOOC . C_6H_4 . SO_2 . C_6H_4 . COOH$: Eq. weight = 153; S = 10.5 %

o-Chlorotoluene. Using a nitric acid of 15 % concentration in a 10—30 % excess at 180—200° C and 25—40 kg/cm² a 75—80 % yield was obtained. The crude products had the equivalent weights 154.1—156.8 (calc. 156.5), melting points 138—139° C and chlorine contents 21.0—22.2 (calc. 22.7). The nitrogen content (Dumas) was found to be about 0.3 %. Using nitric acid of 30 % concentration in a 10 % excess at 200° C and 33 kg/cm² an 80 % yield of a crude product with the equivalent weight 155.8, melting point 138—139.5° C and chlorine content 20.4 % was obtained.

m-Chlorotoluene. The oxidation was performed with nitric acid of 15 % concentration in a 10 % surplus. When oxidizing at 200° C and 37 kg/cm² an 80 % yield of a crude product with the equivalent weight 157.1, melting point 144—148° C, chlorine content 24.0 %

and nitrogen content (Dumas) 0.7 % was obtained. When the reaction was performed at 183° C and 27 kg/cm^2 the yield was 72 % and the equivalent weight 159.8, melting point 138—142.5° C and chlorine content 22.4 %.

p-Chlorotoluene. The oxidations were performed with a nitric acid of 15 % and 30 % concentration respectively. The excess was 30 % in all experiments. The result from representative runs may be seen from Table 6.

Table 6.

HNO_3 conc. %	Temperature °C	Pressure kg/cm^2	Yield %	Eq. weight	Melting point °C	Cl %
15	180	28	87	156.1	242—242.5	22.4
15	200	39	88	156.1	242—242.5	20.4
30	180	31	89	155.5	242—242.5	21.4
30	200	33	91	156.6	242—243	21.3

The nitrogen content (Dumas) was around 0.5 %. The crude chlorobenzoic acids were purified by dissolving in alkali, removing the nitro compounds and precipitating after treatment with active carbon. Pure acids with accurate analytical data were obtained in 80—90 % yields.

3,4-Dichlorotoluene. Using nitric acid of 15 % concentration in a 30 % surplus at 18 kg/cm^2 and 190° C the yield was 76 % of a crude product with the following data: Equivalent weight 189.0 (calc. 190.9) chlorine 34.5 % (calc. 37.2), nitrogen (Dumas) 0.6 %. When the oxydation was performed using nitric acid of 30 % concentration in 30 % excess at 31 kg/cm^2 and 215° C, the yield was 86 % of a product with the following data: Eq.w. 192.0 Chlorine 36.8, nitrogen (Dumas) 0.5 %.

2-Chloro-4-nitrotoluene. The redistilled chloronitrotoluene (freezing point 62.4—62.6°C) was oxidized in the usual way and then the crude product was treated with caustic soda and reprecipitated in order to remove unconverted chloronitrotoluene. In a series of experiments it was found, that a big charge was possible because the reaction proceeded relatively calmly. Thus it was possible to charge as much as 300—400 g in the 2 500 ml autoclave without risking a too violent reaction. Table 7 shows some representative results, obtained with nitric acid of 30 % concentration in a 20 % excess. The acid was charged in two portions.

Table 7.

Temperature °C	Pressure kg/cm^2	Conversion %	Yield %	Melting point °C	Eq. weight
160—165	16	82	70	140—141	203.2
165—170	39	89	79	136—138	206.3
180	38	91	88	140—141	204.6
200	34	95	82	138—139	201.5

Experiments with nitric acid of 20 % concentration at 170—180° C and 25—35 kg/cm^2 gave a 72—76 % conversion and an 87—88 % yield of a product with a good analysis. Chlorine content analyses were in good accordance with the calculated values.

The author wishes to thank Dr. Allan Dahlén for his great interest in this work and for valuable discussions.

SUMMARY

Using dilute nitric acid at temperatures around 200° C and pressures around 30—50 kg/cm^2, *o*- and *p*-xylenes, *p*-cymene, *p*-toluic aldehyde, ditolylsulfone, 2-chloro-4-nitrotoluene and different chlorotoluenes were oxidized to the corresponding carboxylic acids in yields generally between 75 and 90 %. The crude acids were somewhat contaminated with nitrated products.

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Antitubercular Compounds Related to *p*-Aminosalicylic Acid

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Following the demonstration of the tuberculostatic activity of *p*-aminosalicylic acid (PAS) [1] many investigators have prepared derivatives of *p*-aminosalicylic acid [2a, b and c] with the object of obtaining substances with increased potency against the tubercle bacilli.

Until Freire [3] in 1950 published his communication on the exceptionally high *in vitro* activity of the phenyl ester of *p*-aminosalicylic acid, all the derivatives of PAS which had been prepared had exhibited a lower activity or, at most, the same activity as PAS.

As *in vivo* experiments on mice and guinea pigs have also proved the phenyl ester of *p*-aminosalicylic acid to have a higher activity than PAS [4], we have prepared a number of derivatives of the phenyl esters of PAS and *p*-aminothiolsalicylic acid. In addition to the phenyl esters we have in this communication included the preparation of the *p*-aminosalicylic esters of various alcohols. The results of the testing of the activity of these substances, *in vitro* as well as *in vivo*, will be published elsewhere [5].

Tables 2 and 4 list a number of esters of 4-aminosalicylic acid and 4-aminothiolsalicylic acid, respectively, with various phenols. All these esters were prepared by reduction of the corresponding nitro compounds, which are listed in Tables 1 and 3. The preparation of phenyl esters of 4-nitro- and 4-aminosalicylic acid has been described previously [6, 7, 8]. The latter reference also includes the synthesis of the corresponding *p*-cresyl esters.

The simplest of the nitro esters were prepared by fusing together 4-nitrosalicylic acid and the phenol or thiophenol concerned in the presence of phosphorus oxychloride (method A). Later on we found it more convenient to start with 4-nitrosalicoyl chloride and allow this substance to react with the phenol or thiophenol in boiling toluene (method B). Some of the phenols used were, however, so sparingly soluble in toluene, that the reaction was very

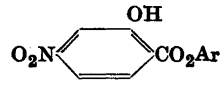


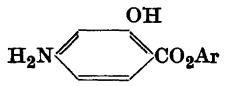
Table 1.

ArOH	Yield %	M. P. °C	Formula	Carbon Calc.	Carbon Found	Hydrogen Calc.	Hydrogen Found	Nitrogen Calc.	Nitrogen Found	Method	Crystallized from
Phenol	79	152 —153.5	$C_{13}H_9NO_5$	60.23	60.35	3.50	3.63	5.40	5.34	A	HAc
p-Cresol	82	123 —124.5	$C_{14}H_{11}NO_5$	61.53	61.78	4.06	4.14	5.13	5.13	A	MeOH
p-(β-Iodoethyl)phenol[a]	67	98 — 99.5	$C_{15}H_{12}JNO_5$	43.57	43.70	2.90	3.24	3.39		B	EtOH
p-Ethoxyphenol	80	108 —109.5	$C_{15}H_{13}NO_6$	59.40	59.21	4.29	4.28	4.62	4.67	B	MeOH
p-Carbomethoxyphenol	80	168 —170	$C_{15}H_{11}NO_7$	56.67	56.77	3.47	3.34	4.41	4.38	B	HAc
p-Carbethoxyphenol	73	115 —118	$C_{16}H_{13}NO_7$	58.00	57.90	3.93	4.12	4.23	4.47	B	EtOH
p-Hydroxypropiophenon	55	96 — 98	$C_{16}H_{13}NO_6$	60.93	61.14	4.16	4.13	4.44	4.13	B	HAc
p-Bromophenol[b]	74	145 —146.5	$C_{13}H_8BrNO_5$	46.14	46.16	2.36	2.51	4.13	4.27	B	HAc
o-Bromophenol[c]	48	122 —123	$C_{13}H_8BrNO_5$	46.14	46.28	2.36	2.49	4.13	4.45	B	Methyl*iso*-butyl-ketone
m-Nitrophenol	73	167 —169	$C_{13}H_8N_2O_7$	51.31	51.47	2.63	2.71	9.22	9.37	B	Methyl*iso*-butyl-ketone
p-Nitrophenol	86	153.5—155	$C_{13}H_8N_2O_7$	51.31	51.34	2.63	2.67	9.22	9.11	A	EtAc
m-Acetaminophenol	55	168.5—169.5	$C_{15}H_{12}N_2O_6$	56.96	57.12	3.79	3.86	8.86	8.90	C	MeOH
p-Acetaminophenol	82	196.5—197.5	$C_{15}H_{12}N_2O_6$	56.96	56.73	3.79	3.87	8.86	9.08	C	HAc
m-Aminophenol	78	158.5—161.5	$C_{13}H_{10}N_2O_5$	56.93	57.35	3.65	3.79	10.22	10.28	—	EtOH
p-Aminophenol	60	177 —179	$C_{13}H_{10}N_2O_5$	56.93	56.87	3.65	3.62	10.22	10.34	—	Acetone-water
m-Succinylaminophenol	46	190 —192	$C_{17}H_{14}N_2O_8$	54.55	54.30	3.75	3.93	7.48	7.76	G	HAc
p-Succinylaminophenol	90	201 —202	$C_{17}H_{14}N_2O_8$	54.55	54.69	3.75	3.77	7.48	7.64	H	Dioxane
p-Phthalylaminophenol		316.5—318.5 (dec.)	$C_{21}H_{14}N_2O_8$	59.72	60.42	3.32	3.48	6.63	6.74	G	HAc
p-Dimethylaminophenol	90	146.5—147	$C_{15}H_{14}N_2O_5$	59.57	59.39	4.64	4.77	9.27	9.50	C	Methyl*iso*-butyl-ketone

		Calc.	Found.
a)	J:	30.74	30.44
b)	Br:	23.64	23.60
c)	Br:	23.64	23.72

slow or did not proceed at all. To overcome this difficulty, it was attempted to use pyridine as a solvent, although Libermann [9] has shown, that in the presence of pyridine, 4-nitrosalicoyl chloride is converted into the corresponding disalicylide. However, when anhydrous pyridine was used, the esterification proved to proceed without complications and provide good yields.

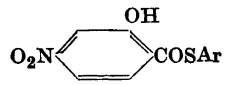
For the preparation of the phenyl esters which contain a free carboxyl group in the phenol component (3′-succinylamino-, 4′-succinylamino- and 4′-phthalylamino phenyl esters) the following method was used:



ble 2.

ArOH	Yield %	M. P. °C	Formula	Carbon Calc.	Carbon Found	Hydrogen Calc.	Hydrogen Found	Nitrogen Calc.	Nitrogen Found	Method	Crystallized from
henol	84	149 —150.5	$C_{13}H_{11}NO_3$	68.12		4.85		6.07	6.12	E	EtOH
Cresol	86	118.5—120.5	$C_{14}H_{13}NO_3$	69.15	68.96	5.41	5.75	5.76	5.73	E	70 % EtOH
(β-Iodoethyl)phenol[a]	66	166 —167.5	$C_{15}H_{14}JNO_3$	47.00	47.32	3.66	3.70	3.66	4.05	F	EtOH
Ethoxyphenol	58	173 —174.5	$C_{15}H_{15}NO_4$	65.94	66.14	5.55	5.53	5.13	5.13	E	*Iso*propanol
Carbomethoxyphenol	91	159 —161	$C_{15}H_{13}NO_5$	62.70	62.65	4.52	4.60	4.87	4.91	E	EtOH
Carbethoxyphenol	73	162 —163	$C_{16}H_{15}NO_5$	63.76	63.40	4.98	5.03	4.65	4.67	F	EtOH
Hydroxypropiophenon	77	167.5—169	$C_{16}H_{15}NO_4$	67.34	67.2	5.31	5.18	4.89	5.02	F	Methyl*iso*butylketone
Bromophenol[b]	77	170.5—171.5	$C_{13}H_{10}BrNO_3$	50.63	50.71	3.24	3.33	4.54	4.99	E	EtOH
Bromophenol[c]	81	133 —135	$C_{13}H_{10}BrNO_3$	50.63	50.50	3.24	3.22	4.54	4.63	F	EtOH
-Aminophenol	67	157 —159	$C_{13}H_{12}N_2O_3$	63.91	63.62	4.96	4.62	11.47	11.25	E	70 % EtOH
Aminophenol	45	183.5—184.5	$C_{13}H_{12}N_2O_3$	63.91	64.20	4.96	5.26	11.47	11.34	E	EtOH
Acetaminophenol	75	203 —204	$C_{15}H_{14}N_2O_4$	62.94	63.16	4.89	5.01	9.80	9.90	E	70 % EtOH
-Succinylaminophenol	70	175 —177	$C_{17}H_{16}N_2O_6$	59.30	59.12	4.65	4.61	8.14	8.16	E	MeOH
Succinylaminophenol	82	191 —192.5	$C_{17}H_{16}N_2O_6$	59.30	59.40	4.65	4.55	8.14	7.93	E	Methylcellosolve-water
Phthalylaminophenol	68	211 —212	$C_{21}H_{16}N_2O_6$	64.28	64.09	4.09	4.30	7.14	7.30	E	Methylcellosolve-water
Dimethylaminophenol	83	185 —186	$C_{15}H_{16}N_2O_3$	66.13	66.13	5.94	5.76	10.28	10.23	E	50 % EtOH

		Calc.	Found.
a)	J:	33.1	32.33
b)	Br:	25.98	26.94
c)	Br:	25.98	26.40



ble 3.

ArSH	Yield %	M. P. °C	Formula	Carbon Calc.	Carbon Found	Hydrogen Calc.	Hydrogen Found	Nitrogen Calc.	Nitrogen Found	Sulfur Calc.	Sulfur Found	Method	Crystallized from
Thiophenol	73	196—198	$C_{13}H_9NO_4S$	56.70	56.8	3.28	3.33	5.09	5.26	11.62	11.40	A	HAc
-Ethoxythiophenol	66	126—127	$C_{15}H_{13}NO_5S$	56.41	56.6	4.08	3.80	4.39	4.43	10.03	9.71	B	HAc

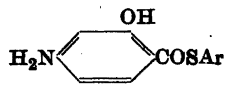


Table 4.

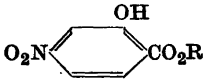
ArSH	Yield %	M. P. °C	Formula	Carbon Calc.	Carbon Found	Hydrogen Calc.	Hydrogen Found	Nitrogen Calc.	Nitrogen Found	Sulfur Calc.	Sulfur Found	Method	Crystallized from
Thiophenol	55	142–144	$C_{13}H_{11}NO_2S$	63.64	63.69	4.49	4.63	5.71	5.59	13.05	12.73	F	EtOH
p-Ethoxythiophenol	70	165–166	$C_{15}H_{15}NO_3S$	62.26	62.01	5.19	5.20	4.84	4.73	11.07	11.06	F	EtOH

4-nitrosalicylic acid was esterified with *m*- or *p*-acetaminophenol. The acetyl group was removed by mild hydrolysis, and the product was finally acylated by means of the anhydride of the appropriate dibasic acid.

The nitro compounds were reduced to the corresponding amino compounds either catalytically by means of Adam's catalyst (method E) or by means of stannous chloride (method F). Most nitro compounds can be reduced according to both methods, but in some cases the stannous chloride method is to be preferred, particularly in the case of the sulphur-containing compounds. In spite of the fact that the reduction with stannous chloride takes place in a liquid of fairly high acidity, the esters are not appreciably hydrolyzed.

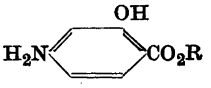
The six esters of 4-aminosalicylic acid with different alcohols which are listed in Table 6 have likewise been prepared by reduction of the corresponding nitro compounds. The reduction was effected catalytically according to method E, with one exception (the benzyl ester) in which the stannous chloride method (method F) proved more convenient. All the nitro compounds which are listed in Table 5 were prepared by treating 4-nitrosalicoyl chloride with an excess of the appropriate alcohol. The ethyl- and *n*-butyl esters of 4-nitrosalicylic acid and 4-aminosalicylic acid have previously been described by Jensen *et al.*[2a]

Table 7 comprises a number of N-substituted compounds of phenyl 4-aminosalicylate. Apart from phenyl 4-carbethoxyaminosalicylate, which was prepared by fusing together 4-carbethoxyaminosalicylic acid and phenol in the presence of phosphorus oxychloride, they were all prepared from phenyl 4-aminosalicylate; the tosyl compound by treatment with toluenesulphochloride in pyridine, the other acyl compounds by the action of the appropriate acid anhydride in boiling glacial acetic acid (method G) or in acetone (method H). Incidentally the acetyl compound has previously been prepared by Marauyama and Imamura [8] by treating N,O-diacetyl-4-aminosalicoyl chloride with phenol.



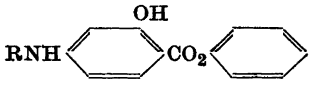
ible 5.

R	Yield %	M. P. °C	Formula	Carbon Calc.	Carbon Found	Hydrogen Calc.	Hydrogen Found	Nitrogen Calc.	Nitrogen Found	Method	Crystallized from
Ethyl	70	88	$C_9H_9NO_5$							D	EtOH
i-Butyl	68	33.5— 34.5	$C_{11}H_{13}NO_5$							D	EtOH
*Iso*hexyl		oil	$C_{13}H_{17}NO_5$							D	
*Cyclo*hexyl	50	92 — 93.5	$C_{13}H_{15}NO_5$	58.83	58.87	5.71	5.71	5.27	5.27	D	HAc
Benzyl	64	69 — 70	$C_{14}H_{11}NO_5$	61.51	61.30	4.03	4.11	5.12	5.34	D	HAc



ible 6.

R	Yield %	M. P. °C	Formula	Carbon Calc.	Carbon Found	Hydrogen Calc.	Hydrogen Found	Nitrogen Calc.	Nitrogen Found	Method	Crystallized from
Ethyl	80	114 —116	$C_9H_{11}NO_3$							E	EtOH
i-Butyl	70	92.5— 93.5	$C_{11}H_{15}NO_3$							E	70 % EtOH
*Iso*hexyl		42	$C_{13}H_{19}NO_3$	65.77	65.94	8.09	7.81	5.91	6.13	E	
*Cyclo*hexyl	89	131 —132.5	$C_{13}H_{17}NO_3$	66.35	66.22	7.30	7.36	5.96	5.85	E	EtOH
Benzyl	79	98 — 99	$C_{14}H_{13}NO_3$	69.10	69.31	5.40	5.35	5.76	5.88	F	EtOH



ble 7.

R	Yield %	M. P. °C	Formula	Carbon Calc.	Carbon Found	Hydrogen Calc.	Hydrogen Found	Nitrogen Calc.	Nitrogen Found	Sulfur Calc.	Sulfur Found	Method	Crystallized from
p-Toluenesulfonyl	57	169 —171	$C_{20}H_{17}NO_5S$	62.66	62.61	4.44	4.54	3.66	3.46	8.35	8.10		
Acetyl	76	181.5—183	$C_{15}H_{13}NO_4$	66.42	66.29	4.84	4.84	5.16	5.14			G	EtOH
Carbethoxy	65	149 —151	$C_{16}H_{15}NO_5$	63.78	63.86	4.98	5.10	4.65	4.74				EtOH
Lactosyl *	20	208 —212	$C_{25}H_{39}NO_{17}$	48.00	48.01	6.30	6.44	2.24	2.02				
Mannosyl	70	191.5—193	$C_{19}H_{21}NO_8$	58.31	58.37	5.42	5.56	3.58	3.83				
Glucosyl **	60	117 —120	$C_{19}H_{33}NO_9$	55.75	55.87	5.67	6.00	3.42	3.26				
Phthalyl	59	181.5—182.5	$C_{21}H_{15}NO_6$	66.83	66.76	3.98	4.09	3.71	3.80			H	Acetone
Succinyl	57	179 —181	$C_{17}H_{15}NO_6$	61.95	62.03	4.56	4.45	4.25	4.40			G	70 % EtOH
Maleinyl	66	188 —191	$C_{17}H_{13}NO_6$	62.37	62.42	3.97	4.12	4.28	4.26			H	EtOH

* Obtained as the tetrahydrate.
** » » » monohydrate.

The catalyst applied for the glucoside formation in the preparation of the three N-glucosides mentioned in the table was either ammonium chloride, as devised by Kuhn [10], or glacial acetic acid, as described by Weygand [11]. The corresponding N-glucosides of 4-aminosalicylic acid have recently been prepared by Haberland [12] in a similar manner.

EXPERIMENTAL

Microanalyses by G. Cornali. All melting points are corrected.

Intermediates. 4-Nitrosalicylic acid was prepared as described by Mc Ghie *et al.*[13], and 4-nitrosalicoyl chloride was prepared by treating 4-nitrosalicylic acid with thionyl chloride [14].

4-Aminosalicylic acid and most of the phenols used were commercially available. The following phenols were prepared according to methods previously described: *p*-(β-iodoethyl)-phenol [15], *p*-ethoxyphenol [16], *p*-propiophenol [17], *p*-dimethylaminophenol [18], and *p*-ethoxythiophenol [19].

4-Carbethoxyaminosalicylic acid was prepared by carbethoxylation of 4-aminosalicylic acid as described by Doub *et al.*[2c]

Method A. A mixture of 0.5 mole of 4-nitrosalicylic acid and 0.75 mole of the appropriate phenol was heated to a temperature of 80—100° C. 30 ml of phosphorus oxychloride was added and the temperature raised to 140° C and this temperature maintained until the evolution of gas had ceased. The mixture was then cooled to 115° C and 10 ml of toluene was added to keep the mixture in a fluid condition during the subsequent cooling process. When the mixture had been cooled to 60° C, 75 ml of methanol was added, and the cooling was continued until room temperature had been reached. The resulting crystalline 4-nitrosalicylic acid ester was filtered and washed on the filter with 75 ml of methanol. After drying, the compound was recrystallized from the solvent mentioned.

Method B. A suspension was made of 0.05 mole of the appropriate phenol in 50 ml of dry toluene and 0.05 mole of 4-nitrosalicoyl chloride was added. The mixture was refluxed until the evolution of hydrogen chloride had ceased (½—2 hours) and was then cooled to room temperature. In most cases the ester separates on standing; if not, 50 ml of petroleum ether should be added. The crystals which separated were filtered, washed with 10 ml of methanol, 10 ml of a saturated sodium bicarbonate solution and 25 ml of water and subsequently recrystallized.

Method C. A solution was prepared of 0.15 mole of 4-nitrosalicoyl chloride and 0.15 mole of the appropriate phenol in 100 ml of dry pyridine *. The mixture was heated to the boiling point and kept at this temperature for 5 minutes.

After cooling to room temperature, 200 ml of water was added to precipitate the crystalline ester. The ester was filtered, washed with saturated sodium bicarbonate solution and water and recrystallized.

Method D. 4-Nitrosalicoyl chloride was suspended in 3—6 times the equivalent amount of the appropriate alcohol. The mixture was heated on the steam-bath until the evolution of hydrogen chloride had ceased (½—2 hours) and subsequently cooled to room temperature. The crystals which separated were filtered, washed with methanol and recrystallized from a suitable solvent. The *iso*-hexylester, which was an oil, was isolated

* Dried by azeotropic distillation with benzene, according to Mitchell and Smith [20].

by removing the excess of *iso*-hexanol *in vacuo*, and hydrogenated without further purification.

Method E. A suspension was made of 0.03 mole of the 4-nitrosalicylic acid ester in 100 ml of methylcellosolve; 0.1 g of Adam's platinum oxide was added, and the mixture was reduced at room temperature and at a hydrogen pressure of 3 at., until the theoretical amount of hydrogen had been absorbed (1—2 hours). After removal of the platinum by filtration, about 50 mg of sodium dithionite was added and the amino compound precipitated by slowly adding 200 ml of water. The 4-aminosalicylate which separated was filtered and recrystallized, if required.

Method F. A solution was prepared of 20 g of stannous chloride dihydrate in a mixture of 55 ml of ethanol and 20 ml of conc. hydrochloric acid. The mixture was heated to the boiling point and 0.025 mole of the nitro compound was added gradually. The reaction mixture was refluxed for a further 5—10 minutes, and then 250 ml of water * (heated to about 50° C) was added. After cooling to room temperature the separated amino compound was filtered and washed with water.

Method G. A solution was prepared of 0.05 mole of the amino compound and 0.066 mole of the appropriate acid anhydride by heating in 75 ml of glacial acetic acid. The mixture was refluxed for 1 hour, cooled to room temperature, and 150 ml of water was added. The solution was allowed to stand, and when the acyl amino compound had crystallized, it was filtered, washed with water and recrystallized.

Method H. A mixture of 0.05 mole of the amino compound and 0.066 mole of the acid anhydride in 150 ml of acetone was refluxed. After a period of about 20 minutes the acyl amino compound begins to crystallize from the clear solution. After refluxing for 2 hours the mixture was cooled, the solid which separated was filtered and washed with acetone.

As regards the phenyl ester of 4-phthalylaminosalicylic acid, the reaction product is not clearly soluble in sodium bicarbonate solution. Consequently it was extracted with 150 ml of a saturated sodium bicarbonate solution at 50° C. A small amount of insoluble material was removed by filtration, the filtrate was acidified with 4 *N* hydrochloric acid, and the phenyl 4-phthalylaminosalicylate which crystallized was filtered and washed with water.

Phenyl 4-(mannosylamino)-salicylate. A mixture of 4.6 g (0.02 mole) of phenyl 4-aminosalicylate and 3.6 g (0.02 mole) of D(—)mannose in 25 ml of absolute ethanol was refluxed for 1 hour, 0.2 g of ammonium chloride being added as a catalyst. After half an hour crystals of the mannoside begin to separate from the hot solution. After cooling, the solid was filtered, washed with absolute ethanol and recrystallized from 70 per cent ethanol.

Yield: 5.45 g ~ 70 %. M.p. 191.5—193° C. $[\alpha]_D^{20}$ in pyridine: $-194° \pm 3°$ ($c = 1$).

Phenyl 4-(lactosylamino)-salicylate. A mixture of 4.6 g (0.02 mole) of phenyl 4-aminosalicylate and 7.2 g (0.02 mole) of lactose in 70 ml of dry methanol was refluxed for 40 hours, 0.2 ml of glacial acetic acid being added as a catalyst. The reaction mixture was filtered while hot, and the filtrate was cooled to room temperature. The lactoside which separated was recrystallized from 70 per cent ethanol.

Yield: 2.55 g ~ 20 %. M.p. 208—212° C (decomp.) $[\alpha]_D^{20}$ in pyridine: $-69° \pm 3°$ ($c = 1$).

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* In the preparation of phenyl 4-aminothiolsalicylate it was necessary, before the addition of water, to remove a certain amount of undissolved substance by filtration.

The analysis corresponds to the tetrahydrate, a finding which was confirmed through a determination of the weight loss by drying at 0.01 mm Hg and 40° C.

Phenyl 4-(glucosylamino)-salicylate. A mixture of 4.6 g (0.02 mole) of phenyl 4-aminosalicylate and 3.6 g (0.02 mole) of glucose in 70 ml of dry methanol was refluxed for 24 hours, 0.25 ml of glacial acetic acid being added as a catalyst. After cooling 70 ml of water was added to precipitate the glucoside. The latter was washed with water and recrystallized from 70 per cent ethanol.

Yield: 9.9 g ~ 60 %. M.p. 117—120° C (decomp.) $[\alpha]_D^{20}$ in pyridine: $-111° \pm 3°$ ($c = 1$).

The analysis corresponds to the monohydrate, a finding which was confirmed through a determination of the weight loss by drying at 0.01 mm Hg and 40° C.

Phenyl 4-toluenesulphonaminosalicylate. Phenyl 4-aminosalicylate, 3.45 g (0.015 mole) and toluenesulphochloride, 3.00 g (0.0158 mole) was dissolved in 25 ml of pyridine. The solution was kept at a temperature of about 50° C for 1 hour, after which dilute hydrochloric acid was added to precipitate the sulphonamide. After recrystallisation from ethanol, the melting point was 169—170° C.

Yield: 3.25 g ~ 57 %.

4'-Aminophenyl 4-nitrosalicylate. A mixture of 9.48 g (0.03 mole) of 4'-acetaminophenyl 4-nitrosalicylate and 100 ml of *N* ethanolic hydrogen chloride was refluxed. After a few minutes a clear solution results, but shortly afterwards the hydrochloride of 4'-aminophenyl 4-nitrosalicylate begins to precipitate. After refluxing for 1 hour, the mixture was cooled, and the hydrochloride filtered off and washed with ether. The hydrochloride was stirred into 60 ml of *N*/2 sodium acetate solution to liberate the base. The latter was filtered, washed with water and reprecipitated from acetone-water.

Yield: 4.9 g ~ 60 %. M.p. 177—179° C.

3'-Aminophenyl 4-nitrosalicylate. This compound was prepared from 3'-acetaminophenyl 4-nitrosalicylate in analogy with the preparation of 4'-aminophenyl 4-nitrosalicylate.

Yield: 78 %. M.p. 158.5—161.5° C.

Phenyl 4-carbethoxyaminosalicylate. A mixture of 9.0 g (0.04 mole) of 4-carbethoxyaminosalicylic acid and 7.5 g (0.08 mole) of phenol was heated to about 100° C, and 2.4 ml of phosphorus oxychloride was added. The temperature was raised to 120—130° C and kept here until the evolution of gas had ceased. The mixture was now cooled to room temperature and 20 ml of methanol was added. The ester which separated was filtered, washed with methanol and recrystallized from ethanol.

Yield: 7.8 g ~ 65 %. M.p. 149—151° C.

SUMMARY

The preparation of a number of esters of 4-nitrosalicylic acid and 4-aminosalicylic acid with various phenols and thiophenols and with a few simple alcohols is described. A number of N-substituted compounds of the phenyl ester of 4-aminosalicylic acid have also been prepared.

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The Interaction between Halogen Acids and Nitro-amines *

II. Investigation of the Reaction Mechanism

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The reaction between 4,9-dinitro-3-aminoretene and halogen acids has been described in a previous paper [1]. It was shown that this amine, and also some other *o*-nitro-amines were quantitatively transformed into a chlorodiazonium chloride when dissolved in glacial acetic acid and treated with concentrated hydrochloric acid. The reaction with excess of hydrochloric acid at 90—100° C was completed very rapidly, and in the case of the retene derivative mentioned above the reaction time was as short as 15—30 seconds under suitable conditions. Owing to the rapidity of this unexpected reaction it was assumed provisionally to be an intramolecular transformation (*i.e.* the direct reaction of the nitro-group with the amino-group in the *o*-position) though no experiments which supported this hypothesis had been carried out in that investigation. The present paper concerns the mechanism of this reaction.

Two alternative mechanisms, one intramolecular (A) and one intermolecular (B) may be suggested (see p. 791) and the experiments described below have been performed in order to decide which actually occurs.

When 4,9-dinitro-3-aminoretene was treated as described above, but in the presence of an equimolecular amount of *p*-chloroaniline, the reaction was considerably retarded, and after 2 minutes a large proportion of the starting material remained unchanged. For complete consumption of the nitro-amine a reaction time of about 5 minutes was required. Before the reaction products were investigated the diazonium salts which had been formed were decomposed by reduction with hypophosphorous acid or by a Sandmeyer reaction (cuprous chloride), and then worked up.

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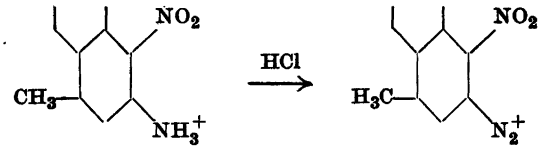
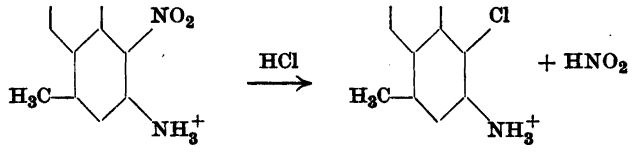
* The previous paper of this series appeared in *Acta Chem. Scand.* **5** (1951) 872 and was entitled "Intramolecular Diazotisation of *o*-Nitroamines".

A. Intramolecular mechanism



B. Intermolecular mechanism

Initiation:



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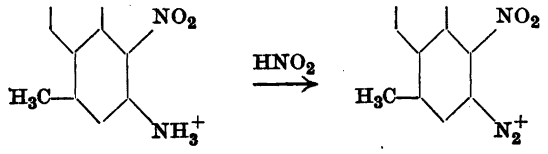

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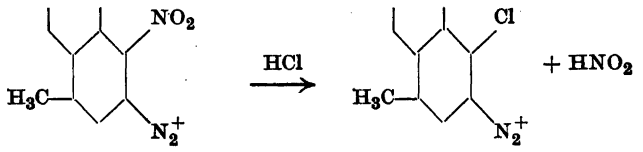
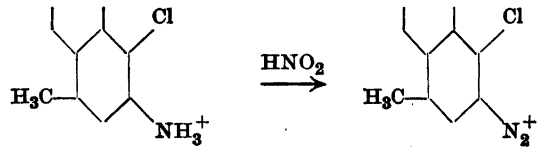


(3)

Termination:



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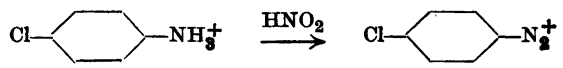

(5)

Reaction scheme

After reduction with hypophosphorous acid two amines, 4-chloro-9-nitro-3-aminoretene and 9-nitro-3-aminoretene and a neutral substance, 4-chloro-9-nitroretene were isolated in about 8 % yield each, and after the Sandmeyer reaction *p*-dichlorobenzene and 3,4-dichloro-9-nitroretene were isolated in yields of about 10 % and 16 % respectively.

These results strongly support the intermolecular mechanism (B). The diazotisation of the *p*-chloroaniline demonstrates that a diazotising agent (indicated in the reaction scheme by HNO_2) must be formed from dinitroaminoretene. The fact that the addition of *p*-chloroaniline decreases the rate of the reaction indicates that it proceeds by a chain mechanism — initiation (reaction 1), propagation (reactions 2 and 3), and termination (reaction 4). By addition of an easily diazotable amine a new termination reaction, the diazotisation of the amine (reaction 5), occurs, and the total velocity is decreased. On the other hand the velocity should be increased by the addition of a small amount of nitrous acid and this was also demonstrated: a suspension of 4,9-dinitro-3-retylamine hydrochloride in a mixture of glacial acetic and concentrated hydrochloric acids was perfectly stable at room temperature (*cf.* part I[1]) but when one tenth of the equivalent amount of nitrite was added, the amine was transformed quantitatively into 4-chloro-9-nitroretene-3-diazonium chloride within 3 minutes. It is to be expected that the presence of a diazonium-group will facilitate the elimination of the nitro-group (reaction 3) to a greater extent than will the ammonium ion (reaction 1), for the diazonium-group is the more powerful electron attracting substituent.

The possibility that the *p*-chloroaniline was diazotised by 4-chloro-9-nitroretene-3-diazonium chloride, formed by intramolecular diazotisation, *via* the diazoaminocompound,

$$RN_2^+ + H_2NR' \rightleftharpoons R\overset{\overset{+}{\frown}}{-NH\cdots N\cdots NH}-R' \rightleftharpoons RNH_2 + R'N_2^+$$

is rather improbable, as the reactions are carried out in an extremely acid medium, but the point has nevertheless been investigated: *p*-chloroaniline was added to a hot (90° C) solution of 4-chloro-9-nitroretene-3-diazonium chloride in a mixture of glacial acetic and concentrated hydrochloric acids, the proportions of the components being the same as in the experiment described above, and after 5 minutes the mixture was treated with cuprous chloride. From the resulting mixture no *p*-dichlorobenzene could be obtained, indicating that the postulated mechanism does not operate.

As mentioned above 9-nitro-3-aminoretene was isolated when the reaction mixture from 4,9-dinitro-3-aminoretene, *p*-chloroaniline and hydrochloric acid in acetic acid was reduced by hypophosphorous acid; it was also formed on treatment of the reaction mixture with cuprous chloride or by heating only.

A possible source for this product could be 4-chloro-9-nitro-3-aminoretene which can be dechlorinated by cuprous chloride. In agreement with this fact 9-nitro-3-aminoretene was obtained in good yield (*ca.* 70 %) when 4,9-dinitro-3-aminoretene was heated with excess of cuprous chloride in a

mixture of glacial acetic and concentrated hydrochloric acids. This reductive dechlorination, however, could not be effected with hypophosphorous acid, nor did it take place when 4-chloro-9-nitro-3-aminoretene was added to a solution of 4-chloro-9-nitroretene-3-diazonium chloride, *p*-chloroaniline, *p*-chlorobenzene diazonium chloride and hydrochloric acid in acetic acid (proportions approximately the same as in the experiment that yielded 9-nitro-3-aminoretene), and the diazonium salts were decomposed by heating. The intention with the latter experiment was to find out if products formed in the reaction mixture could have effected the dechlorination, but this was obviously not the case. A possible explanation for the formation of 9-nitro-3-aminoretene is that 4,9-dinitro-3-aminoretene may undergo denitration, just as sulphonic acids can be desulphonated; this remains to be investigated further. Though not quite comparable, a reaction applicable to certain nitrohalobenzenes may be mentioned. These compounds on treatment with potassium cyanide in alcoholic solution exchange the nitro-group with hydrogen and at the same time a cyano-group is introduced in the ortho-position to the eliminated nitro-group (the von Richter reaction, recently investigated and discussed by Bunnett, Cormack, and McKay [2]). A reaction discovered by Kohn [3] may also be mentioned. He found that *m*-dinitrobenzene and 2,4-dinitrotoluene on treatment with aqueous hydroxylamine exchange a nitro-group with hydrogen yielding nitrobenzene and *o*-nitrotoluene respectively.

Since the first paper [1] on this subject was published, Dey, Krishna Maller, and Pai [4] have reported an analogous reaction. They found that on heating in a mixture of glacial acetic and concentrated hydrochloric acids to 100° C 3-chloro-4,6-dinitroaniline loses its nitro-groups. The present author has investigated the six isomeric dinitroanilines with respect to this reaction and the results will be published in a forthcoming paper.

EXPERIMENTAL

The interaction between hydrochloric acid and 4,9-dinitro-3-aminoretene in the presence of *p*-chloroaniline

A hot mixture of glacial acetic acid (10 ml) and conc. hydrochloric acid (14 ml) containing *p*-chloroaniline (0.50 g; 0.004 mole) was added to a hot (*ca.* 90° C) well-stirred solution of 4,9-dinitro-3-aminoretene (1.35 g; 0.004 mole) in glacial acetic acid (50 ml). After five minutes at *ca.* 90° C the reaction solution was divided into two equal parts. One part was reduced with hypophosphorous acid (20 ml 50 % acid were added) and the other was decomposed by the Sandmeyer reaction (cuprous chloride in hot conc. hydrochloric acid).

The former was mixed with hypophosphorous acid and kept at room temperature for five hours, then diluted, made alkaline, and extracted with ether. After washing with

water and drying over sodium sulphate the ether solution was saturated with hydrogen chloride whereupon the amines present were precipitated as their hydrochlorides. These were collected, converted into the corresponding amines (yield 0.14 g), dissolved in benzene and adsorbed on a column of alumina (10 cm^2 × 20 cm). On development with benzene many zones appeared. The lowest, orange-coloured zone contained a compound (0.05 g, 8 %) which crystallized from ethanol in orange-yellow needles, m.p. 129—130 °C*. By its conversion to 9-nitro-3-aminoretene and 3,4-dichloro-9-nitroretene it was shown to be 4-chloro-9-nitro-3-aminoretene (these experiments are described below). [Calc. for $C_{18}H_{17}O_2N_2Cl$ (328.8): C 65.8; H 5.21. Found: C 65.8; H 5.30.]

An acetyl-derivative of this amine is described below.

Another compound was collected from an orange-red zone, separated from the lowest one by a violet zone which was not investigated. Reddish-yellow needles (0.05 g, 8 %), m.p. 156—157 °C, undepressed on admixture with 9-nitro-3-aminoretene ** were obtained.

The acetyl-derivative, prepared as described for 9-nitro-3-acetylaminoretene [5], crystallized from glacial acetic acid as scales, m.p. 290—291 °C, undepressed on admixture with 9-nitro-3-acetylaminoretene.

About the same amount of 9-nitro-3-aminoretene has been obtained in another experiment where the diazonium salts were decomposed by heating only.

The ether filtrate saturated with hydrogen chloride was washed with water and dried. The residue obtained after evaporation of the ether was adsorbed, from a benzene solution, on a column of alumina (10 cm^2 × 20 cm). A yellow zone could be seen among those which appeared on development with a mixture of benzene (1 vol.) and petroleum ether (10 vol., b.p. 40—50 °C). This yielded on elution a yellow well-crystalline compound (0.05 g, 8 %), m.p. 130—131 °C. Crystallized from glacial acetic acid it was obtained as yellow flat needles, m.p. 133—134 °C, undepressed on admixture with 4-chloro-9-nitroretene.

The second part of the reation mixture, in which the diazonium salts were decomposed by the Sandmeyer reaction, was diluted with water and extracted with ether which then was repeatedly washed with dilute sodium hydroxide until the water-phase was nearly colourless. The ether solution was washed with water, dried over sodium sulphate and saturated with hydrogen chloride. The precipitate formed was collected and the filtrate washed with water and dried. After evaporation of the ether the residue was steam distilled and gave colourless crystals, m.p. 51.5—53 °C, undepressed when mixed with *p*-dichlorobenzene. Yield 0.03 g, *ca.* 10 %. [Calc. for $C_6H_4Cl_2$ (147.0): C 49.0; H 2.74. Found: C 49.6; H 2.84.]

The residue from the steam distillation was dissolved in benzene and adsorbed on a column of alumina (10 cm^2 × 20 cm). Development with benzene caused many zones to separate. Only the compound from the most quickly moving, light-yellow zone was collected, yield 0.14 g, m.p. 146—148 °C. The product was again chromatographed but now, a mixture of benzene (1 vol.) and petroleum ether (10 vol., b.p. 40—50 °C) was used. Two yellow zones appeared. The lower zone containing the main product yielded 0.11 g (16 %), m.p. 155—156 °C. Crystallized from glacial acetic acid the compound was obtained as light-yellow needles, m.p. 158—159 °C, undepressed on admixture with 3,4-dichloro-9-nitroretene.

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* All melting points are corrected.

** In a previous paper [5] the m.p. is incorrectly reported to be 152—153 °C.

Experiments giving information about the rate of the reactions

I. A hot (80 °C) mixture of glacial acetic acid (4 ml) and conc. hydrochloric acid (3 ml) was added to a hot (80 °C) solution of 4,9-dinitro-3-aminoretene (0.20 g) in glacial acetic acid (10 ml). A small amount of the amine hydrochloride separated but was consumed within 20 seconds when a clear solution was obtained. The amine hydrochloride is slightly soluble in this mixture even at 80° C and therefore, no greater amount of it can be present in the clear solution.

II. Experiment (I) was repeated in the presence of *p*-chloroaniline (0.20 g) which was dissolved in the mixture of glacial acetic and conc. hydrochloric acids before the solutions were mixed. Within 10 seconds a large amount of the amine hydrochloride precipitated. The reaction mixture was kept at 80 °C for 2 minutes with stirring, and the amine hydrochloride still present was collected from the hot suspension, washed with a mixture of glacial acetic acid (5 ml) and conc. hydrochloric acid (2 ml), dried and suspended in ether. When ammonia was passed through the suspension a light-yellow coloured solution was obtained. On evaporation of the ether unchanged 4,9-dinitro-3-aminoretene (0.16 g, 80 %), m.p. 208—209 °C, undepressed on admixture with an authentic sample, was obtained.

III. Experiment (II) was repeated with a reaction time of 5 minutes. In this case 0.07 g (35 %) of unchanged 4,9-dinitro-3-aminoretene was recovered.

IV. Experiment (II) was allowed to proceed until all 4,9-dinitro-3-aminoretene was consumed (no crystals of the amine hydrochloride was present in the reaction solution). A reaction time of 12—14 minutes was required.

V. 4,9-Dinitro-3-aminoretene (0.34 g, 0.001 mole) was dissolved in glacial acetic acid (20 ml) at 30 °C. Conc. hydrochloric acid (4 ml) was added and the resultant suspension (a large amount of the amine hydrochloride separated as colourless needles) was cooled to room temperature (22 °C). A solution of sodium nitrite (*ca.* 0.0001 mole) was added to the mixture with stirring. After 3 minutes a clear solution was obtained, indicating that all 4,9-dinitro-3-aminoretene had been diazotised. The diazonium salt was decomposed by the Sandmeyer reaction (cuprous chloride). By chromatographic purification (Al_2O_3, benzene-petroleum ether) of the reaction product, rather pure 3,4-dichloro-9-nitroretene was obtained, (yield 0.28 g, 80 %, m.p. 157—158 °C). Crystallized from glacial acetic acid it melted at 159—160 °C, undepressed on admixture with an authentic sample of 3,4-dichloro-9-nitroretene.

Derivatives prepared from 4-chloro-9-nitro-3-aminoretene

4-Chloro-9-nitro-3-acetylaminoretene was obtained by adding acetic anhydride to a solution of 4-chloro-9-nitro-3-aminoretene in benzene. The solution was kept at room temperature for 8 hours when light-yellow needles appeared in the reaction mixture. Petroleum ether was added to cause complete precipitation. Crystallized from ethanol the acetyl-derivative melted at 211—212 °C. [Calc. for $C_{20}H_{19}O_3N_2Cl$ (370.8): C 64.8; H 5.16. Found: C 65.6; H 5.31.]

3,4-Dichloro-9-nitroretene. 4-Chloro-9-nitro-3-aminoretene (0.10 g) was dissolved in glacial acetic acid (2 ml). The amine hydrochloride, precipitated by the addition of conc.

hydrochloric acid (0.5 ml), was diazotised at room temperature with sodium nitrite (0.03 g) dissolved in a small amount of water. After 5 minutes the clear diazonium salt solution, thus obtained, was added to a hot solution of cuprous chloride (1 g) in conc. hydrochloric acid (10 ml). The reaction mixture was diluted with water and the precipitate collected (yield 0.10 g, m.p. 152—154 °C). After purification by chromatography and crystallization from glacial acetic acid the compound melted at 159—160 °C, undepressed on admixture with 3,4-dichloro-9-nitroretene.

9-Nitro-3-aminoretene. A hot (90 °C) solution of 4-chloro-9-nitro-3-aminoretene (50 mg) in glacial acetic acid (15 ml) was mixed with a hot solution consisting of glacial acetic acid (3 ml), conc. hydrochloric acid (4 ml), and cuprous chloride (0.3 g). The reaction mixture was shaken at 90 °C for 7 minutes, then cooled and diluted with hydrochloric acid to ensure complete separation of the reaction product. The colourless crystals, thus obtained, were collected, washed with hydrochloric acid and suspended in ethanol. On the addition of sodium acetate a red solution was obtained, from which the reaction product was precipitated by dilution with water (yield 40 mg, m.p. 154—155 °C). Crystallized from ethanol it was obtained as reddish-yellow needle-shaped crystals, m.p. 156—157 °C, undepressed on admixture with 9-nitro-3-aminoretene.

In another experiment a solution of 4-chloro-9-nitro-3-aminoretene (50 mg) in glacial acetic acid (15 ml) was mixed with a mixture of 50 % hypophosphorous acid (10 ml) and conc. hydrochloric acid (3 ml). On heating the suspension to 65 °C a clear solution was formed. After 5 minutes at 65 °C the solution was allowed to cool and was kept at room temperature for 10 hours. From the reaction mixture pure 4-chloro-9-nitro-3-aminoretene was obtained quantitatively.

The interaction between hydrochloric acid and 4,9-dinitro-3-aminoretene in the presence of excess of cuprous chloride

4,9-Dinitro-3-aminoretene (0.68 g) was dissolved in hot glacial acetic acid (30 ml). A solution of cuprous chloride (1.2 g) in a mixture of glacial acetic acid (5 ml) and conc. hydrochloric acid (7 ml) was added at 90 °C and the reaction mixture was kept at this temperature for 10 minutes with stirring. At the end of this time a large amount of the reaction product had precipitated as colourless needles giving the reaction mixture a pulpy appearance. After the addition of dilute hydrochloric acid (200 ml) the reaction product was collected, washed with dilute hydrochloric acid, suspended in ether and washed with aqueous alkali. Evaporation of the ether yielded a reddish-brown product (0.60 g) which was dissolved in benzene and adsorbed on a column of alumina (10 cm^2 × 20 cm). From the orange-red zone which appeared on development with benzene, 9-nitro-3-aminoretene (0.40 g, 68 %) was obtained. Crystallized from ethanol it melted at 156—157 °C, undepressed on admixture with an authentic sample.

SUMMARY

In a previous paper[1] on this subject it was reported that certain nitroamines are converted to chlorine substituted diazonium salts by treatment with a halogen acid alone. This reaction, conjectured as an intramolecular

diazotisation, has now been investigated more closely. On the basis of the experimental evidence a probable mechanism representing the reaction as intermolecular is put forward and discussed.

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Synthetic Inhibitors of Hyaluronidase

I. Demonstration of the High Inhibitory Power of some Diphenylmethane and Triphenylmethane Derivatives

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Some 25 years ago Duran-Reynals [1] demonstrated in rabbits that vaccinial infection is considerably enhanced by a simultaneous injection of testis extract into the skin. Two years later, Hoffman and Duran-Reynals [2] and McClean [3] showed that the testis extract revealed its enhancing effect by promoting the spread of the virus. The occurrence of spreading factors was later demonstrated in certain pathogenic bacteria [4], in poisonous snakes and in insects [5]. In 1939 Chain and Duthie [6] observed that testis extract contains an enzyme capable of hydrolysing hyaluronic acid. They suggested that the hyaluronidase may be responsible for the spreading properties of the extract. These observations stimulated extensive investigation of aspects bearing on the problem complex *spreading factor-hyaluronidase-hyaluronic acid.*

Already before Chain and Duthie's publication Meyer, Dubos and Smyth [7] had demonstrated the occurrence of a hyaluronic acid splitting enzyme in pneumococcal autolysate. Such hyaluronidases have since been recovered from many other species of pathogenic bacteria. This occurrence of bacterial hyaluronidases together with the knowledge of the presence of hyaluronic acid in the connective tissue, where it is believed to form an essential part of the interfibrillar cement substance, gave rise to wide speculation on a possible relationship between the hyaluronidase-hyaluronic acid system and certain pathological changes, particularly those seen in rheumatic diseases.

A possible way of checking whether any imbalance of the hyaluronidase-hyaluronic acid system is involved in the etiology or course of such diseases, is to study the response, if any, to the administration of inhibitors of hyaluronidase.

The inhibition of hyaluronidase by serum or certain serum fractions has been the subject of much research [8]. It is generally accepted that inhibition is ascribable to thermolabile substances of high molecular weight. Some well-defined chemical compounds such as dicoumarol and rutin are also known to exert an inhibitory action *in vitro* [9], and heparin is claimed to inhibit hyaluronidase activity *in vivo*, too [10].

In 1948 an extensive search for inhibitors of hyaluronidase was started at this laboratory. Below a report will be given of an investigation of the inhibitory effect of a number of organic compounds on the splitting of hyaluronic acid by testis hyaluronidase *.

METHOD

Substrate solution. 2 g sodium hyaluronate prepared from human umbilical cord by the method of Blix [12] were dissolved in 1 litre $M/6.5$ phosphate buffer solution of pH 7. The solution thus prepared was stored in the refrigerator.

Enzyme solution. Hyaluronidase was extracted from the bull's testes and purified by the method earlier described [13]. The stock solution was kept in the refrigerator and dilutions were prepared with isotonic saline once a week for laboratory use. The concentration of the diluted enzyme solution was such that on addition of 0.3 ml enzyme to 2.7 ml substrate solution at 37° C, the viscosity value of the latter was reduced by half in about 2 minutes.

Preparation of the test substances. The substances to be tested for inhibition of hyaluronidase activity were dissolved in distilled water or in dilute solutions of hydrochloric acid or sodium hydroxide and the pH was adjusted to 7. The solutions were made isotonic by the addition of saline.

Method of determination of inhibitory power. The initial viscosity of the substrate solution was determined by adding 0.3 ml isotonic saline to 2.7 ml substrate solution in an Ostwald viscosimeter submerged in a water bath at 37° C and noting the flow time. Enzyme activity was then estimated by the following procedure: 1.8 ml enzyme solution was mixed with 0.2 ml isotonic saline and stored at 37° C. A volume of 0.3 ml of this mixture was added to 2.7 ml of the substrate solution provided in the viscosimeter, through which nitrogen was allowed to bubble in order to secure good intermixture. The flow times were recorded in rapid succession, and on the basis of these recordings the time necessary for the viscosity to decrease by half (half-viscosity time) was calculated. This procedure was also used for determining any inhibitory power of the test substances, but here 1.8 ml enzyme solution was mixed with 0.2 ml of a test solution of arbitrary concentration instead of with isotonic saline. Any inhibitory power was reflected by an increase in the halfviscosity time.

If a substance tested produced such a prolongation, it was retested, but in concentrations as close as possible to that estimated to produce a fivefold increase in the half-viscosity time. The results of these trial and error tests were plotted and a curve was

* Recently, Forrest, Overell, Petrow and Stephenson [11] published an interesting paper on the inhibitory power *in vitro* of oxidation products of gentisic acid.

Table 1.

Compound	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)	Compound	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)
Methyl alcohol	< 0.1	4,4′-Dihydroxy-3,3′-dicarboxy-D,L-benzoin	3.8
Propyl alcohol	< 0.1	2,5-Dihydroxyterephtalic acid	1
Sodium formiate	0.1	3,4-Dimethoxy-*a*-carboxystilbene	0.1
Sodium oxalate	< 0.1	2-Iodo-4-amino benzoic acid	0.1
Sodium succinate	< 0.1	4-Methyl-1-(*p*-carboxy-*m*-hydroxy-phenyl)-thiosemi-carbazide	0.2
Acetone	0.4	4-Phenyl-1-(*p*-carboxy-*m*-hydroxyphenyl)-thiosemi-carbazide	2.5
Acetonylacetone	< 0.1	4,4′-Dihydroxy-diphenylsulfone	0.1
Furfurole	0.8	4,4′-Dihydroxy-diphenylsulfone disulfonic acid	0.1
Glucosamine-HCl	< 0.1	5-Formylsalicylic acid	1.8
N-acetylglucosamine	< 0.1	Stearylaminosalicylic acid	2
Pentaacetylglucose	< 0.1	Fluoresceine	8
Ethyl acetate	< 0.1	*β*-Dimethylaminoethyl-benz-hydryleter-hydrochlorid	0.1
2-Aminoethanol	0.5	Inosite	0.5
Urea	< 0.1	Pyridine	0.4
Urethane	< 0.1	Sodium-diethyl-barbiturate	0.4
Sodium diethyl-dithio-carbamate	< 0.1	Alloxan	0.2
Acetoxime	< 0.1	Triamino-hydroxy-pyrimidine sulfate	0.2
Aconitic acid	< 0.1	3-Hydroxy pyridine	0.2
m-Aminophenol	< 0.1	Piperidine	0.2
Pyrocatechol	< 0.1	Isonicotinic acid	0.1
Hydroquinone	0.5	Succinylamino-benzene-sulfonamide	0.2
Phloroglucinol	0.2	Quinoxaline	1
1,2,4-Benzenetriol	< 0.1	Quinoline-HCl	1.2
Salicylic acid	0.2	Quinhydrone	1
3-Hydroxy benzoic acid	0.4	N-iodoethyl-quinoline	< 0.1
4-Hydroxy benzoic acid	< 0.1	Sodium taurocholate	< 0.1
4-Aminosalicylic acid	0.5	D-Gluconic acid	< 0.1
4-Methylaminosalicylic acid	< 0.1	Protocatechualdehyde	1.3
Hippuric acid	0.5	Bis-(4-hydroxy-coumarinyl)-acetic acid	1.2
Gentisic acid	0.8	Methylene-bis-(4-hydroxy-7-carboxycoumarin)	2.5
a-Resorcylic acid	0.4	N,N′-Dianthranilomethane	1.5
β-Resorcylic acid	1.5		
γ-Resorcylic acid	1.9		
2,4,6-Trihydroxy benzoic acid	1.5		
Gallic acid	1.0		
Mandelic acid	0.5		
Saccharin	0.4		
Benzenesulfonic acid	< 0.1		
4-Benzoylaminosalicylic acid	0.1		
3-Carboxy-4-hydroxy-succinic acid monoanilide	0.9		

fitted to the plottings. The concentration necessary to produce a fivefold increase in the half-viscosity time was read off the diagram.

The reciprocal values of these concentrations of the test substances were taken as a measure of their inhibitory effect on hyaluronidase activity. As the absolute values thus obtained vary with the degree of polymerization of the hyaluronic acid and the age of the enzyme solution, all the recordings were compared with those of a standard solution and recalculated as relative units.

RESULTS

A number of organic compounds of various types were tested for any inhibitory action on hyaluronidase activity (Table 1). The inhibitory power of the compounds investigated was expressed in relative units, the inhibitory activity of resorcinol being taken as unity. The inhibitory effect of this substance has been described earlier by Calesnick and Beutner [12]. Under the conditions used in the present investigation resorcinol in a concentration of 0.2 per cent produces a fivefold increase in the half-viscosity time and complete inhibition occurs in a concentration of 0.8 per cent. None of the compounds first tested showed an inhibitory power of more than 8 times that of resorcinol.

In 1949 it was found in collaboration with K.-G. Rosdahl at this laboratory that the inhibitory power of methylene-disalicylic acid is about 40 times as high as that of resorcinol. This finding prompted us to prepare a number of related

Table 2. Hyaluronidase-inhibiting power of condensation products of diphenylmethane type.

Compound condensed with formaldehyde	Condensation product	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)
4-Hydroxy benzoic acid	Compound 2	380
Gentisic acid	Compound 7	1 200
α-Resorcylic acid	Compound 8	760
β-Resorcylic acid	Compound 9	400
γ-Resorcylic acid	Compound 19	980
2,5-Dihydroxy-terephtalic acid	Compound 6	2
2,4,6-Trihydroxy benzoic acid	Compound 3	780
Gallic acid	Compound 1	250
4-Methylamino salicylic acid	Compound 11	19
Acetylsalicylic acid	Compound 4	25
o-Thymotic acid	Compound 5	30
Anthranilic acid	Compound 13 [2]	2
Phloroglucinol	Compound 12	— [1]

[1] Saturated solution at pH 7 gives no inhibition.

[2] Prepared from N,N′-dianthranilomethane by rearrangement with hydrochloric acid.

Table 3. Hyaluronidase-inhibiting power of condensation products of triphenylmethane type obtained on reaction of diphenylmethane derivatives with phenol derivatives in the presence of an oxidizing agent.

Diphenylmethane derivative	Phenol derivative	Condensation product	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)
Compound 2	4-Hydroxy benzoic acid	Compound 20	1 100
Compound 7	Gentisic acid	Compound 21	1 450
Compound 1	Gallic acid	Compound 18	200
Compound 4	β-Resorcylic acid	Compound 10	32
Compound 9	Gallic acid	Compound 16	1 070
Compound 9	Salicylic acid	Compound 15	900
Compound 12	Salicylic acid	Compound 14	750
Compound 12	4-Hydroxy benzoic acid	Compound 17	640

diphenylmethane derivatives by condensation of hydroxy benzoic acids and other benzoic acid derivatives with formaldehyde.

0.2 mole of the substituted benzoic acid was suspended in 90 g 50 per cent sulfuric acid. 0.1 mole of formaldehyde as a 40 per cent solution was added and the mixture was heated on a water bath for 5 hours. Vigorous stirring throughout the reaction insured a thorough intermixture. The reaction mixture was cooled and the precipitate was filtered off and washed with hot water until free from sulfuric acid. The product was then dissolved in a slight excess of diluted sodium hydroxide solution and reprecipitated with diluted sulfuric acid. The precipitate was washed free from mineral acid and dried *in vacuo*.

Most of the condensation products prepared showed pronounced inhibitory activity (Table 2). The condensation product of gentisic acid with formaldehyde proved the most powerful inhibitor of this group. The corresponding derivatives of 2,4,6-trihydroxy benzoic acid, α-, β- and γ-resorcylic acid and 4-hydroxy benzoic acid were also found to exert a strong inhibitory effect.

Further investigation showed that the inhibitory power of most of the condensation products described above is potentiated by substitution of a hydrogen atom of the methylene bridge with a carboxy-hydroxy phenyl group (Table 3).

5 g of sodium nitrite were ground and thoroughly mixed with 10 g of a condensation product of diphenylmethane type and 5 g of a hydroxy benzoic acid. 105 g of concentrated sulfuric acid was then gradually added and the mixture agitated until it gave off red nitrogen oxide gas. The temperature of the mixture was kept below 10° C throughout the reaction. The solution

Table 4.

Compound	Hyaluronidase-inhibiting power in relative units ($resorcinol_{30\ min.} = 1$)	
	after 30 minutes' incubation	after 24 hours' incubation
2	380	900
7	1 200	2 200
8	760	1 200
9	400	920
3	780	1 200
20	1 100	2 100
21	1 450	2 700
16	1 070	2 000
15	900	1 750
14	750	1 520

obtained was poured into cold water. The precipitate thus obtained was filtered off and washed with cold water until free from sulfuric acid. It was then dissolved in a slight excess of diluted sodium hydroxide solution and reprecipitated with diluted sulfuric acid. The precipitate was washed with cold water until free from sulfuric acid and finally with hot water. It was dried in a vacuum desiccator.

In this group, too, the condensation product prepared from gentisic acid was the most active inhibitor.

In order to study any influence of the incubation period on the inhibition of hyaluronidase the mixture of enzyme and inhibitor was sometimes incubated for 24 hours, instead of for 30 minutes, before being added to the substrate. This prolonged incubation was found to produce a varying increase in the inhibition of hyaluronidase by the test substance (Table 4).

The most active compounds were tested for chronic toxicity in rats. Daily oral administration of compounds 3, 7, 14, 19 and 21 in doses of 200 mg/kg bodyweight produced no manifest toxic side effects. A similar daily dose of compounds 15, 16 and 20 caused toxic symptoms in the form of diarrhoea and loss of weight.

COMMENTS

The investigation of various groups of organic compounds for any inhibitory effect on testis hyaluronidase activity showed that certain diphenylmethane and triphenylmethane derivatives built up of nuclei substituted with 1—3 hydroxy groups are powerful inhibitors of hyaluronidase. On the other hand, corresponding uninuclear compounds such as phenols, mono-, di- and tri-

hydroxy benzoic acids inhibited hyaluronidase only when they were used in such concentrations as would cause denaturation of proteins.

Knowledge of new diphenylmethane and triphenylmethane derivatives with a strong inhibitory effect on hyaluronidase and only slight toxicity opens up a new approach to the treatment of diseases characterized by pathological changes in the ground substance of the mesenchymal tissues. It seems reasonable to suppose that these inhibitors may be able to control any imbalances of the hyaluronidase-hylauronic acid system of the body due to (a) hyaluronidase produced by infective agents, particularly bacteria (b) disturbances of the hyaluronic acid metabolism or (c) insufficient production of hyaluronidase inhibitors by the body.

In infections with hyaluronidase-producing bacteria, the spread of these micro-organisms in the body is facilitated by the hydrolytic action of the bacterial hyaluronidase on the hyaluronic acid in the interfibrillar substance of the connective tissue. Furthermore, the resultant increase in the permeability of the connective tissue invites invasion by other nonhyaluronidase-producing micro-organisms. This suggests that the inhibitors may also be of value in the prophylaxis and therapy of certain bacterial and viral infections.

SUMMARY

More than 90 organic compounds were investigated for any inhibitory effect on testis hyaluronidase *in vitro*. As standard use was made of resorcinol, whose inhibitory power was taken as unity. Resorcinol produced practically complete inhibition when employed in a concentration of 0.8 per cent.

Salicylic acid, gentisic acid, γ-resorcylic acid and other hydroxy benzoic acids showed an inhibitory power of less than 2. Diphenylmethane derivatives obtained by condensation of dihydroxy and trihydroxy benzoic acids with formaldehyde proved strong inhibitors. Of these, the condensation product of gentisic acid (compound 7) possessed the strongest inhibitory power (1 200 rel. units). The corresponding derivative of 4-hydroxy benzoic acid showed also a pronounced inhibitory activity.

Triphenylmethane derivatives were prepared by substitution of condensation products of diphenylmethane type in the methylene bridge with hydroxy-carboxy phenyl radicals. The inhibitory capacity of three of these compounds exceeds 1 000 units. The most active derivative of this group was the one made up of gentisic acid (compound 21), which showed an inhibitory power of 1 450. Compound 7 and compound 21 produced no demonstrable toxic effects in rats on oral administration for 30 days in total daily doses of 200 mg/kg bodyweight.

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Synthetic Inhibitors of Hyaluronidase

II. New Polycondensed Diphenylmethane and Triphenylmethane Derivatives

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In the preceding paper [1] we described the hyaluronidase inhibiting activity of some diphenylmethane derivatives obtained by condensation of hydroxy benzoic acids with formaldehyde. Analogous triphenylmethane derivatives were found to be even more active.

In a search for still more active inhibitors we studied the reaction between hydroxy benzoic acids and formaldehyde under varying conditions to find out any relation between the conditions under which the condensation was carried out and the inhibitory effect of the products obtained. It was found that the inhibitory effect of the condensation products varies with the ratio between the amounts of hydroxy benzoic acid and formaldehyde entering the reaction. With an excess of formaldehyde very active compounds of high molecular weight were obtained.

From the diphenylmethane derivatives thus prepared triphenylmethane derivatives were obtained by condensation with a phenol or a hydroxy benzoic acid under the influence of nitrous acid. Some triphenylmethane derivatives were also prepared in a similar way from condensation products of phenols with formaldehyde.

The purpose of this paper is to report an investigation of the inhibitory effect of these new substances on hyaluronidase *in vitro* and their oral toxicity in mice.

METHOD

Substrate solution. 2 g sodium hyaluronate prepared from human umbilical cord by the method of Blix [2] were dissolved in 1 litre $M/6.5$ phosphate buffer solution of pH 7. The solution thus prepared was stored in the refrigerator.

Enzyme solution. Unless otherwise stated, use was made of hyaluronidase extracted from the bull's testes and purified by the method of Hahn [3]. The stock solution was kept in the refrigerator, and dilutions were prepared with isotonic saline once a week for laboratory use.

Preparation of the test substances. The substances to be tested for inhibition of hyaluronidase activity were dissolved in diluted sodium hydroxide solution, and the pH was adjusted to 7. The solutions were made isotonic by the addition of saline.

Method of determination of inhibitory power. The estimations were made by the method described in the preceding paper. In the present assays a product obtained by the condensation of gentisic acid with formaldehyde (compound 7) was used as standard. The relative inhibitory power of this compound was 1 200 (resorcinol = 1) *.

Toxicity **. The test substance was emulsified in an aqueous solution of gum arabic and sugar. The concentration of the emulsion varied from 2 per cent to 30 per cent according to the size of the dose used. Single doses of 0.01—0.05 ml of the emulsion per g bodyweight were given by stomach tube to female mice weighing 20—24 g. Six dosage levels of every substance were studied. Each dosage level was tested on 6 mice. The animals were then closely observed for one week. When death occurred it usually happened within 48 hours. The LD_{50} values were determined by the Behrens method, as modified by Kärber [4] and expressed in mg/g.

RESULTS

As mentioned in the preceding paper, the inhibitor built up of gentisic acid units was the most powerful of the condensation products of diphenylmethane type studied. Therefore, when studying the effect that modifications of the conditions under which hydroxy benzoic acids are condensed with formaldehyde might exert on the inhibitory power of the resultant product, gentisic acid was chosen as a test substance. Observations made in these trials showed that the inhibitory power of the condensation products within certain limits varies with the ratio between the amount of gentisic acid and formaldehyde entering the reaction. Thus, when 4 parts of gentisic acid were allowed to react on the boiling water bath for five hours with 1 part of 40 per cent formaldehyde (molecular ratio 1 : 0.5) in the presence of 20 parts of 50 per cent (v/v) sulfuric acid, the inhibitory power of the substance obtained was 1 200 rel. units, while the corresponding value of a product prepared with twice the amount of formaldehyde but under otherwise identical conditions was 2 000. This last-mentioned condensation product (compound 7 P, "Digentisic acid") consisted mainly of compounds of high molecular weight, as demonstrated by dialysis of a solution of its sodium salt through cellophane at pH 7, when only about 15 per cent passed through the membrane. A further increase of the amount of formaldehyde was found to have no appreciable effect on the activity of the product.

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* *Cf* Table 2 in the preceding article.

** The toxicity tests were carried out by Dr. M. Fabinyi-Szebehely.

Table 1. Hyaluronidase-inhibiting power of polycondensed diphenylmethane derivatives

Compound condensed with formaldehyde in the molecular proportion of 1 : 1	Condensation product Code name	No.	Hyaluronidase inhibiting power in relative units (resorcinol = 1)*	Acute oral toxicity in mice LD_{50}
Salicylic acid	"Disalicylic acid"	23	220 (50)	4.8
p-Hydroxy-benzoic acid	"Di-*p*-hydroxy benzoic acid"	2 P	400 (380)	4.5
Gentisic acid	"Digentisic acid"	7 P	2 000 (1 200)	10.0
Protocatechuic acid		22	1 120	10.0
α-Resorcylic acid		8 P	800 (760)	
β-Resorcylic acid	"Di-*β*-resorcylic acid"	9 P	560 (400)	
γ-Resorcylic acid	"Di-*γ*-resorcylic acid"	19 P	1 700 (980)	4.6
Gallic acid	"Digallic acid"	1 P	350 (250)	
2,3,4-Trihydroxy benzoic acid		63	510	
2,4,6-Trihydroxy benzoic acid		3 P	900 (780)	
2,4,5-Trihydroxy benzoic acid		58	620	
Vanillic acid		24	360	
Hydroquinone	"Dihydroquinone"	27	—	
Pyrogallol	"Dipyrogallol"	25	—	
Resorcinol	"Diresorcinol"	26	—	
Phloroglucinol	"Diphloroglucinol"	12 P	—	

* Bracketed figures denote the inhibitory power of the corresponding product obtained on condensation with formaldehyde in the molecular ratio of 1 : 0.5.

These results prompted us to condense other hydroxy benzoic acids with formaldehyde in the molecular proportion of 1 : 1 under conditions otherwise identical with those described in the preceding article. The products thus obtained consisted to a great part of high molecular compounds. The condensation product of *β*-resorcylic acid and formaldehyde (compound 9 P), for example, showed a content of 79 per cent non-dialyzable substance and the corresponding figure for the condensation product of *p*-hydroxy benzoic acid (compound 2 P) was found to be 63 per cent. As a rule the inhibitory powers of these products were appreaciably higher than those of the products prepared by condensation of the corresponding hydroxy benzoic acid with formaldehyde in a molecular ratio of 1 : 0.5 (Table 1). Condensation in proportions $1 : > 1$ did not yield products of still higher activity. Some phenols were also condensed with formaldehyde in a molecular proportion of 1 : 1. None of the products thus obtained were soluble enough to permit any estimation of their inhibitory power *in vitro*.

Polycondensed products built up of triphenylmethane units were prepared by oxidation of the above-mentioned polycondensed diphenylmethane derivatives in the presence of a phenol or a hydroxy benzoic acid with nitrous acid. Use was made of the method described in the preceding article. The inhibitory powers of all of the substances tested are summarized in Table 2. Many of these compounds possess a higher inhibitory power than any substance hitherto described. The most active product was compound 21 P ("Trigentisic acid").

The triphenylmethane derivatives listed in Table 2 differ from one another by the number and position of the hydroxy and carboxy groups and presumably by the degree of condensation. This applies to the diphenylmethane derivatives, too (Table 1).

As yet the exact chemical formulas of these condensation products of diphenylmethane and triphenylmethane type cannot be given. The first-mentioned products may be conceived as long chain molecules built up of substituted benzene rings linked together by methylene groups. Except for the condensation products of trihydroxy benzoic acids these chains may also be branched. As to the structure of the triphenylmethane derivatives described here, it must be borne in mind that when building up triphenylmethane molecules by the introduction of a radical into the methylene bridge of diphenylmethane molecules, the nuclei of the diphenylmethane molecules themselves may compete with the phenol or carboxyphenol molecules.

Observations hitherto made in the present material do not allow of any definite conclusions regarding the possible relationship between the number and position of the substituents in the aromatic nuclei of the condensation products and the inhibitory activity of these substances. The most active diphenylmethane derivatives are the condensation products of dihydroxy benzoic acids. The most powerful condensation products of triphenylmethane type have 5—7 hydroxy groups per triphenylmethane unit. This suggests that the number of hydroxy groups is of importance for the inhibitory power of the compounds. Furthermore, the diphenylmethane and triphenylmethane derivatives built up of salicylic acid possessed but weak inhibitory power and were much less active than the corresponding derivatives of *p*-hydroxy benzoic acid. This indicates that the inhibitory power of the products may vary with the position of the hydroxy groups. This suggestion is supported by the difference observed between the inhibitory power of corresponding condensation products of different dihydroxy benzoic acids.

Work is in progress at this laboratory [5] to clear up the question whether the degree of condensation influences the inhibitory power of the condensation products described here. Suffice it here to mention that an increase in the quantity of formaldehyde in the condensation of phenols or hydroxy benzoic

Table 2. Hyaluronidase-inhibiting power of triphenylmethane derivatives obtained on reaction of a polycondensed diphenylmethane derivative with a phenol derivative in the presence of an oxidizing agent.

Diphenylmethane derivative	Phenol derivative	Condensation product No.	Hyaluronidase inhibiting power in relative units (resorcinol = 1)	Acute oral toxicity in mice LD_{50}
"Disalicylic acid"	Salicylic acid	35	< 200	
"Di-*p*-hydroxy-benzoic acid"	*p*-Hydroxy benzoic acid	20 P*	1 000	2.7
"Di-*p*-hydroxy-benzoic acid"	Gentisic acid	36	1 550	6.3
"Di-*p*-hydroxy-benzoic acid"	β-Resorcylic acid	38	1 300	3.8
"Di-*p*-hydroxy-benzoic acid"	Gallic acid	37	2 000	5.6
"Di-*p*-hydroxy-benzoic acid"	Hydroquinone	39	1 100	9.0
"Di-*p*-hydroxy-benzoic acid"	Phloroglucinol	40	2 100	18.0
"Digentisic acid"	Salicylic acid	28	2 100	8.7
"Digentisic acid"	*p*-Hydroxy benzoic acid	29	1 950	> 20.0
"Digentisic acid"	Gentisic acid	21 P**	2 500	> 20.0
"Digentisic acid"	β-Resorcylic acid	31	2 250	6.5
"Digentisic acid"	Gallic acid	30 ***	2 200	12.5
"Digentisic acid"	Resorcinol	32	1 650	> 20.0
"Digentisic acid"	Hydroquinone	33	1 900	18.0
"Digentisic acid"	Phloroglucinol	34	1 600	19.5
"Di-β-resorcylic acid"	*p*-Hydroxy benzoic acid	42	2 000	4.6
"Di-β-resorcylic acid"	Gentisic acid	41	1 900	4.4
"Di-β-resorcylic acid"	β-Resorcylic acid	43	1 300	3.5
"Di-β-resorcylic acid"	Gallic acid	16 P ****	1 300	5.4
"Di-β-resorcylic acid"	Hydroquinone	45	2 300	4.5
"Di-β-resorcylic acid"	Phloroglucinol	44	2 200	5.0
"Di-γ-resorcylic acid"	Gentisic acid	59	2 350	3.8
"Di-γ-resorcylic acid"	γ-Resorcylic acid	60	1 900	4.0
"Di-γ-resorcylic acid"	Hydroquinone	61	1 800	
"Di-γ-resorcylic acid"	Phloroglucinol	62	2 300	5.0
"Dipyrogallol"	Salicylic acid	53	1 350	
"Dipyrogallol"	*p*-Hydroxy benzoic acid	54	650	
"Dipyrogallol"	Gentisic acid	51	700	
"Dipyrogallol"	β-Resorcylic acid	52	1 700	18.5
"Dipyrogallol"	Gallic acid	50	2 000	17.5
"Diresorcinol"	Gallic acid	47	300	
"Dihydroquinone"	Salicylic acid	55	300	
"Dihydroquinone"	Gentisic acid	56	250	
"Dihydroquinone"	β-Resorcylic acid	57	480	
"Diphloroglucinol"	*p*-Hydroxy benzoic acid	17 P	700	
"Diphloroglucinol"	Gentisic acid	48	750	
"Diphloroglucinol"	β-Resorcylic acid	49	1 650	6.0
"Digallic acid"	β-Resorcylic acid	46	100	

* "Tri-parahydroxy-benzoic acid"
** "Trigentisic acid"
*** "Digentisic-gallic acid"
**** "Di-β-resorcylic-gallic acid"

acids, which appears to favour the formation of products of high molecular weight, resulted in the formation of more active diphenylmethane and triphenylmethane derivatives. In this connection it should perhaps be pointed out that the number and position of hydroxy groups may influence the mechanism of the condensation and thereby also the degree of polycondensation.

Inhibition of staphylococcal hyaluronidase. Whether the compounds described above inhibit hyaluronidase derived from sources other than mammalian testes is a question of great importance in the investigation of the mechanism by which hyaluronidase of varying origin breaks down hyaluronic acid and for defining the range of indications for these inhibitors in the clinic. The inhibitory action of some of the compounds here described on hyaluronidase obtained from different strains of pathogenic bacteria will be the subject of a later paper. It may be sufficient here to mention that hyaluronidase obtained from a strain of *Staphylococcus pyogenes* was inhibited to roughly the same extent as testis hyaluronidase. For example, compound 21 P was investigated in a concentration which in our test with testis hyaluronidase gives a fivefold prolongation of the half-viscosity time. Using staphylococcus hyaluronidase under strictly identical conditions a 3.5 fold prolongation was obtained. The corresponding figures for compound 7 P and 20 P were 3.6 and 2.1 respectively.

Toxicity. The products with the most pronounced inhibitory power were tested for acute oral toxicity in mice (Tables 1 and 2). 8 of the compounds showed LD_{50} values above 15 mg/g, compound 21 P and two related compounds being the least toxic. The remarkably low toxicity of some of these substances is important to the possible clinical value of the hyaluronidase inhibitors mentioned in the preceding paper. A more detailed study of the toxicity of compound 21 P and some other compounds as well as of their inhibitory action on hyaluronidase *in vivo* is the subject of another paper [6]. The results of a clinical trial with compounds 7 P, 16 P and 21 P in rheumatoid arthritis have been published elsewhere [7].

SUMMARY

A further study was made on the inhibition of testis hyaluronidase by diphenylmethane and triphenylmethane derivatives with hydroxy and carboxy groups in the nuclei.

A number of mono- and polyhydroxy benzoic acids were condensed with formaldehyde in the molecular ratio of 1 : 1. Dialysis of the diphenylmethane derivatives obtained showed that they mainly consist of high molecular polycondensation products. They possessed higher inhibitory power than the corresponding products obtained by condensation in the molecular ratio of

1 : 0.5 as described earlier. The reaction product of gentisic acid with formaldehyde, compound 7 P ("Digentisic acid"), was found to be most powerful (relative inhibitory activity: 2 000; resorcinol = 1).

Polycondensation products built up of triphenylmethane units were produced by the oxidation of polycondensed diphenylmethane derivatives with nitrous acid in the presence of a phenol or a hydroxy benzoic acid. Altogether 38 substances of triphenylmethane type were synthesized and examined for their inhibitory action on hyaluronidase *in vitro*. Of these, 9 possessed an inhibitory power of more than 2 000 rel. units. Compound 21 P ("Trigentisic acid"), a product obtained by oxidizing compound 7 P in the presence of gentisic acid, proved most active with an activity of 2 500 relative units.

Compounds 21 P, 7 P and 20 P ("Tri-parahydroxy benzoic acid", a *p*-hydroxy benzoic acid analogous to compound 21 P) were also tested for inhibitory effect on hyaluronidase from a strain of *Staphylococcus pyogenes*. The inhibitory action found was roughly the same as on testis hyaluronidase.

The acute oral toxicity in mice of the most active polycondensed diphenylmethane and triphenylmethane derivatives was assessed. Compound 21 P and two closely related derivatives, obtained by the oxidation of compound 7 P in the presence of *p*-hydroxy benzoic acid and resorcinol respectively, proved least toxic ($LD_{50} > 20$ mg/g body weight). The LD_{50} values of further 5 triphenylmethane derivatives exceeded 15 mg/g.

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On the Thermodynamics of Interfaces and Its Application to Detergency

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1. DETERGENCY

One of the fields in which interfacial phenomena play a great role in practice is detergency, for instance of clothes (laundering) or of glass and porcelain (dish washing). In the production of detergents the problem arises to evaluate their efficiency, and this problem led to the following considerations.

There seems to be to some extent a general inclination to regard a surface tension-concentration curve combined with an analysis of the product for contents of "active" substance as a sufficient basis for an evaluation of the mentioned nature, the word surface being taken to mean the interface against air. The quantity which is essential to accomplish detergency must, however, be the mechanical work which must at the temperature of detergency and atmospheric pressure be done upon the system if the process is carried through reversibly. Perhaps this is expressing it rather schematically, but if we confine ourselves to consider dish washing (where the soil at detergency temperature can practically be considered liquid) *, such factors as the possible deleterious effect of the detergent on the object of detergency will not influence the evaluation, and the detergent permitting detergency to be carried out completely involving the smallest amount of work must be said to be best.

After these considerations it would be natural to resort to Dupré's expression for adhesion work.**

We shall consider a cylinder or other body built up of two phases 1 and 2, and surrounded by a third, 3 (Fig. 1). 3 may be a detergent, 2 fat, and 1 porce-

* This assumption is mentioned with a view to the following considerations.

** The writer has planned to discuss the use of this expression more in detail in another paper.

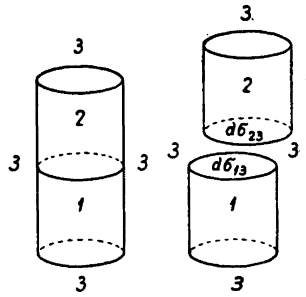

Fig. 1 A. *Fig. 1 B.*

lain. If 1 and 2 are separated, the interface 12 disappears, and new interfaces 13 and 23 appear. Using the terms γ for interfacial tension, σ for interfacial area with pertinent indices, and in putting $-d\sigma_{12} = d\sigma_{13} = d\sigma_{23} = 1$ and W_A = adhesion work, we obtain according to a paper from 1869 by Dupré

$$W = -\gamma_{12} + \gamma_{13} + \gamma_{23} \tag{1}$$

For phase 1 = phase 2 we have specifically adhesion work = cohesion work = $2\gamma_{13}$ $(= 2\gamma_{23})$.

The use of the expression requires some comments as it will appear from the following. First it might, however, be said that in laundering, which takes longer time in practice than dish washing, the overcoming of adhesion is not the sole object, as in the former case redeposition of the soil must also be prevented. But dish washing will not allow time enough for redeposition phenomena to occur. The ideal case would be $W_A < 0$ as 1 and 2 would then be separated simply by being placed in the medium 3. In that case redeposition on the object of detergency would not occur. The important factor in evaluation would then be the time required for the process of separation.

If values for $-W_A$ are used as a measure of the "efficiency", difficulties are immediately encountered. The reason is that it is not surface tensions which are involved, but on the contrary interfacial tensions liquid-liquid and liquid-solid. Surface tensions and tensions liquid-liquid can be measured directly, but for tensions liquid-solid no general methods are stated in the literature. In the following it will be shown that at any rate in principle it will be possible to measure the quantity W_A.

2. ON THE POSSIBILITY IN PRINCIPLE TO MEASURE THE ADHESION WORK

Equation (1) includes the tensions in interface 13 and 12, where 1 is the solid phase; but they are included as a difference which it will be possible in principle to measure.

We shall consider a drop of 2 in equilibrium on a plane layer of 1. (Fig. 2). 1 and 2 are surrounded by 3. A virtual displacement during which the contact angle $= \Theta$ and the volume of the drop is kept constant (constant volume in order to avoid a spatial contribution to the work) gives at once the known condition of equilibrium stated by Young (1805)

$$\cos \Theta = \frac{\gamma_{13} - \gamma_{12}}{\gamma_{23}} \tag{2}$$

This relation can be used for our purpose. For the sake of clearness we shall designate

Phase 1 by P (porcelain)
Phase 2 by O (oil)
Phase 3 by D (detergent) or when phase 3 is gaseous Phase 3 by G (gas).

Furthermore, Θ^{c}_{ab} is the contact angle between phase a and phase b, a and b being surrounded by c.

(2) now gives the following relations for O-drops in equilibrium on plane P-layers:

$$\cos \Theta^{G}_{PO} = \frac{\gamma_{PG} - \gamma_{PO}}{\gamma_{OG}}$$

$$\cos \Theta^{G}_{PD} = \frac{\gamma_{PG} - \gamma_{PD}}{\gamma_{DG}}$$

whence we obtain if P has been surrounded by the same G-phase during the two determinations so that γ_{PG} is the same

$$\gamma_{PD} - \gamma_{PO} = \gamma_{OG} \cos \Theta^{G}_{PO} - \gamma_{DG} \cos \Theta^{G}_{PD} \tag{3}$$

With the new symbols (1) is written

$$W_A = -\gamma_{PO} + \gamma_{PD} + \gamma_{OD}$$

which combined with (3) gives

$$W_A = \gamma_{OG} \cos \Theta^{G}_{PO} - \gamma_{DG} \cos \Theta^{G}_{PD} + \gamma_{OD}$$

in which W_A is expressed by the contact angles for O and D-drops against plane P-layers and easily measurable tensions (liquid-liquid and liquid-gaseous).

C_1 and C_2 denoting measurable constants dependent on oil and detergent, respectively, and both dependent on porcelain, we can write

$$W_A = \gamma_{OG} C_1 - \gamma_{DG} C_2 + \gamma_{OD}$$

For a given oil and a given object of detergency it is thus possible to express the adhesion work by the surface tensions of oil and detergent and their interfacial tension and C_1 and C_2.

From experience we know that the ordinary detergents wet glass and porcelain particularly well so that in general we shall have small values for Θ^{G}_{PD}. With approximation we shall thus be able to put $C_2 = 1$, and we then obtain

$$W_A = \gamma_{OG}\, C_{PO} - \gamma_{DG} + \gamma_{OD} \qquad (4)$$

C_1 being designated C_{PO} because it is dependent on the natures of O and P.

It is hardly feasible to eliminate C_{PO} from the expression, as this would indicate that all kinds of oil and porcelain (and glass) were having the same contact angle against gas (air). In other words: If it is wanted to evaluate a detergent, W_A must be found for a number of representative kinds of O and P, which means that one figure for W_A will not suffice. This procedure would involve a differentiation between detergents suited for washing certain objects of detergency. The O-kinds may be considered fairly constant. If this latter assumption holds, (4) can be written

$$W_A = C_P - \gamma_{DG} + \gamma_{OD}\,,$$

where C_P is a constant only dependent on the nature of the object of detergency. The members at disposal in (4) are at any rate γ_{DG} and γ_{OD}, which can be varied through choice of detergent and its concentration.

From the above it will be seen that it will be advantageous for the separation of P and O to choose γ_{DG} high (which is probably contrary to the most usual view in practice) * if at the same time γ_{OD} can be low. Here the problem arises whether there is any relationship between γ_{DG} and γ_{OD} or, more in general, between γ_{OG}, γ_{DG} and γ_{OD}. In that case W_A could be rewritten so that it contained only *surface* tensions. The question whether it is possible from the *surface* tensions of two liquids to compute their interfacial tension has occupied many research workers in the course of time. The assumption that such a relation exists is based by its supporters on a paper from 1907 by Antonoff [1].

This relation which is usually called Antonoff's rule will now be discussed.

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* In practice there is a tendency to consider only the wetting of P and O, *i. e.* replacement of the surrounding gaseous phase by D, and making γ_{DG} only responsible for this process, which is not in the present paper regarded as any important problem.

3. ANTONOFF'S RULE

Antonoff's rule is as follows:

$$\gamma_{AB} = \gamma_A - \gamma_B \tag{5}$$

where γ_{AB} is the interfacial tension between the two liquids A and B with surface tensions γ_A and γ_B. From a formal viewpoint this seems immediately objectionable; for when $\gamma_{AB} = \gamma_A - \gamma_B$, we must have $\gamma_{BA} = \gamma_B - \gamma_A$, but γ_{AB} and γ_{BA} are identical quantities. In other words (5) is wanting in symmetry with respect to the way in which the surface tensions of the two liquids are included in the equation. For example the question can be put: Is $\gamma_{OD} = \gamma_{DG} - \gamma_{OG}$ or $\gamma_{OD} = \gamma_{OG} - \gamma_{DG}$?

In the former case (4) can be written

$$W_A = \gamma_{OG}(C_{PO} - 1) , \tag{6}$$

which would be a convenient solution to the problem of evaluating detergents, as it would mean that they are all equally good and equally good in large concentration intervals (or practically equally good, equation (4) being based upon $\Theta^G_{PD} \sim 0$).

As it is, however, known from experience that $\gamma_{DG} > \gamma_{OD}$ and $\gamma_{OG} > 0$, (6) must be a conclusion from Dupré's and Antonoff's expressions; for $-\gamma_{OG}$ must be negative, and the expression $\gamma_{OD} = \gamma_{OG} - \gamma_{DG}$ would give absurdly high values for γ_{OG}, *viz.* $\gamma_{OD} + \gamma_{DG}$. If for example D = water, the result would be obtained that the surface tension of oil were higher than that of water. This result is evidently inconsistent with experience *.

To the above it may at once be said that Antonoff states that his rule will only give correct results if certain conditions are met: Phase A must be saturated with B and vice versa. In other words the system must be in chemical equilibrium, and the mutual solubility may change the values inserted in (5) materially. We can illustrate this fact with an example. In the literature we find

$$\left.\begin{array}{ll} \gamma_{\text{water, air}} = 72.8 & \gamma_{\text{benzene, air}} = 28.9 \\ & \gamma_{\text{water, benzene}} = 35.0 \end{array}\right\} \text{measured in dynes/cm at } 20^\circ \text{ C.}$$

But if we form $\gamma_{\text{water, air}} - \gamma_{\text{benzene, air}}$, we obtain $\gamma_{\text{water, air}} - \gamma_{\text{benzene, air}} = 43.9$, which is inconsistent with the measured value.

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* As an example we have at 20° C

$\gamma_{\text{water, air}} = 73$ dynes/cm; $\gamma_{\text{oleic acid, air}} = 32.5$ dynes/cm;

$\gamma_{\text{water, oleic acid}} = 15.6$ dynes/cm.

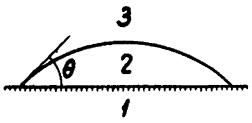

Fig. 2

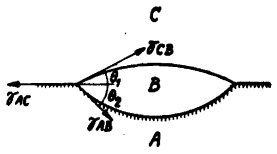

Fig. 3

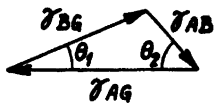

Fig. 4

But Antonoff saturates water and benzene mutually, and for the aqueous solution A he finds [1]

$$\gamma_{AG} = 70.6 \text{ against air}$$
$$\gamma_{AG} = 60.0 \text{ against vapours}$$

and

$$\gamma_{BG} = 28.2 \text{ (B = "benzene phase")}$$

and the rule holds beautifully when we choose γ_{AG} against vapours.

On the basis of corresponding measurements for the systems *iso*butylalcohol-water, *iso*amylalcohol-water, ether-water and chloroform-water Antonoff sets forth his rule as a general rule, on condition that regard is being had to establishment of equilibrium. For the system ether-water he even takes measurements at three temperatures: 14° C, 20° C, 24° C. But he omits to point out that the rule only holds at 14° C. This fact (in the opinion of the writer) invalidates the rule. A "rule" which holds only at a random temperature — or rather a temperature dependent on the system but not predictable — is no rule.

Finally, from a theoretical standpoint there is no reason why the rule should hold exactly at chemical equilibrium; for the conditions of equilibrium can never involve any relationship between the quantities included in (5). Let us consider a system consisting of a liquid phase A with a drop of B on the surface, all surrounded by a gaseous phase C (Fig. 3). With respect to the *mechanical* stability of the system we have the alternative: Either the drop represents a stable form of equilibrium, or stable mechanical equilibrium will not occur until B has spread over the surface AC. If in the latter case the drop is spreading in a layer of a certain thickness, the final result will be such a layer and B-surplus in drop form, *e.g.* monomolecular films [2].

In the former case we can find the mechanical conditions of equilibrium by making virtual displacements corresponding to those mentioned in the description of Fig. 2. But illustrating the case more clearly, we shall obtain them by observing that γ_{AC}, γ_{CB} and γ_{AB} must form a closed polygon of forces (triangle of forces) as shown in Fig. 4, where γ_{AC} is designated γ_{AG} and

γ_{BC} is designated γ_{BG}, while C = gas (air). It appears clearly that here γ_{AB} cannot be $= \gamma_{AG} - \gamma_{BG}$. By a calculation it is at once seen from the cosine relations, which give

$$\gamma_{AB}^2 = \gamma_{AG}^2 + \gamma_{BG}^2 - 2\,\gamma_{AG}\,\gamma_{BG}\,\cos\Theta_1$$
$$\gamma_{BG}^2 = \gamma_{AB}^2 + \gamma_{AG}^2 - 2\,\gamma_{AG}\,\gamma_{AB}\,\cos\Theta_2 \qquad (7)$$

whence by inserting $\gamma_{AB} = \gamma_{AG} - \gamma_{BG}$ we obtain

$$\cos\Theta_1 = 1 \qquad \cos\Theta_2 = 1$$
$$\Theta_1 = 0 \qquad \Theta_2 = 0$$

This mean that the triangle and consequently the drop cannot exist in a stable form, which we assumed. Thus Antonoff's rule cannot hold in this case, and it should be noted, neither at chemical equilibrium nor at chemical non-equilibrium; for if the system begins in chemical non-equilibrium with a mechanically stable drop and ends in chemical and mechanical equilibrium with a drop, a triangle of forces must exist at the initial as well as the final states, it must be admitted with triangle sides varying from beginning to end.

(7) shows that γ_{AB} must in this case be higher than determined by Antonoff's rule.

If the other possibility occurs, *i.e.* that the drop is spreading, neither in that case will there be any reason why (5) should hold. For spreading does not require $\cos\Theta_1 = 1$ and $\cos\Theta_2 = 1$ as a necessary condition. The latter is only a specific case, and in this case Antonoff's rule will hold, but it can never be known beforehand whether this specific case is occurring. The condition for using the cosine relations was mechanical equilibrium with drop, and when the condition is not fulfilled, the cosine relations cannot be applied to the phenomenon.

Finally it should be mentioned that when we do not find higher but nearly always lower values for γ_{AB} than supposed by Antonoff's rule, it is due to the fact that as a rule the drop is not in equilibrium with the interface AC proper, but with a spread surface layer on the latter, and the surface layer is so thin that its tension is a special film tension and not γ_{AC}. This phenomenon is dealt with in detail by Harkins [2].

With regard to an application of Antonoff's rule to oil-detergent (particularly oil-pure water) solutions, the example mentioned in the footnote on p. 817 shows clearly that for non-equilibrium values it does not hold. Moreover, as the mutual solubility of oleic acid-water is very slight, it probably will not hold if the liquids are mutually saturated.

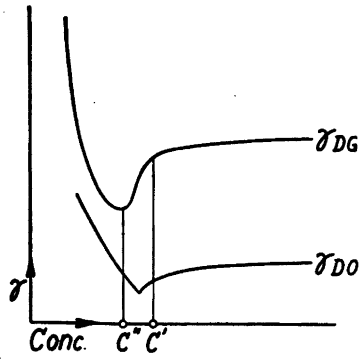

Fig. 5 A

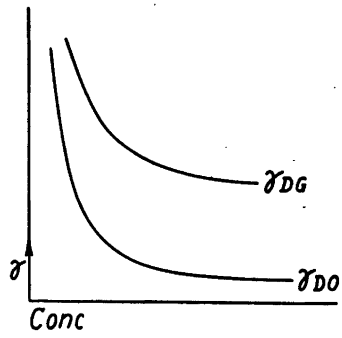

Fig. 5 B

4. EVALUATION OF D BASED ON THE PRECEDING CONSIDERATIONS

In the detergency literature many examples are found of surface tension and interfacial tension-concentration curves, *e.g.* in Niven's book on the subject [3]. It appears that γ_{DG} and γ_{OD} curves for the various known detergents do not show great variations, and the possibilities of variation through the nature of detergents in accordance with (4), *i.e.* high γ_{DG} and low γ_{OD} do not seem as large as might have been expected.

Figures 5 a and b indicate a general course of such tension-concentration-curves *. It is, however, an open question whether these curves can be of any assistance in evaluating which concentration should be chosen. As Dupré's formula applies to reversible separation, it requires equilibrium values for the γ's.

But if importance is attached to the curves on the assumption that mutual solubility is slight, it is interesting that in view of the preceding considerations concentration c'', Fig. 5 a, should not be chosen for detergents having the represented course of the curve. c'' corresponds to the lowest surface tensions, and is consequently the concentration considered to be best in practice. As we should, however, have γ_{DG} as high and γ_{OD} as low as possible, c' must be chosen. However, c'' and c' are not very far apart; but it is also remarkable that the detergency curve in Fig. 14—2, page 232 in Niven's book [3] *reaches its maximum at* c' after which it remains practically level, and that *a major part of the steep increase of the curve occurs in the interval from* c'' *to* c'.

* The writer has seen such tension-concentration curves verified by measurements against air with du Noüy's tensiometer.

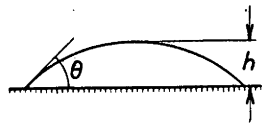

Fig. 6

Finally it is evident that if the values from such curves are required together with γ_{OG} against air to satisfy Antonoff's rule, the latter is invalidated, as $\gamma_{DG} - \gamma_{DO} = \gamma_{OG} =$ constant according to the rule.

With regard to the possibility of measuring the contact angle Θ it is a condition that no phenomena of hysteresis occur for this angle, Θ must have the same value when the drop of O or D is brought to a state of equilibrium by spreading and by contraction.

The actual measurement is easily carried out by microphotography of the drop from the side. Such measurements are often described in the literature, *e.g.* by Talmud and Lubman [4] for a drop surrounded by a liquid phase resting on a plane solid phase.

The writer has, however, tried a method which is not to be found in the literature, and which only requires a very simple apparatus.

With a Carlsberg pipette a small drop of known volume corresponding to that of the pipette is slowly blown on to a plane P layer which is placed horizontally. Such small drops (10—40 mm^3) may with good approximation be considered spherical segments of one base. As we have one of the drop's geometric parameters, *viz.* volume *, only one more is required for determination of the configuration. For this purpose the maximum height above the P-layer was chosen. With a spherometer ** this height can be measured with the accuracy of a few per cent. The method only requires little time and cheap apparatus, but with regard to accuracy it is inferior to microphotography. It may, however, give better values than the »tilting plate-method», where a P-plate is tilted 90°—contact angle until the meniscus adjoins the latter horizontally. h being the maximum height, v being volume of the drop, and Θ being the contact angle sought (Fig. 6), we find

$$\cos \Theta = \frac{v - 2/3\pi h^3}{v + 1/3\pi h^3} \tag{8}$$

* There is actually an additional pressure in a drop, but a calculation on the basis of the dimensions occurring in such measurements compared with the order of magnitude of the compressibility coefficients of liquids shows that the compression will have no measurable influence on volume.

** We get a very sharp indication that the screw of the spherometer touches the drop in that as soon as they touch, the drop changes its form and is spreading over the end of the screw.

For small Θ (small h), the members including h^3 will be insignificant compared to the v which can be produced with Carlsberg pipettes, and sin Θ must be used in an approximated form. r being the radius in the base of the spherical segment of one base, we obtain

$$\sin \Theta = \frac{2rh}{r^2 + h^2}$$

and

$$r^2 = \frac{2v}{\pi h} - \frac{h^2}{3}$$

For small h the latter may be approximated

$$r^2 = \frac{2v}{\pi h}$$

and hence

$$\sin \Theta = \frac{\sqrt{2v\pi h^3}}{v + \frac{h^2}{2}} \tag{9}$$

which may again for very small h be approximated

$$\sin \Theta = \sqrt{\frac{2\pi h^3}{v}} \tag{10}$$

For an increasing contact angle equations (10), (9) and (8) are to be used successively.

The following can be stated as an example of such measurements:

Sheets of plate glass were cleaned by standing in a potassium dichromate-sulphuric acid solution for 24 hours, whereupon they were rinsed in tap water, distilled water, alcohol and ether in the succession mentioned.

Drops of distilled, boiled water were placed on the sheets with a Carlsberg pipette of 40 mm^3.

Five experiments were made, at the beginning of each five drops were measured, and h varied in the interval 0.37 mm $\leqq h \leqq$ 0.40 mm. Thus it seems that we have here a well defined contact angle *. The measurements were made at 18° C—20° C and against air.

The sample sheets remained in the same room, and measurements on new drops were made regularly and frequently. In all cases the initial value proved to last only a few minutes, and h increased at a decreasing rate, until after

* This contact angle is not given, because correction was not made for appendage to the pipette at these preliminary investigations. For each new liquid such a correction must be made by weighing the pipette when filled and after the drop has been blown out in order to ascertain the volume of liquid which remains in the pipette.

about two hours h was found to be in the interval 0.96 mm $\leqq h \leqq$ 100 mm. This value was found to be the same after 24 hours.

Therefore, measurements made in the air of an ordinary room must be made very quickly after cleaning of the glass sheet as the character of its surface evidently changes quickly, the surface probably being coated with a film of air, water vapour and dust. Since final value as well as initial value seem reproducible in this case, both are of interest.

Corresponding measurements were tried with a Carlsberg pipette of 80 mm^3, and it was found that h of drops from this pipette, h_{80} were related to h_{40} in the ratio $\sqrt[3]{\frac{80}{40}}$, which shows that the drops of water have been uniform, and that gravity has not influenced the configuration even of drops having as large a volume as 80 mm^3.

On the other hand the reproducibility seemed less good for drops of peanut oil and solutions of sulphonated products, and certain observations seemed to indicate phenomena of hysteresis. However, a systematic investigation according to the principles outlined might be of interest.

It has been shown above that measurements of such capillary-chemical quantities as interfacial tensions and contact angles can at any rate in principle be used for solution of the practical task of evaluating detergents.

Naturally this task could also be tackled from a different angle by simply carrying out dish washing on standard soiled plates in a fixed way, and research work on this principle is also done [5]. But it would save time in practice if an evaluation could be made on the basis of quantities measurable in a quick and exact way.

SUMMARY

1. The reversible isothermal work in Dupré's equation is put forward as a measure of the efficiency of detergents.
2. The conditions for use and measurement of this quantity are discussed.
3. Antonoff's rule is shown to be invalid.
4. A new, simple method for measurement of contact angles is described.

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Studies on Aspartase

I. Quantitative Separation of Aspartase from Bacterial Cells, and Its Partial Purification

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In 1926 Quastel and Woolf[1] described a deamination of aspartic acid to fumaric acid in the presence of resting cells of *Escherichia coli*. The enzyme responsible for this reaction, which was reversible, was called aspartase [2] and was found to be present in several facultative anaerobes [3]. Virtanen and Tarnanen [4] obtained a cell-free aspartase preparation by extracting dry bacteria of *Pseudomonas fluorescens* under toluene at 37° C (pH 7). This was the first enzyme to be separated from cells, that caused the synthesis of an amino acid. Attempts to prepare cell-free aspartase solutions from dry bacteria and from an autolyzed bacteria medium of *Propionibacterium* and *Lactobacillus casei* met with no success. From *Pseudomonas fluorescens* only a part of the original aspartase activity found in the dry bacteria could be recovered in solution.

It is a known fact that many enzymes are firmly bound to the cell structure. Different methods have been used in the attempt to break up the association between proteins and insoluble particles in order to obtain larger yields of enzyme, but they have had only a limited application. Recently, however, noticeable improvement seems to have been reached by using *n*-butanol as a reagent in the dissociation of the lipidprotein complex [5].

In the present investigation *n*-butanol has been used in preparing cell-free extracts from dry bacteria of *Pseudomonas fluorescens* and *Propionibacterium peterssonii*, whereby larger enzyme yields were obtained. A partial purification of the enzyme was carried out with this extract.

EXPERIMENTAL

Enzyme material. As enzyme preparation finely ground dry preparations of *Ps. fluorescens* (strain of the Biochemical Laboratory, Univ. of Helsinki) and *Propionibacte-*

rium peterssonii (strain of this laboratory) were used. In culturing *Ps. fluorescens* a nutrient solution of the following composition was employed:

100 l tap water 800 g meat extract-peptone powder (Bacto Nutrient Broth Dehydrated, Difco Laboratories U.S.A.) 300 g K_2HPO_4 100 g $MgSO_4 \cdot 7\ H_2O$	pH 7

The culture of the bacterial mass was performed in an aluminium vat containing 100 litres of nutrient solution at 18° C. After 48 hours the bacteria were harvested by means of a milk separator, and washed with tap water. The bacterial mass was dried on porous plates and the dry mass was ground to a dust-like powder.

The nutrient solution for *Propionibacterium* was prepared as follows:

50 litres of skim milk was coagulated at 35° C with rennet, heated to 96° C to destroy the enzyme, filtered and sterilized at 120° C. After chilling to 45° C the whey was inoculated with *Lactobacillus helveticus* (strain of this laboratory). The lactic acid produced was neutralized with sterilized chalk. The whey culture was incubated at 42° C until all the lactose was fermented to lactic acid, which was checked with the Fehling test. The incubation had to be continued for 5—6 days. The bacteria were then separated from the solution. To 50 litres of fermented whey 1 000 g pressed yeast and 100 g peptone were added. The mixture was heated to 120° C for 30 min. in an autoclave, and centrifuged. The pH was adjusted to 6.5 and the solution was portioned out into 8-litre Erlenmeyer flasks and sterilized at 120° C for 30 min. The culture of the bacterial mass was performed in these Erlenmeyer flasks for the first 20 h at 37° C and later at 25° C. After 45 h the bacteria were separated from the solution with an airdriven Sharples Supercentrifuge. The washed mass was dried on porous plates and ground to a dust-like powder.

Activity determinations. The activity of the preparations was determined by the rate at which aspartic acid was deaminated. The most rapid and convenient method for this purpose is the determination of ammonia.

The experiments were performed in 10 ml measuring cylinders, in which the following test solution was incubated at 37° C for 24 h:

100	mg	dry bacterial mass
26.6	mg	aspartic acid (in 2 ml, pH 7.2)
2	ml	phosphate buffer *M*/15 (pH 7.2)
6	ml	distilled water
10	ml	total volume (0.3 ml toluene was used as antiseptic)

When enzyme extracts were used instead of dry bacteria they were used in amounts corresponding to 100 mg dry bacterial mass.

During the incubation, 2 ml samples were taken for the determination of the liberated ammonia, which was distilled with an apparatus modified from that of Pucher *et al.*[6] The ammonia was trapped in the receiver by 0.01 *N* H_2SO_4, and the excess of acid was titrated iodometrically. The solution to be analyzed was made alkaline in the distillation flask with a sodium carbonate buffer (5 g Na_2CO_3 + 5 g NaCl in 100 ml distilled water) [7].

The rate of reaction is linear at first and then falls off as the substrate concentration decreases and the products are formed. The initial velocities when the reaction rate is

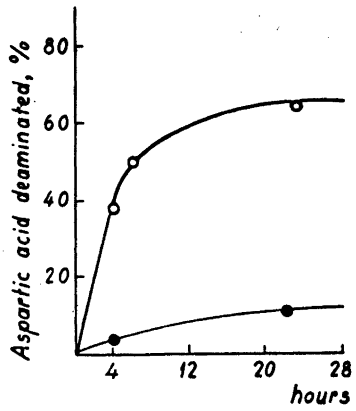

Fig. 1. Deamination of aspartic acid at 37° C by preparations of P s. fluorescens.

○ *dry bacteria*
● *corresponding amount of water extract of dry bacteria*

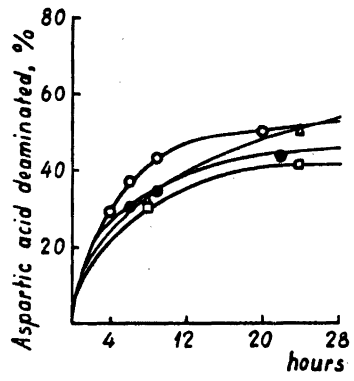

Fig. 2. Deamination of aspartic acid at 37° C by dry bacteria of P s. fluorescens. Bacteria suspension incubated at 37° C.

for 0 days ○
» *3* » ●
» *4* » □
» *5* » △

linear may be expressed as change in aspartic acid concentration per unit time, and as there is a direct correlation between liberated ammonia concentration and aspartic acid concentration, the initial velocity can be expressed in NH_3-N per hour in 10 ml.

Protein-N was determined by the micromodification of the Kjeldahl method. The ammonia was distilled by Klingmüller's method [8] into a receiver containing 10 ml of 0.01 *N* sulphuric acid and titrated iodometrically.

RESULTS

Preliminary experiments were performed according to Virtanen and Tarnanen [4] extracting the dry bacteria with distilled water at 37° C for 24 h. By determining the protein-N in the bacteria and in the extract, it could be shown that 38.3 % of the nitrogen in the dry bacteria was present in the cell extract. Fig. 1 illustrates the poor yield of active aspartase using this extraction procedure. The poor recovery of the active enzyme in the cell extract is not connected with any heat inactivation of the preparation during incubation at 37° C, since no loss of activity could be observed in a suspension of dry bacteria incubated at 37° C for several days, as is shown in Fig. 2.

No improvement in yield was obtained by using larger volumes of water or repeated extractions. Nor did the raising of the pH of the extraction medium have any effect on the extractability of the enzyme. The enzyme is probably

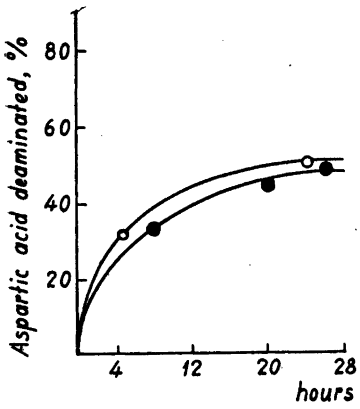

Fig. 3. Deamination of aspartic acid at 37° C by Ps. fluorescens preparations.

○ *dry bacteria*

● *corresponding amount of bacteria extract obtained by butanol treatment*

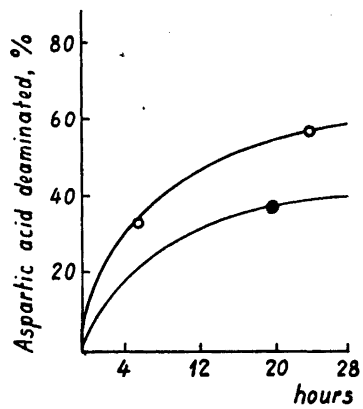

Fig. 4. Deamination of aspartic acid at 37° C by Propionibacterium petersonii.

○ *dry bacteria*

● *corresponding amount of bacteria extract obtained by butanol treatment*

associated with insoluble particles, and till this association is broken the enzyme cannot be extracted with water.

n-Butanol was now used in an attempt to obtain a better yield. The experiments were performed as follows: The dry bacteria were stirred thoroughly with *n*-butanol at a low temperature (0 to −2°) for 30 min. The bacteria were harvested by centrifuging at a low temperature (−2° C), and suspended in water at 0° C. The butanol was removed by dialysing against tap water of low temperature (+ 2° C). After 24 hours' dialysis the bacteria were centrifuged off and aspartase was obtained in a cell-free solution. On determining the protein-N in the dry bacteria and the extract obtained after butanol treatment, it was found that 48.2 % of the nitrogen in the dry bacteria was in the extract.

Fig. 3 shows the practically quantitative yield of active aspartase obtained after *n*-butanol treatment of dry bacteria of *Ps.* fluorescens and Fig. 4 illustrates the yield from *Propionibacterium* after the same treatment.

Precipitation. From *Pseudomonas* extract aspartase was precipitated by lowering the pH to 4.5 according to Virtanen and Erkama [9]. The precipitate was centrifuged off and dissolved in *M*/15 phosphate buffer, pH 7.2. The result of the procedure can be seen in Fig. 5.

With an extract of *Propionibacterium* no precipitation occurred on lowering the pH to 4.5. Thus, the precipitation seems to be specific to *Ps. fluorescens* extract. Fractionation with ammonium sulphate was tried. Solid ammonium

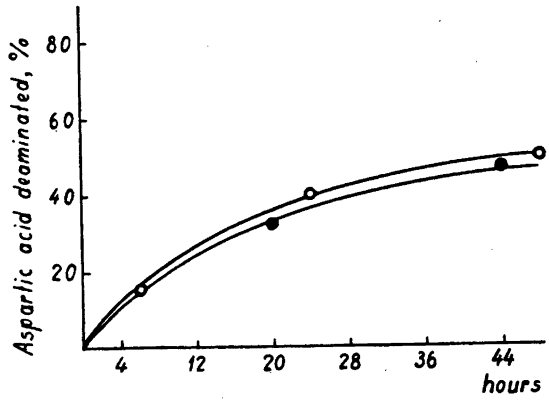

Fig. 5. Deamination of aspartic acid by aspartase preparations.
○ *bacteria extract (butanol treatment)*
● *corresponding amount of aspartase precipitated at pH 4.5*

sulphate was added to the enzyme extract to 6.5 % saturation. After 6 h at 0° the precipitate was centrifuged off. The ammonium sulphate saturation in the mother extract was raised to 30 %, and the extract set aside at 0° for 6 h. The precipitate was again separated, and the ammonium sulphate saturation in the solution was raised to 70 %, *etc.* Thus the extract was fractionated with ammonium sulphate in four fractions of different ammonium sulphate saturation. The different precipitates were dissolved in distilled water, dialyzed overnight against tap water and made up to the same volume. Accordingly, the determinations of the reaction velocities of the different fractions are comparable *inter se.*

Fig. 6 shows the distribution of enzyme activity as a function of ammonium sulphate saturation. Since the recovery of enzyme activity is very poor with ammonium sulphate precipitation (~ 10 %), several other salts were tried ($MgSO_4$, sodium citrate, sodium phosphate) but without any improvement in yield. On the contrary, sodium citrate, for example, gave completely inactivated precipitations though the pH during the precipitation was watched and adjusted to 7.0.

It was found that ethanol precipitation gave poor yields but acetone gave better results. To the enzyme solution was added 0.1 per cent of its volume of a phosphate buffer ($\mu = 0.1$, pH 7.1). The precipitations with acetone were carried out at 0° to −5° C. The precipitate between 28—45 % acetone by volume was taken and dialyzed against tap water for one night. 40 % recovery of the original activity was obtained.

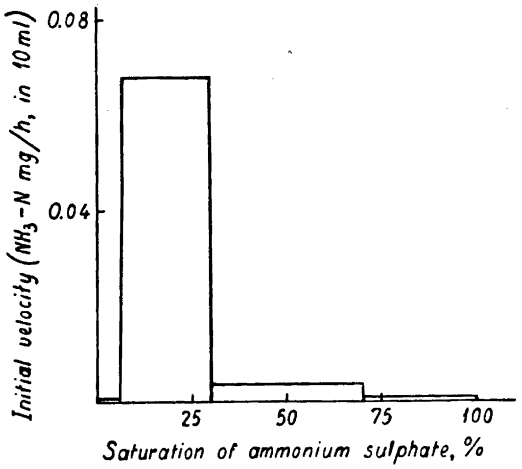

Fig. 6. Distribution of aspartase activity as a function of ammonium sulphate saturation.

DISCUSSION

The results show that aspartase is associated with insoluble lipids in the cell structure. *n*-Butanol, as a somewhat specific reagent in the dissociation of the lipid-protein complex, can successfully be used to separate the water-soluble enzyme from the cells of *Propionibacterium* and *Pseudomonas fluorescens*.

It should be noticed that no inactivation seems to occur during the dialysis of the bacterial suspension after butanol treatment. When, however, preparations precipitated with different salts or with organic solvents were dialyzed, great loss of activity was observed. A quantitative recovery was obtained when the enzyme was precipitated by lowering the pH to 4.5 and dissolving the precipitate formed in neutral buffer without subjecting it to dialysis.

These facts seem to point to the existence of a dissociable active group in the enzyme. In this connection it should be considered that an inactivation of the preparations may be explained as an inhibition of reactive and sensitive groups in the enzyme molecule. Sulfhydryl groups, for instance, are very easily poisoned by traces of heavy metals, which may be added to the solution as impurities with the precipitating salt.

Aspartase from *Ps. fluorescens* was precipitated in the globulin fraction with ammonium sulphate. Here is a contradiction of the results of Gale [10], who reported aspartase I to be precipitated in the albumin fraction (between 50 to 100 % ammonium sulphate saturation).

The precipitation obtained by lowering the pH is largely a precipitation by means of nucleic acids, a method used by Warburg and his school [11]. The difference between *Propionibacterium* and *Ps. fluorescens* in this respect may result from a difference of extractability of nucleic substances from the cells.

SUMMARY

Using *n*-butanol it has been possible to separate aspartase from insoluble particles and obtain the enzyme in solution from the bacteria with a quantitative recovery of the activity. Different precipitation techniques were attempted and acetone precipitation in the cold seems to give the most satisfactory yield.

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The Oxidation of Cysteine by Cytochrome Oxidase and Cytochrome c

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In connection with investigations on the interaction between catalase and cysteine[1] it appeared to be necessary to compare the oxidation of cysteine as catalyzed by catalase with the oxidation catalyzed by the cytochrome system of heart muscle. The latter catalysis, discovered by Keilin[2] in 1930, has been the subject of more recent investigations[3–5] with other tissues as well. The amperometric technique for the estimation of cysteine and cystine used in the present investigation gave some new aspects since it requires smaller concentrations of the reactants than the manometric technique.

MATERIAL AND METHODS

The heart muscle preparation was obtained essentially according to Keilin and Hartree[6]. Pig hearts were collected within four hours after death, freed from fat, ligaments etc., and ground two times in a meat mill. The mince was washed for four hours with running tap water with occasional squeezing in cheese cloth. Portions of 300 g were rapidly homogenized in a "Turmix" with 500 ml cold 0.02 *M* phosphate buffer pH 7.4, the homogenizer being previously chilled with ice. Then followed centrifugation for 20 min. at 2 000 r.p.m. in an angle centrifuge. The supernatant was decanted, and the remaining material again homogenized as above. The two supernatants were combined, and cold, molar acetic acid was added during agitation to pH 5.6–5.7. After centrifugation the sediment was washed with cold water and centrifuged again, and suspended in cold 0.25 *M* phosphate buffer pH 7.4 to a volume of 60–75 ml. The suspension was shaken slowly in a flask until all lumps had disappeared. Sodium nitrate (1 mg/ml) was added. The material was centrifuged for one hour at top speed in a Sorvall centrifuge (about 12 000 r.p.m.). The precipitate was suspended in 0.02 *M* buffer pH 7.25 to give

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the original volume, and ground in a glass homogenizer. After a second high speed centrifugation in the Sorvall centrifuge the bottom layer was suspended in 0.1 *M* phosphate buffer pH 7.25. The Q_{O_2} of such preparations when tested with succinate, varied between 100 and 250 μl O_2/mg dry weight/h (Warburg manometers with air as gas phase, pH 7.25, 37°, 100 osc./min.). In the legends the amounts of heart muscle preparation are given as mg dry weight, corrected for buffer salts.

The preparations were tested for catalase, but no measurable activity was found. Moreover, when catalase was added to give a final concentration of 6.1×10^{-7} *M*, no measurable increase in the rate of cysteine oxidation was found.

Different preparations of cytochrome c with iron contents from 0.347 to 0.427 % were used. They were obtained from beef hearts with the technique usually employed in this laboratory. As suggested by Tsou [7] the *per cent* "autoxidizable cytochrome c" was calculated from the spectral changes upon CO treatment at pH 7.4 of reduced cytochrome. His values for molar absorptions were used.

Cysteine hydrochloride Eastman Kodak or Merck was used.

The oxidation tests were performed at the selected temperatures (25° or 37° C) in test tubes immersed in a water thermostat. The tubes, containing all reactants except the heart muscle preparation, were left in the bath for five minutes to equilibrate temperature. Meantime oxygen from a tank *via* a washing flask (to equilibrate temperature and humidity) was bubbled through the solution. Then the heart muscle preparation (1/15—1/30 volumes of the original content of the tube) was added. The reaction was stopped by rapidly pouring the content of the tube into the titration vessel, thereby diluting it ten times with a solution containing sodium sulfite and no oxygen. Some of the solution in the vessel was withdrawn to rinse the test tube. The titration was carried out within two minutes in a nitrogen atmosphere at pH 9. As an alternative procedure, giving the same results as above, the reaction was stopped by the addition of sulfuric acid in an amount sufficient to bring the content of the test tube to about pH 3. The acid was neutralized with an equivalent amount of ammonia before the titration. Blanks without enzymes were always made.

The amount of cysteine oxidized by the enzyme system was taken as

$$\text{cysteine oxidized} = c_0 - c_i - c_b$$

where c_0 = cysteine present at time zero, c_i = remaining cysteine at time t, and c_b = the amount of cysteine oxidized in the blank test. Usually the latter amount was low (< 5 %), especially at 25° and in the rapid experiments.

Cysteine was determined amperometrically with silver nitrate [8] or copper (II) sulfate [9] according to Kolthoff and Stricks. The latter method was preferred for low cysteine concentrations.

The experiments on the reduction of cytochrome c by cysteine were made in a Beckman spectrophotometer at 550 mμ.

RESULTS

The oxidation of cysteine requires the presence of both cytochrome oxidase and cytochrome c, as already shown by Keilin [2]. Cytochrome c alone has a very low catalytic activity, small in comparison to the activity of the system (heart muscle preparation + cytochrome c). Boiling destroys the catalytic activity of the heart muscle preparation in this reaction (Table 1).

The appearance of cystine in a molar ratio of 1 : 2 to vanished cysteine fully accounts for the disappearance of the latter under our experimental conditions. Medes [3] had earlier found the same under similar conditions. We used the following procedure to confirm that the oxidation does not go further than to cystine. After the addition of heart muscle preparation and cytochrome c to a cysteine solution sufficient time was allowed to pass to let all cysteine be oxidized. The end of the reaction was well indicated by the disappearance of the absorption bands of ferrocytochrome c. An aliquot of the solution was titrated in the presence of sodium sulfite: during this condition both RSH and RSSR compounds react with silver ions in a molar ratio of 1 : 1, and with cupric ions in a ratio of 1 : 2. This is explained by the reaction between RSSR and sulfite (Clark [10]).

$$RSSR + SO_3^{--} - = RS^- + RSSO_3^- \qquad (1)$$

Thus a titration with a silver or copper salt in the presence of sulfite does not give a measure of the amount of RSH but of RSH + RSSR. As it is seen in Table 2 the total amount of RSH + RSSR had dropped to half of its original

Table 1. The oxidation of cysteine by the various components of the system. A: *2.23 × 10^{-3} M cysteine.* B: *Heart muscle preparation (1.55 mg/ml) in M/15 phosphate buffer pH 7.3.* C: *4.48 × 10^{-4} M cytochrome c. Temp. 25°, pH 7.50, M/15 phosphate buffer. Reaction time 1 min.*

Experiment no.	Reactants	Remaining amount of cysteine in μmoles	% oxidized of initial amount of cysteine
1	3 ml. A	6.68	0.3
2	3 ml. A + 0.2 ml. B	6.68	0.3
3	3 ml A + 0.2 ml. C	6.61	0.5
4	3 ml A + 0.2 ml B + 0.2 ml C	2.77	58.6
5	3 ml A + 0.2 ml B + 0.2 ml boiled O	6.57	1.8

Table 2. Identification of reaction product. Content of reaction vessel: 5 ml 1.143 × 10^{-3} M cysteine solution + 0.6 ml 1.88 × 10^{-5} M cytochrome c + 0.03 ml heart muscle preparation of dry weight 21.2 mg per ml. Temp. 25°, pH 7.25.

Titration no.	Titrated material	μmoles of (RSH + RSSR) per ml
1	Original cysteine solution	1.143
2	At the end of the oxidation	0.578
3	After reduction with Na-amalgam	1.130

value when all the cysteine had been acted upon by (cytochrome oxidase + cytochrome c). Another aliquot of the solution was acidified, thoroughly treated with sodium amalgam, and titrated. The amalgam reduced the cystine back to cysteine, and the original concentration at time zero was restored (after correction for the dilution with acid).

For the oxidation of four moles of cysteine one mole of oxygen is required under the actual conditions. The oxidation of cysteine during one minute in experiment 4 in Table 1 would thus correspond to the Q_{O_2}-value of 4 670 μl O_2/mg/h. However, the extrapolation, on which this calculation of Q_{O_2} is based, is not justified in this case, since the reaction is not zero but first order with respect to the disappearance of cysteine (*cf.* below). Thus Q_{O_2}-values, calculated from successive periods, will decrease with time. It was consistently

Table 3. Influence of oxygen supply. Initial cysteine concentration: 2.5 × 10^{-3} M (total amount of cysteine = 12.5 μM). Cytochrome c: 2.69 × 10^{-5} M. Heart muscle preparation: 0.093 mg per ml of final solution. Total volume 5 ml Temp. 37°, pH 7.50, M/15 phosphate. Reaction time one minute.

Experiment no.	Oxygen supplied by	Cysteine oxidized	
		μmoles	% of initial amount
1	Air without shaking	2.10	17
2	Air with shaking	2.99	24
3	Air bubbling	3.85	31
4	Oxygen bubbling	5.29	42

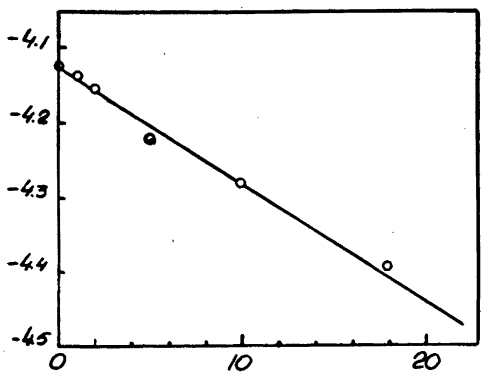

Fig. 1. Kinetics of cysteine oxidation. Ordinate: log (total amount of cysteine in solution). Abscissa: Time in minutes. Cytochrome c : 1.74 × 10^{-5} M. Heart muscle preparation: 0.108 mg per ml of final solution. 25°, pH 7.25, M/15 phosphate.

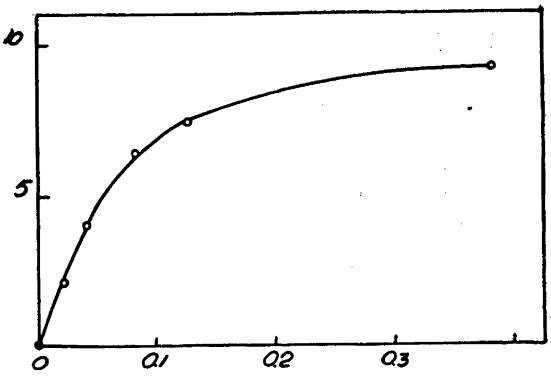

Fig. 2. Influence of [heart muscle preparation]. Ordinate: μmoles of cysteine oxidized in one minute. Abscissa: Mg heart muscle preparation per ml of final solution. Cysteine: 1.08 × 10^{-2} M (initial concentration). Cytochrome c: 1.22 × 10^{-5} M. 25°, pH 7.25, M/15 phosphate.

found that the oxidation of cysteine proceeded faster in the amperometric than in the manometric experiments. The cytochrome oxidase preparation, employed in the experiments in Table 1, gave manometrically $Q_{O_2} = 227$ with 0.02 *M* succinate and 200 with 0.015 *M* cysteine as substrates, both determinations being made at 37°. Here the oxygen uptake was linear with time for the period of the experiment (18 min.). The rate of cysteine oxidation is highly dependent on the oxygen tension (Table 3).

Kinetics of the reaction. Fig. 1 illustrates the kinetics of the oxidation of cysteine by (heart muscle preparation + cytochrome c). It appears that the

Table 4. Influence of temperature. Initial cysteine concentration: 2.5 × 10^{-3} M. Cytochrome c: 2.69 × 10^{-5} M. Heart muscle preparation: 0.093 mg per ml of final solution. Reaction time 1 min., pH 7.25, M/15 phosphate buffer.

Experiment no.	Temperature	Average *k*-value
1, 2, 3	25° C	0.038
4, 5, 6	37	0.082

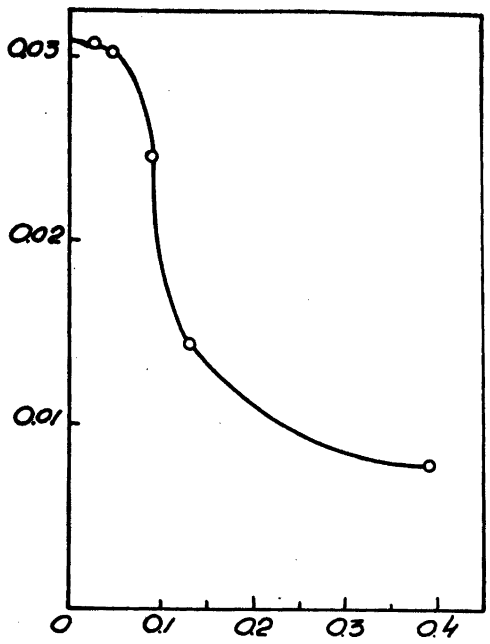

Fig. 3. Influence of [heart muscle preparation]. Ordinate: Values of k (Formula 2). Abscissa, reaction mixture etc., cf. Fig. 2.

reaction is of order one with respect to the concentration of cysteine. Hence in the sequel the first order constant of the reaction

$$k = \frac{1}{\text{(sec.) (mg preparation/ml)}} \times \ln \frac{x_0}{x_t} \qquad (2)$$

will be employed, where x_0 and x_t denote the concentrations of cysteine at times zero and t, respectively. "μ"[11] of the over-all reaction under these experimental conditions is calculated as 11 800 cal. $\times$ mole^{-1} from the values of the constants at 25° and 37° (Table 4). This is an order of magnitude frequently found in biological reactions.

The effect of the concentration of cytochrome oxidase. Fig. 2 shows the oxidation at different concentrations of the heart muscle preparation. For small additions of the preparation the rate of oxidation increases rapidly, while further additions are less effective until at high oxidase concentrations a saturation effect is evident. In Fig. 3 the same data are plotted in the form of the first order constant per mg preparation *versus* the concentration of the oxidase.

The effect of the concentration of cysteine. According to the results given in Fig. 4, there is an optimal concentration of cysteine at which a maximal number of cysteine molecules are oxidized per unit of time. However, if the relative

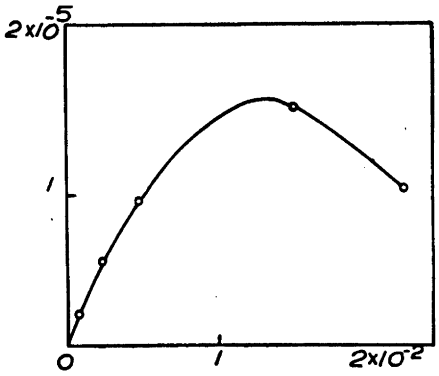

Fig. 4. Influence of [cysteine], Ordinate: Moles of cysteine oxidized in 5 min. Abscissa: Initial concentration of cysteine in M. Cytochrome c: 1.74 × 10⁻⁵ M. Heart muscle preparation: 0.10 mg per ml of final solution. Reaction time 5 min., 24°, pH 7.25, M/15 phosphate.

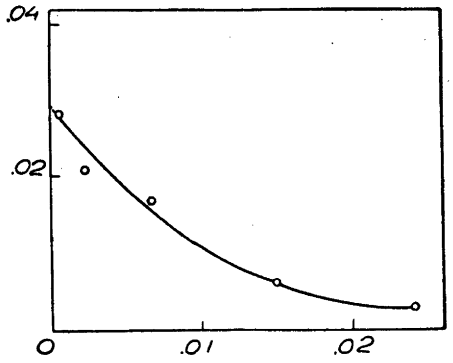

Fig. 5. Influence of [cysteine], Ordinate: First order constant of disappearance of cysteine. Abscissa: reaction mixture etc., cf. Fig. 4.

amounts are considered (Fig. 5) it is seen that the lower the cysteine concentration is, the higher will be the fraction of the cysteine molecules, oxidized per unit of time. Fig. 5 shows clearly an inhibition by cysteine, acting already at low cysteine concentrations. This observation is in agreement with earlier results obtained by Potter and Dubois [12] and Ames and Elvehjem [13] (*cf.*, however, also Barron and Singer [14]).

Since the oxidation of cysteine is first order with respect to cysteine without any sign of increased reaction velocity with the gradual disappearance of cysteine from the solution, the inhibition by cysteine must occur rapidly and be only slowly reversible.

The effect of the concentration of cytochrome c. Fig. 6 shows a plot of the inverse of the amount of cysteine oxidized in a 4 min. experiment *versus* the inverse of the cytochrome c concentration [15]. Fig. 7 is a plot of the first order

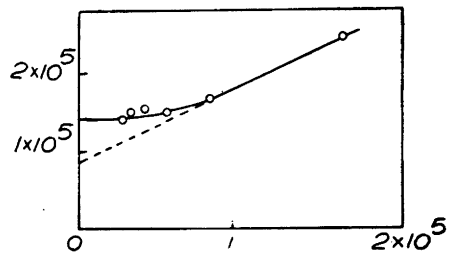

Fig. 6. Influence of [cytochrome c]. Ordinate: 1/(moles of cysteine oxidized in 4 min.). Abscissa: 1/M of cytochrome c. Cysteine: 2.09 × 10⁻³ M. 24°, pH 7.25, M/15 phosphate. Heart muscle preparation: 0.10 mg per ml of final solution.

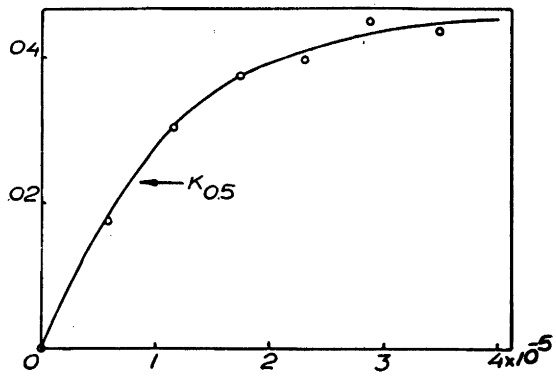

Fig. 7. Influence of [cytochrome c]. Ordinate: Value of k (Formula 2). Abscissa: M of cytochrome c. Same experiment as in Fig. 6.

constant (Formula 2) *versus* the concentration of cytochrome c, and shows that there is an accessible concentration of cytochrome c after which a further addition does not increase the rate very much. Fig. 7 permits also the calculation of $k_{0.5}$, *i. e.* that concentration of cytochrome c at which, under the actual conditions, the oxidation of cysteine proceeds at a half maximal rate. Its numerical value is 0.8×10^{-5} *M*. This constant has not the meaning of a Michaelis constant. Employing different experimental conditions Slater[16] found $k_m = 1.2 \times 10^{-5}$ *M*, and 6.1×10^{-5} *M* with *p*-phenylenediamine and ascorbic acid as reducing agents. The fact that no hyperbolic relation correlates in our

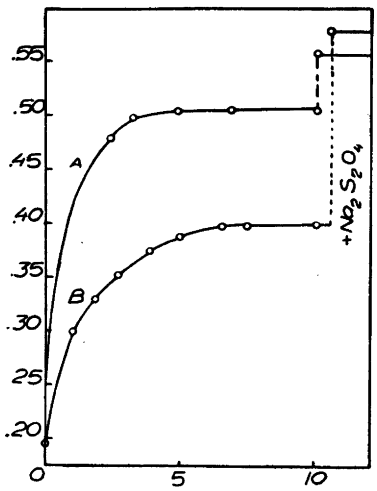

Fig. 8. Comparison between normal and autoxidizable cytochrome c. Ordinate: log $\frac{I_0}{I}$ *at 550 mμ. Abscissa: Time in min. Cytochrome c: 1.88 × 10⁻⁵ M. Curve A: Cytochrome containing 0.348 % Fe, 2 % autoxidizable cytochrome. Curve B: 0.437 % Fe, 100 % autoxidizable. At time zero addition of cysteine to a concentration of 5.7 × 10⁻⁴ M. At ten min. addition of dithionite. 24°, pH 7.25, M/15 phosphate.*

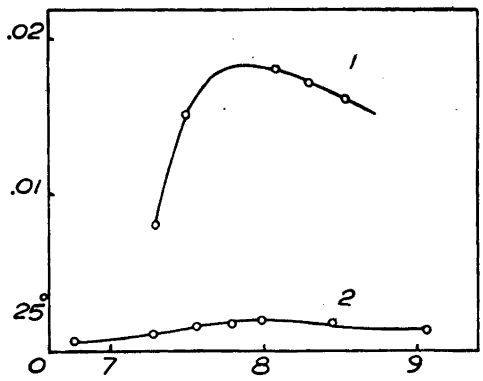

Fig. 9. Effect of pH on the oxidation of cysteine by heart muscle preparation + cytochrome c. Ordinate: Values of k (Formula 2). Abscissa: pH. Cytochrome c: 2.62 × 10^{-5} M. Cysteine: 2.0 × 10^{-3} M (Curve 1), 2.0 × 10^{-2} M (Curve 2). 25°, M/15 phosphate.

instance the substrate concentration to the reaction velocity might be due to the influence of the inhibiting effect of cysteine.

Another preparation of cytochrome c, spectroscopically normal but completely autoxidizable, proved to be quite inactive under the same conditions. With *p*-phenylenediamine or succinate as substrates Keilin and Hartree [6] had found the same, and stated, as the reason for the inactivity of autoxidizable cytochrome c, that neither succinic acid nor *p*-phenylenediamine could reduce it with sufficient velocity. Also with cysteine as reducing agent the autoxidizable cytochrome c is reduced more slowly than is normal cytochrome (Fig. 8). A spectrophotometric assay of the activity of a cytochrome c preparation can be based on this fact.

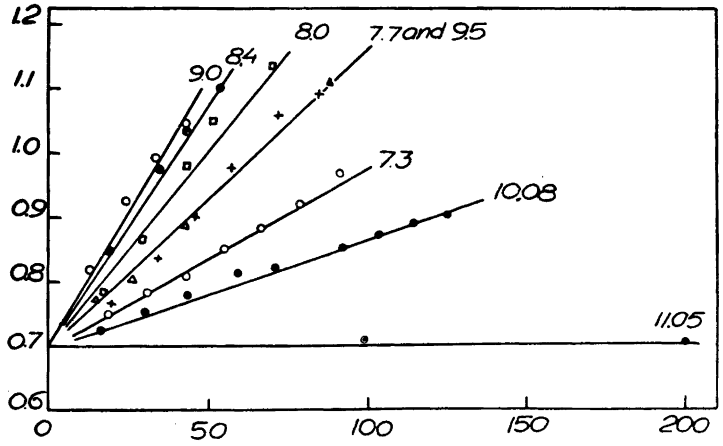

Fig. 10. Ordinate: log ([cysteine] / [ferricytochrome c]). Abscissa: Time in sec. Cytochrome c: 1.49 × 10^{-5} M (initially all oxidized). Cysteine: 7.45 × 10^{-5} M (initial concentration). M/15 prim. phosphate, brought to desired pH values with 0.10 M NaOH.

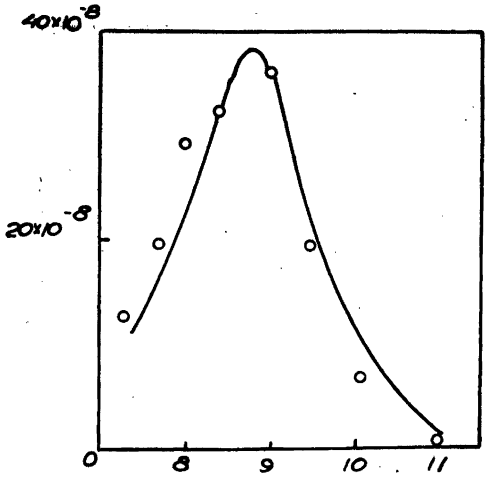

Fig. 11. Ordinate: Rate of reduction of cytochrome. Abscissa: pH. O *experimental values given in Fig. 10. Full drawn line: Theoretical rate of reduction, calculated from* $pK = 8.20$ *for the thiol group of cysteine and* $pK = 9.35$ *for the transformation of ferricytochrome III to IV, and assuming that the reaction takes place according to*

$$\frac{d\,[cyt.Fe^{++}]}{dt} = 550\,[RS^-]\,[cyt.\ III\ Fe^{+++}]$$

The effect of pH on the rate of the oxidation of cysteine by (heart muscle preparation + cytochrome c) is given in Fig. 9. For two different concentrations of cysteine the optimum was found at about pH 8 under the conditions actually employed.

Some determinations of the influence of pH on the reduction of ferricytochrome c by cysteine were also made (Fig. 10 and 11). The plotting of log ([cysteine]/[ferricytochrome c]) against time gives straight lines at all examined values of pH. pH 8.8 was found to be optimal for this reaction.

DISCUSSION

The aerobic, "oxidatic", oxidation of cysteine involves the components: oxygen-cytochrome oxidase (as heart muscle preparation)—cytochrome c—cysteine. The interactions between the components are complex in nature.

In a system containing *oxygen + cysteine* no oxidation to cystine occurs in the absence of a catalyst [17,18]. When *e. g.* copper ions act as catalyst in this reaction, a pronounced pH maximum is found [18,19], the position of which depends to some extent upon the nature of the buffer system. Hydrogen peroxide is formed during this reaction. Correction for the oxidation of cysteine directly by oxygen in our experiments is given by the blank determinations.

In the system *oxygen + cytochrome oxidase + cysteine* a slow oxidation of cysteine takes place (Table 1). In the case of *oxygen + cytochrome c + cysteine* it is obvious that the oxidation goes further than what can be accounted for by a stoichiometric reaction between the two latter components. This may be

attributable to an unspecific metal catalysis by the cytochrome c or to the presence of non-cytochrome iron in the preparation.

In a system *oxygen + cytochrome oxidase + cytochrome c* there is a rapid oxidation of ferrocytochrome. Some indirect information on this reaction may be gained from a comparison of Fig. 9 and Fig. 11, which shows that the optimal pH for the oxidation of cysteine shifts from 8.8 to 8.0 when cytochrome oxidase is introduced into the system. The shift could depend either upon an alkali denaturation of the cytochrome oxidase or upon the presence of an essential group with pK around 7 in it, where only the acidic form is active. The latter hypothesis is supported by the results of Wainio *et al.*[20] who found the optimal pH for the oxidation of cytochrome c by cytochrome oxidase to be 6.67 in 0.033 M phosphate.

Molecular *oxygen + ferrocytochrome c* do not react except at pH<4 or >13[21]. There is a not yet sufficiently studied modification of cytochrome c, the "autoxidizable cytochrome c", which is oxidized by molecular oxygen at any pH. It has been shown by Keilin and Hartree [6], Tsou [7], and in the present investigation that autoxidizable cytochrome c is inactive in promoting oxidation of various substrates by (molecular oxygen + heart muscle preparations). The reason, as given by Keilin and Hartree, is that the substrates reduce this modified cytochrome c more slowly than normal cytochrome.

The reaction between *ferricytochrome c and cysteine* is bimolecular, one mole of cytochrome being reduced by one mole of cysteine. It proceeds only within a confined pH range (Fig. 11). Towards the acid side the region may be limited by the dissociation of the sulfhydryl group of cysteine. The oxidation of cysteine could be depicted as a two-step process:

$$2RSH \rightleftharpoons 2RS^- + 2H^+ \quad (3)$$

$$2RS^- \rightleftharpoons RSSR + 2e^- \quad (4)$$

There seems to be some uncertainty about the pK of reaction (3). The treatise of Schmidt [22] favours the value of pK = 8.18, whereas Cohn and Edsall [23] give pK = 10.25. The most recent investigation [24] discloses the difficulties in attributing the two pK:s visualized in titration curves, to either of the thiol or the amino groups, respectively. Our results fit with pK = 8.18, but would be difficult to explain if the thiol group should dissociate with pK above 10.

Three factors enter on the alkaline side of the optimal pH. The decrease of the rate of reduction of cytochrome parallels the transformation of ferricytochrome type III to type IV (pK 9.35 [21]). At present it is not possible to eluci-

diate what structural changes in this transformation would prevent the reduction of type IV by cysteine. Another possibility, although less probable, is that the change in the charge of the substrate molecule due to the ionization of the amino group (pK 10.28)[25] would influence. Third, the dissociation of a proton (pK 8.18) from the reductant in the (cystine/cysteine)-system would decrease the slope of the E_o'/pH-curve of the system, since no cancelling dissociation occurs in the oxidant. The E_o'/pH-slope of cytochrome is — 0.06 mV/pH from pH 7 to 11 [25a]. Obviously the distance between E_o' of the two systems must decrease at pH$>$8.18, which also could account for the decrease in reaction velocity.

In the complete system *oxygen + cytochrome oxidase + cytochrome c + cysteine* the latter is oxidized more rapidly than was believed from previous manometric experiments. The discrepancy between the Q_{O_2}-values from manometric and amperometric experiments may be accounted for by the following facts.

1. The substrate concentration in the amperometric experiments was much lower than in the manometric determinations. This must of course be of importance when substrates are employed which inhibit the reaction, *e. g.* cysteine.

2. The oxygen supply was much better in the amperometric than in the manometric experiments. This is in agreement with the wellknown observation that an increase in the number of oscillations per minute of the manometers will increase the Q_{O_2}-value. It must be considered that to oxidize completely to cystine 45 μmoles of cysteine in 3 ml of water at 37° with air as the gas phase, the oxygen, dissolved in the liquid, must be renewed more than 20 times.

In the oxidation of succinate by (heart muscle preparation + cytochrome c) also other catalysts are involved (succinic dehydrogenase and Slater's factor), which may have been partly damaged during the preparation. Since these catalysts are probably not acting in the oxidation of cysteine a direct comparison is not justified between our amperometric and manometric Q_{O_2}-values with cysteine as substrates and those of Keilin and Hartree and Slater with succinate. Slater [16] found the Q_{O_2} of cytochrome oxidase to be 3 400 (38°, fat free dry weight, ascorbate as substrate).

The pH optimum of the over-all reaction from oxygen to cysteine via(cytochrome oxidase + cytochrome c) was found as 8.0, whereas the optimum of the part-reactions, cytochrome oxidase / cytochrome c and cytochrome c/ cysteine, were found to be 6.67 [20] and 8.8 (present investigation), respectively. Since the three values have been obtained with different techniques a coordination will not be attempted. The position of the rate-determining step in the

reaction chain will, as is evident from the above, be dependent upon the pH at which the reaction is carried out.

Fromageot [26] has pointed out that the term cysteine oxidase should be considered as no more than a general expression which covers enzymes whose action involves simple elementary transformations. With regard to this and to the fact that in the oxidation of cysteine by (cytochrome oxidase + cytochrome c) oxygen is the ultimate electrone acceptor with the formation of water, it is correct to say that (cytochrome oxidase + cytochrome c) functions as a cysteine oxidase. Preliminary experiments have shown that not only glutathion but also numerous other low-molecular thiols can substitute cysteine as a reducer of cytochrome c. The expression cysteine oxidase could therefore just as well be exchanged for thiol oxidase in this case.

Succinic dehydrogenase contains one or two SH-groups, essential for its activity [27,28]. It is tempting to think that (cytochrome oxidase + cytochrome c) could oxidize directly thiol groups in enzymes in a way similar to what it does with the simple thiols.

SUMMARY

1. Using an amperometric technique for the determination of cysteine and cystine it has been confirmed that a system containing oxygen + heart muscle preparation + cytochrome c + cysteine oxidizes cysteine only to cystine.

2. The reaction is dependent on the available amount of oxygen. If oxygen is bubbled through the vessel, and low concentrations of cysteine are used, the reaction proceeds more rapidly than found in manometric experiments.

3. The reaction is of order one with respect to the concentration of cysteine. μ is around 11 800 cal $\times$ mole^{-1}.

4. An increase in the concentration of cysteine decreases the rate constant because of the inhibition of the oxidase by cysteine.

5. The maximum efficiency (rate constant per mg of oxidase preparation) is obtained at low concentration of the oxidase preparation. These low concentrations are to be used in any assay of the cytochrome oxidase activity of a preparation when cysteine is the substrate.

6. The addition of cytochrome c increases the rate constant, but not along a hyperbolic function.

7. Autoxidizable cytochrome c oxidizes cysteine at a significantly lower rate than normal cytochrome c does.

8. Only non-autoxidizable cytochrome c is effective in promoting the catalysis of cysteine oxidation by oxidase and oxygen.

9. Both at low and high concentrations of cysteine the optimal pH for the activity of the system containing oxygen + oxidase + cytochrome c + cysteine lies around pH 8.0.

10. Cysteine reduces ferric cytochrome c with a typical bimolecular kinetics. The reaction has a pH optimum at 8.8,

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2,5,5-Trimethoxytetrahydropyran

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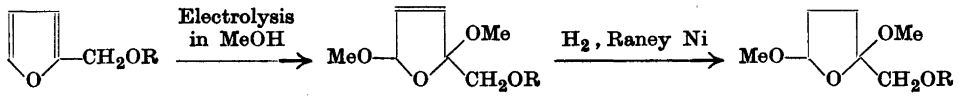
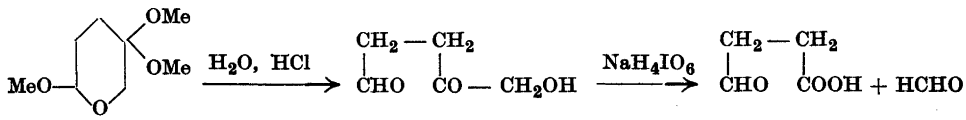
The preparation of 2,5-dimethoxy-2-hydroxymethyl-2,5-dihydrofuran (I) and 2,5-dimethoxy-2-acetoxymethyl-2,5-dihydrofuran (II) from furfuryl alcohol and furfuryl acetate, respectively, has recently been described [1]. These dihydrofurans have been catalytically hydrogenated to the corresponding new tetrahydrofurans III and IV. Their structures follow from synthesis and from analyses.



I (R = H); II (R = Ac) III (R = H); IV (R = Ac)

When III or IV are heated under reflux with methanolic hydrogen chloride a compound with the formula $C_5H_7O(OCH_3)_3$ is formed. It is a colorless liquid with a characteristic terpene-like odor. We believe this compound to be the hitherto unknown 2,5,5-trimethoxytetrahydropyran (V) since: (a) refluxing over sodium under atmospheric pressure as well as shaking with Raney nickel in methanol under 100 atmospheres of hydrogen at 80° for four hours leaves it unaffected (b) hydrolysis with boiling $N/10$ sulfuric acid for one minute gives a neutral compound, which is cleaved by oxidation with one mole of sodium periodate into β-formylpropionic acid and formaldehyde.

If one accepts formula V for the new compound, the neutral compound formed by hydrolysis should be 1-hydroxy-2,5-dioxo-pentane (VI). The formation of β-formylpropionic acid and formaldehyde from V may therefore be formulated as follows:



V VI

EXPERIMENTAL

Microanalyses by A. Grossmann, E. Boss and K. Glens.

2,5-Dimethoxy-2-hydroxymethyl-tetrahydrofuran (III). 2,5-Dimethoxy-2-hydroxymethyl-2,5-dihydrofuran [1] (I) (50.0 g) and methanol (75 ml) were shaken (3 h) with Raney nickel (2.0 g) under hydrogen (100 atm). The product was isolated by distillation.

Fraction (g)	B.p. $_{13}$ °C	n_D^{25}	OCH_3 Calc. 38.3 %
1 (7.0)	104–105	1.4472	37.2
2 (14.0)	104–106	1.4472	37.7
3 (15.2)	106–107	1.4477	38.1
4 (12.4)	107–110	1.4477	38.0

The yield (all fractions) was 48.6 g (96 %) of III (colorless liquid). A portion of fraction 3 was also analyzed for carbon and hydrogen.

$C_5H_8O_2(OCH_3)_2$ (162.2) Calc. C 51.8 H 8.7
Found » 52.3 » 8.6

2,5-Dimethoxy-2-acetoxymethyl-tetrahydrofuran (IV). 2,5-Dimethoxy-2-acetoxymethyl-2,5-dihydrofuran [1] (II) (20.2 g) and methanol (25 ml) were shaken (2 h) with Raney nickel (0.5 g) under hydrogen (100 atm). The product was isolated by distillation.

Fraction (g)	B.p. $_{13}$ °C	n_D^{25}
1 (0.8)	113–114	1.4355
2 (7.0)	114–115	1.4356
3 (6.4)	114–115	1.4358
4 (5.0)	115–116	1.4359

The yield (all fractions) was 19.2 g (96 %) of IV (colorless liquid). A portion of fraction 3 was analyzed.

$C_5H_7O_2(OCH_3)_2(COCH_3)$ (204.2) Calc. C 52.9 H 7.9 OCH_3 30.4 $COCH_3$ 21.1
Found » 53.3 » 8.0 » 30.7 » 21.7

2,5,5-Trimethoxytetrahydropyran (V). (a) Preparation from III. III (16.2 g, 0.10 mole) was heated under reflux (1 h) with methanol (80 ml, dried with magnesium) to which had been added acetyl chloride (2.00 ml). Sodium (3.0 g) was added and the dark-

red mixture heated further under reflux (20 h); this treatment with sodium methoxide, which probably causes the destruction of some carbonyl compounds, was necessary in order to obtain a pure product. After cooling, the precipitated sodium chloride was removed by filtration and the methanol distilled through a Vigreux column. Ether (50 ml) was added to the residue and a voluminous precipitate removed by centrifugation. The precipitate was washed twice with ether and the combined etheral solutions distilled. The yield was 12.2 g (69 %) of V (colorless liquid, $b.p._{13-14} = 79-84°$, $n_D^{25} = 1.4352$). 2—4 g of a black residue remained in the flask.

$C_5H_7O(OCH_3)_3$ (176.2)	Calc.	C	54.5	H	9.2	OCH_3	52.8
	Found	»	54.5	»	9.1	»	52.5

Upon redistillation of the above product through a 48 cm fractionating column packed with Dixon gauze rings the boiling point and the refractive index remained constant throughout the whole distillation.

40 g of V was recovered unaffected ($b.p._{760} = 203-205°$, $n_D^{25} = 1.4351$) after reflux (5 min) over sodium under atmospheric pressure followed by distillation.

10 g of V was recovered unaffected ($n_D^{25} = 1.4352$) after shaking (2 h) with methanol (20 ml) and Raney nickel (0.8 g) under hydrogen (100 atm, 80°).

(b) Preparation from IV. IV (20.4 g, 0.10 mole) was heated under reflux with methanolic hydrogen chloride as above. Sodium (700 mg) was added, a precipitate of sodium chloride removed by filtration, and the low-boiling products distilled through a Vigreux column. This was done in order to remove the methyl acetate formed during the reaction. The residue was heated under reflux (20 h) with a solution of sodium methoxide (from 1.5 g of sodium) in methanol (50 ml) and worked up as above. The yield was 12.8 g (73 %) of V (colorless liquid, $b.p._{12} = 82-84°$, $n_D^{25} = 1.4352$).

	Found	C	54.7	H	9.2	OCH_3	52.6

(c) Hydrolysis and reaction with periodate. V (1.00 g, 0.00568 mole) was heated under reflux (1 min) with sulfuric acid (0.1 *N*, 13.0 ml) and the colorless solution allowed to stand (30 min, room temperature). Monosodium paraperiodate (0.00568 mole) was added and dissolved. After standing (30 min) at room temperature half of the solution was continuously extracted (4 h) with ether and the extract evaporated in vacuum (200 mm, 45°). A solution of 2,4-dinitrophenylhydrazine (0.80 g) in perchloric acid (30 %, 34 ml) was added and the mixture allowed to stand overnight. Filtration, washing — first with perchloric acid (30 %) and then with water — and drying gave 0.69 g (86 %) of β-formylpropionic acid dinitrophenylhydrazone [m.p. 196—198° (Hershberg apparatus, corr.)]; previously found 198—200° [2].

$C_{10}H_{10}N_4O_6$ (282.2)	Calc.	C	42.6	H	3.6	N	19.9
	Found	»	42.5	»	3.4	»	20.1

Crystallization from acetone-ligroin did not change the m.p. or the analytical values.

To the second half of the above solution was added 5 ml of water. About 8 ml was distilled into a solution of dimedone (1.00 g) in water (300 ml) and the resulting precipitate of the dimedone derivative of formaldehyde isolated in the usual way [3]. The yield was 0.77 g (93 %) (white crystals, m.p. 189—190°).

$C_{17}H_{24}O_4$ (292.4)	Calc.	C	69.8	H	8.3
	Found	»	69.6	»	8.1

In a parallel experiment it was found that 12.9 ml of *N*/10 sodium hydroxide was necessary to neutralize the hydrolysate of V (phenolphthalein indicator). Hence no acid products were formed by hydrolysis.

SUMMARY

2,5-Dimethoxy-2-hydroxymethyl-2,5-dihydrofuran (I) and 2,5-dimethoxy-2-acetoxymethyl-2,5-dihydrofuran (II) have been catalytically hydrogenated to 2,5-dimethoxy-2-hydroxymethyl-tetrahydrofuran (III) and 2,5-dimethoxy 2-acetoxymethyl-tetrahydrofuran, respectively. Both tetrahydrofurans gave the new 2,5,5-trimethoxytetrahydropyran (V) upon boiling with a methanolic solution of hydrogen chloride.

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Untersuchungen am System MgO-Al_2O_3-SiO_2 mit BaO als Zusatzmittel

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Ziel der Untersuchungen war die Aufklärung der Wirkung von BaO-Zusätzen als Flussmittel in dem für die Herstellung von Elektroporzellan interessanten System MgO-Al_2O_3-SiO_2. Zu diesem Zwecke wurden unter Variation der Zusammensetzung in den Systemen BaO-MgO-Metakaolin bezw. BaO-Talk-Metakaolin folgende Bestimmungen durchgeführt:

a. Schmelzpunktsisothermen für 1 250 und 1 300° C,
b. Temperaturen der Sinterung für Zusammensetzungen entlang dieser Isothermen, und
c. Temperaturen für eine gewisse Scherben-Dichte bei diesen Zusammensetzungen.

Die Schmelzpunktsisothermen wurden durch Bestimmung der Kegelschmelzpunkte für eine genügend grosse Anzahl verschiedener Zusammensetzungen im Dreiecksdiagram BaO-MgO-Metakaolin bezw. BaO-Talk-Metakaolin erhalten.

Bisherige Erfahrungen über die Wirkung von MgO und BaO in keramischen Massen[1]

Magnesiumoxyd ist für die Herstellung keramischer Massen und Glasuren von grösster Bedeutung. Es ist ein wichtiger Bestandteil des Magnesiaporzellans (Melanit), der Steatitmassen und einer Reihe feuerfester Stoffe. Es gleicht in diesen Stoffen in seiner Wirkung dem Kalziumoxyd, macht diese aber eher schwerschmelzbar. Erst bei Temperaturen oberhalb von etwa 1 200° macht sich eine gewisse Sinterwirkung bemerkbar. Zusätze von Magnesiumoxyd zu Porzellanmassen erteilen diesen eine Reihe wertvoller Eigen-

schaften, vor allem hohe Festigkeit und geringe Schwindung. Ferner wird die Beständigkeit moderner keramischer Produkte auf das Auftreten von Cordierit zurückgeführt.

Bei Zusätzen von Steatit zu Kaolin sinkt bis 70% Zusatz der Kegelschmelzpunkt der Massen auf 1 280° C. In analoger Weise werden die Sinterungstemperaturen der Massen bei Steatitzusätzen verändert, doch nähern sich mit steigendem Steatitgehalt Sinterungstemperatur und Kegelschmelzpunkt immer mehr um bei einem Steatitgehalt von 50 bis 70% praktisch zusammenzufallen. In diesem Bereich liegen auch die niedrigst schmelzenden Eutektika des Systems MgO-Al_2O_3-SiO_2 (1 345, 1 360, 1 425 und 1 460° C). Beim Brennen auf etwa 1 200° C enthalten alle derartige Massen Cristobalit, dessen Gehalt bei hohen Brenntemperaturen, besonders in Kaolinreichen Massen, immer mehr abnimmt. Massen mit 20—30 % Steatit sind sehr beständig gegen Temperaturschock und finden dementsprechende Anwendung. Von technischem Interesse sind ferner Bestrebungen die Schmelztemperatur in derartigen Massen durch Erhöhung des MgO-Gehaltes über den durch Steatit zugeführten hinaus zu erhöhen.

Im Gegensatz zu der grossen Bedeutung von MgO ist die Anwendung von Bariumoxyd in keramischen Massen bisher gering [2]. Von Interesse ist hier nur zu nennen: Elektroporzellan, dessen Durchschlagsfestigkeit durch BaO-Zusätze erhöht wird, witterungs- und säurebeständiges Material, z.B. das englische Steingut Wedgewood, ferner gewisse Glasuren, besonders für Steingut, in denen BaO das Bleioxyd ganz oder teilweise ersetzt. Als Flussmittel war BaO in der Hauptsache auf Glasphasen beschränkt (PbO-Ersatz) und erst in letzter Zeit wurde die Verwendung von BaO in hochfeuerfesten Stoffen versucht.

EXPERIMENTELLES

Verwendete Präparate

Sämtliche verwendeten Präparate wurden von *Akt. Ges. Gustavsbergs Porslinfabrik* zur Verfügung gestellt.

Kaolin war Zettlitzer Kaolin, der bei den verschiedenen Gemischen mit theoretischer Zusammensetzung entsprechend einem Gehalt von 86,0 % Metakaolin in Rechnung gestellt wurde.

BaO wurde in Form von chemisch reinem Bariumkarbonat mit einem Gehalt von 77,8 % BaO eingeführt.

MgO wurde in Form von Magnesit mit einem analytisch bestimmten Gehalt von 47,9 % MgO verwendet.

Der *Talk* enthielt laut Analyse 31,9 % MgO und 63,4 % SiO_2.

Sämtliche Präparate wurden gemahlen, durch ein Sieb mit 6 400 Maschen per cm^2 gesiebt und schliesslich bei 110° C getrocknet.

Durchführung der Versuche

Die eingewogenen Gemische wurden in einem Mischgefäss mit einer zweiprozentigen Gummi-arabicum-Lösung aufgeschlämmt und nach halbstündiger Mischung entweder auf einer Gipsplatte entwässert, geknetet und zu Pyramiden geformt oder zur Herstellung von Stäben unmittelbar in Gipsformen gegossen. Die Kegeln, mit einer Basislänge von 8 mm und einer Höhe von 12 mm, wurden daraufhin unmittelbar bei 110° C getrocknet, während die Stäbe von 12 × 1 × 0,7 cm Kantenlängen vor der Trocknung bei 110° C zur Vermeidung von Deformierung bei Zimmertemperatur vorgetrocknet wurden.

Die Bestimmung der Kegelschmelzpunkte wurde in einem Heraeus-Rohrofen durchgeführt. Die Kegeln wurden auf sorgfältig gereinigten Platinblechen in den Ofen geschoben und bei in jedem Versuch exakt gleichem Erhitzungsverlauf die Temperatur der Rundung der Kegelspitze bestimmt. Die Erhitzung wurde über diesen Punkt hinaus fortgesetzt. Im allgemeinen sank der Kegel dabei etwa 10° oberhalb dieser Temperatur zusammen und bildete etwa 20° oberhalb eine halbkugelförmige Schmelze. Nur bei einzelnen Zusammensetzungen, und zwar besonders für die 1 250°-Isotherme des Systems BaO-MgO-Metakaolin, waren die Schmelzverhältnisse weniger definiert, und in diesen Fällen konnte sich das Temperaturintervall zwischen der Rundung der Spitze und der des vollständigen Zusammenschmelzen bis auf nahezu 100° erstrecken. In einzelnen Fällen traten Schwierigkeiten auch dadurch auf, dass Sinterung und Schmelzen zu nahe bei einander lagen. Dass diese Komplikationen nicht auf Zufälligkeiten beruhen, zeigte sich darin, dass sie nur bei bestimmten Mischungsverhältnissen und bei diesen in reproduzierbarer Weise auftraten.

Auf die Bedeutung der Einhaltung eines bestimmten Erhitzungsverlaufs wurde bereits hingewiesen, da der Kegelschmelzpunkt von der Erhitzungsgeschwindigkeit abhängig ist. Bei den hier besprochenen Versuchen wurde der Ofen innerhalb 20 Minuten auf 700° C gebracht, dann mit einer Geschwindigkeit von 10°/Minute auf 1 100° und oberhalb dieser Temperatur mit 5°/Minute erhitzt. Eine Erhöhung der Erhitzungsgeschwindigkeit auf 10—15° per Minute bei den höheren Temperaturen ergab 10 bis 20° höhere Kegelschmelzpunkte.

Zur Bestimmung der zufolge Sinterung erzielten Festigkeit und der Scherbendichte der untersuchten Gemische in Abhängigkeit von der Brenntemperatur wurden die getrockneten Stäbe nach exakt eingehaltenem Erhitzungsschema im Silitstabofen erhitzt. Durch Bestimmung des Temperaturgradienten in Ofen war die Brenntemperatur jedes einzelnen Punktes in den Stäben bekannt. Zufolge dieses Gradienten erhielten die Stäbe eine charakteristische Form mit deutlich markiertem Einsatz der Schwindung bei einer

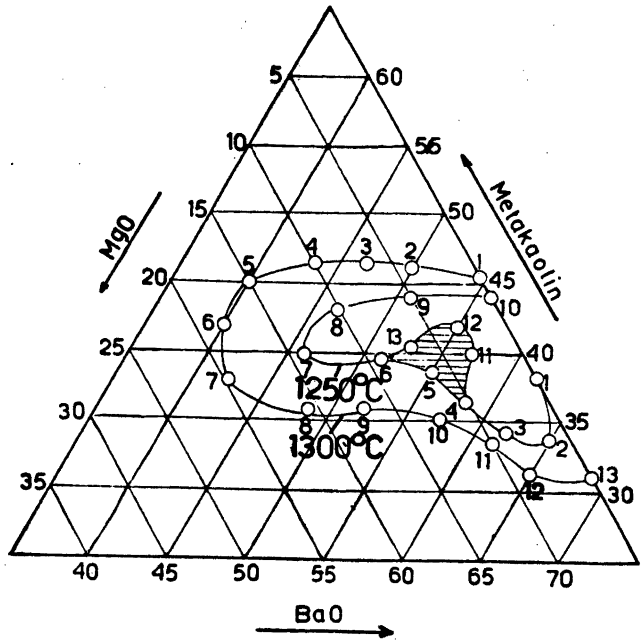

Fig. 1. Kegelschmelzpunktisothermen des Systems BaO-MgO-Metakaolin.

bestimmten Temperatur (bezw. sehr engem Temperaturbereich). Der Teil des Stabes zwischen diesem Punkt und dem am höchsten erhitzen Ende war vollständig zusammengesintert und dicht. Zur Bestimmung dieser Sinterung und der Scherbendichte wurden teils eine Bestimmung der Aufsauggrenze für eine gefärbte Flüssigkeit und teils Nadeldruckversuche ausgeführt.

Bei der Bestimmung der Aufsauggrenzen — im folgenden Temperaturen einer bestimmten Scherbendichte oder einfach *Dichtepunkte* genannt — wurden die Stäbe mit dem porösen, auf niedrigere Temperatur erhitzten Ende zu einer Tiefe von einem halben Zentimeter in eine mit roter Tinte gefüllten Schale gestellt, eine halbe Stunde lang evakuiert und dann die Höhe der aufgesaugten Flüssigkeit ausgemessen. Die Temperatur, welche wie erwähnt durch Bestimmung des Temperaturgradienten bekannt ist, wurde als Dichtepunkt der betreffenden Zusammensetzung notiert. Die Evakuierung ist nicht unbedingt erforderlich, da die gleichen Dichtepunkte auch ohne Evakuieren erhalten werden, allerdings erst nach längerer Verweilzeit in der Flüssigkeit.

Nadeleindruckversuche zur Bestimmung einer gewissen Festigkeit — im folgenden *Sinterungspunkt* genannt — ergaben reproduzierbare Werte

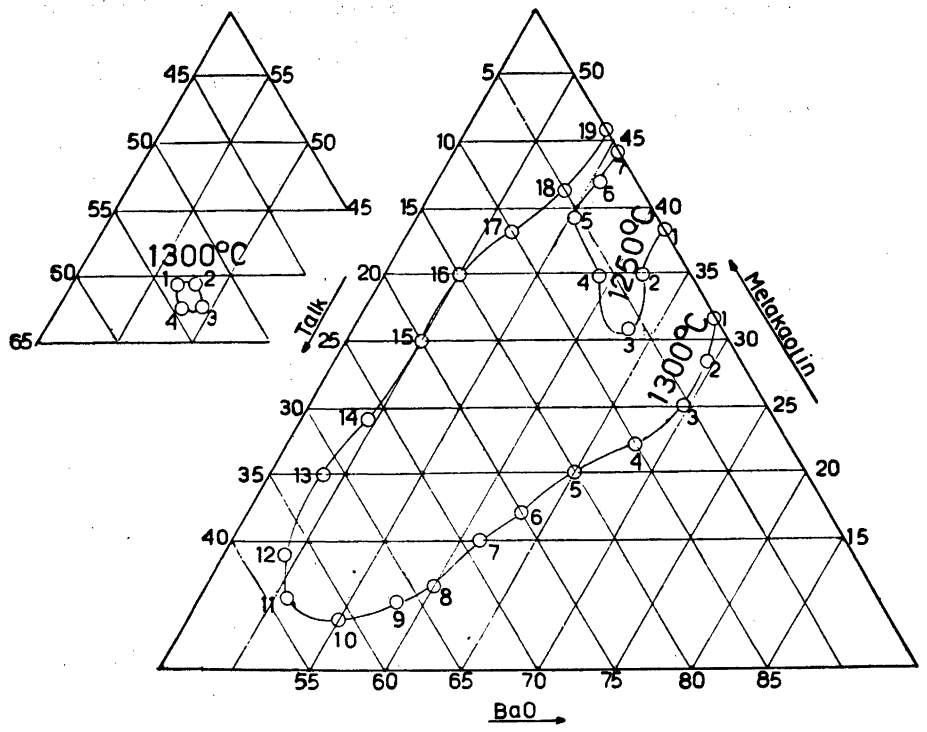

Fig. 2. Kegelschmelzpunktisothermen des Systems BaO-Talk-Metakaolin.

erst nach Anwendung automatisch konstant gehaltener, bei allen Versuchen angewendeten gewissen Belastungen. Das Verfahren ist dadurch objektiv im Gegensatz zu Nadeleindrucken mit freier Hand, wenn auch die Werte relativ sind, da der so erhaltene Sinterungspunkt von der Grösse der Belastung abhängig ist. Bei unseren Versuchen wurde eine Belastung von 3 480 g gewählt. Die Genauigkeit dieser Bestimmungen ist dadurch gross und das Resultat praktisch allein von der Einhaltung der Brennbedingungen abhängig; diese bedingen eine Fehlergrenze für die Sinterungspunkte von etwa $\pm$ 10° C.

VERSUCHSRESULTATE

Das Resultat der Kegelschmelzpunktsbestimmungen für die Temperaturen 1 250 und 1 300° C wird in den Figuren 1 und 2 als Isothermen in Dreiecksdiagrammen wiedergegeben. Das Ergebnis der Bestimmung der Sinterungsvorgänge wird im Anhang in den Tabellen 1 bis 5 zusammengefasst. In

Tabelle 1. Sinterungs- und Dichtepunkte bei Zusammensetzungen der 1 300°-Isotherme im System BaO-MgO-Metakaolin.

Nr.	Zusammensetzung in %			Sinterungspunkt	Dichtepunkt
	BaO	Metakaolin	MgO	°C	°C
1	54,3	45,7	—	1 094	1 246
2	50	46,4	3,6	1 063	1 220
3	47	47	6	1 132	1 242
4	43	47	10	1 140	1 256
5	40	45	15	1 126	1 228
6	40	42	18	1 132	1 228
7	42	38	20	1 136	1 228
8	48	36	16	1 136	1 232
9	52	36	12	1 138	1 237
10	57,4	35	7,6	1 132	1 232
11	62	33	5	1 126	1 218
12	65	31,5	3,5	1 154	1 232
13	68,5	31,5	—	1 200	1 252

diesen sind die Sinterungs- und Dichtepunkte zusammen mit den zugehörigen Zusammensetzungen der in den Dreiecksdiagrammen mit arabischen Ziffern bezeichneten Punkten aufgenommen.

Das Aussehen der Kegeln und Stäbe variierte stark mit ihrer Zusammensetzung: bei hohen BaO-Gehalten wurden sie beim Brennen weiss bis bläulich und teilweise glasig, während bei höheren Kaolingehalten gelbliche Farbtöne auftraten. Massen mit hohem MgO- und BaO-Gehalt hatten geringe Plastizität und waren schwer zu formen.

Tabelle 2. Sinterungs- und Dichtepunkte bei Zusammensetzungen der 1 250°-Isotherme im System BaO-MgO-Metakaolin.

Nr.	Zusammensetzung in %			Sinterungspunkt	Dichtepunkt
	BaO	Metakaolin	MgO	°C	°C
1	62	38	—	1 190	1 205
2	65	34	1	1 215	1 240
3	62	34	4	1 200	1 230
4	58	37	5	1 200	1 225
5	55	39	6	1 200	1 235
6	51	39,5	9,5	1 220	1 245
7	46,5	40	13,5	1 210	1 230
8	47	43	10	1 220	1 248
9	51	44	5	1 235	1 248
10	56	44	—	1 180	1 205
11	57	40	3	1 205	1 240
12	55	42,5	2,5	1 200	1 230
13	52,5	41,5	6	1 205	1 240

Tabelle 3. Sinterungs- und Dichtepunkte bei Zusammensetzungen der 1 300°-Isotherme im System BaO-Talk-Metakaolin.

Nr.	Zusammensetzung in %			Sinterungspunkt	Dichtepunkt
	BaO	Metakaolin	Talk	°C	°C
1	68,5	31,5	—	1 200	1 252
2	69,5	28,5	2	1 100	1 210
3	69,5	25	5,5	1 120	1 230
4	67,5	22,5	10	1 195	1 250
5	65	20	15	1 230	1 260
6	63	17	20	1 245	1 265
7	62	15	23	1 250	1 265
8	60	12	28	1 254	1 265
9	58	10	32	1 255	1 267
10	55	8	37	1 254	1 265
11	51	10	39	1 200	1 230
12	49	14	37	1 190	1 225
13	48	20	32	1 188	1 230
14	49	24	27	1 200	1 238
15	50	30	20	1 215	1 260
16	50	35	15	1 210	1 275
17	52	38	10	1 150	1 260
18	53,5	41,5	5	1 100	1 230
19	54,3	45,7	—	1 094	1 246

Tabelle 4. Sinterungs- und Dichtepunkte bei Zusammensetzungen der zweiten 1 300°-Isotherme im System BaO-Metakaolin-Talk.

Nr.	Zusammensetzung in %			Sinterungspunkt	Dichtepunkt
	BaO	Metakaolin	Talk	°C	°C
1	7	39	54	1 150	1 290
2	8	39,1	52,9	1 160	1 285
3	9,3	37,7	53	1 175	1 285
4	8	37,7	54,3	1 185	1 280

Tabelle 5. Sinterungs- und Dichtepunkte bei Zusammensetzungen der 1 250°-Isotherme im System BaO-Talk-Metakaolin.

Nr.	Zusammensetzung in %			Sinterungspunkt	Dichtepunkt
	BaO	Metakaolin	Talk	°C	°C
1	62	38	—	1 190	1 205
2	62,6	34,5	3	1 210	1 235
3	63	31	6	1 213	1 245
4	59	35	6	1 215	1 235
5	55,8	39	5,2	1 218	1 240
6	56	41,5	2,5	1 205	1 230
7	56	44	—	1 180	1 205

Geringe Zusätze von MgO respektive Talk (5—10 %) hatten in allen Fällen einen günstigen Einfluss auf das Verhältnis zwischen Schmelz- und Sinterungstemperatur — der Temperaturunterschied war bei diesen Zusammensetzungen sehr gross.

Die Sinterungskurven entlang der Zusammensetzungen der Schmelzpunktisothermen zeigen im allgemeinen viel grössere Fluktuationen als die entsprechenden Kurven der Dichtepunkte. Bei niedrigen Dichtetemperaturen ist der Unterschied zwischen diesen und den Sinterungstemperaturen relativ gross und kann bis zu 100—150° C betragen. Liegt hingegen die Aufsauggrenze bei hoher Temperatur, dann ist der Unterschied zwischen dieser und der Sinterungstemperatur gering — in einzelnen Fällen sogar nur etwa 5° C. Dies ist besonders bei den Zusammensetzungen der 1 300°-Isotherme des Systems BaO-Talk-Metakaolin der Fall.

Im System BaO-MgO-Metakaolin traten, wie oben erwähnt, bei gewissen Zusammensetzungen der 1 250°-Isothermen Unregelmässigkeiten auf. In diesem Gebiet wurden Kegelschmelzpunkte zwischen 1 250 und 1 260° C erhalten ohne diese genauer bestimmen zu können. Im Dreiecksdiagramm wurde in diesem Gebiet daher statt einer Isothermenlinie eine Schmelzpunktsfläche eingezeichnet. In diesem Gebiet ist ausserdem der Temperaturunterschied zwischen Sinterungs- und Schmelzpunkt sehr gering, die Schwindung setzt knapp unterhalb des Schmelzpunktes ein und der Schmelzverlauf selbst erstreckt sich über ein grosses Temperaturintervall. Die Kegeln zeigen ferner eine sonst nicht beobachtete Tendenz von der Basis her zu schmelzen und die Farbe der Schmelzen ist gelb-grün.

Im System BaO-Talk-Metakaolin wirkt nicht nur das Magnesiumoxyd sondern auch die Kieselsäure des Talkes als Flussmittel, was sich offenbar darin äussert, dass in diesem System *zwei* 1 300°-Isothermen auftreten. Gleichzeitig tritt eine Verschiebung der Isothermen — verglichen mit dem System BaO-MgO-Metakaolin — in der Richtung zu BaO-reicheren und kaolinärmeren Zusammensetzungen ein.

In technischer Hinsicht ist die 1 300°-Isotherme des Systems BaO-Talk-Metakaolin von grösstem Interesse: die hohen MgO- und Metakaolingehalte entlang der Isotherme und die dementsprechend niedrigen BaO-Gehalte bedingen die Anwendbarkeit dieser Zusammensetzungen für die Herstellung von Elektroporzellan. Eine gewisse Schwierigkeit dürfte nur darin liegen, dass die Dichtepunkte dieser Zusammensetzungen den Schmelzpunkten sehr nahe liegen — der Unterschied beträgt nicht mehr als etwa 5° C. Andererseits liegen jedoch die entsprechender Sinterungstemperaturen relativ niedrig, und es ist auch möglich, dass eine Erhöhung der Brenndauer über die hier verwendeten 30 Minuten hinaus bereits bei niedrigeren Temperaturen zu höherer Scherbendichte führt.

ZUSAMMENFASSUNG

Der Einfluss von BaO auf die Sinterungs- und Schmelztemperaturen im System $BaO-MgO-Al_2O_3-SiO_2$ wurde systematisch bestimmt und seine technische Bedeutung diskutiert.

Die vorliegende Untersuchung wurde mit Unterstützung von *Chalmers Forskningsfond* und *Statens Tekniska Forskningsråd* durchgeführt.

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Separation of Adenosine and Inosine Phosphates by Paper Chromatography

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In the course of a study on the dephosphorylation and deamination of adenosine triphosphate (ATP) by actomyosin, the need arose for the separation of the different adenosine- and inosine-5-phosphates, *viz.* ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine triphosphate (ITP), inosine diphosphate (IDP) and inosine monophosphate (IMP). Ion-exchange chromatography [1], paper chromatography [2-10] and electrophoresis on filter paper [11] or by a moving boundary method in the Tiselius apparatus [12] have been proposed for the separation of ATP, ADP and AMP but as far as we are aware, no separation of the different inosine phosphates from each other and from the corresponding adenosine phosphates has been hitherto described, except for the monophosphates [1,3,4].

In the present communication, our results on the separation of adenosine and inosine phosphates by paper chromatography are reported.

In spite of numerous trials with different solvent systems including those already reported in the literature together with a great number of other solvents, no satisfactory method has so far been found for the separation of all the adenosine and inosine phosphates by one-dimensional chromatography, although recent experiments with the *n*-propanol-1% ammonium sulphate solution-acetic acid (45 : 35 : 20) system [13] show promise in this respect. Mixtures containing only adenosine phosphates or only inosine phosphates are resolved by most of the methods recommended for the separation of adenosine phosphates. In our hands the procedure of Hanes and Isherwood [2], namely descending chromatography with *n*-propanol-ammonia (sp.gr. 0.880)-water (60 : 30 : 10) gave the best results. Mixtures, containing both adenosine phosphates and inosine phosphates are not resolved satisfactorily by this method, as ATP and IDP on the one hand and ADP and IMP on the other, give nearly identical spots (Fig. 1). It has been found, however, that a solvent

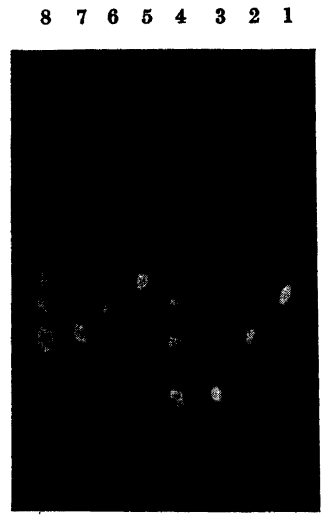

Fig. 1. One-dimensional chromatogram (Munktell No. OB paper) in n-*propanol-ammonia-water (60 : 30 : 10), 20 hours. (1) ATP; (2) ADP; (3) AMP; (4) mixture of ATP, ADP and AMP; (5) ITP; (6) IDP; (7) IMP; (8) mixture of ITP, IDP and IMP; approx. 50 μg of each compound.*

system, consisting of saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2), which had been devised [14] for the separation of nucleotides obtained by the hydrolysis of nucleic acids, separates the adenosine series of phosphates from the inosine series, although no adequate resolution occurs of the individual members in either series (Fig. 2). Combination of the two solvent systems to a two-dimensional method gives complete resolution of mixtures containing all six phosphates (Fig. 3). In the first direction, the chromatogram is developed with *n*-propanol-ammonia-water and in the second

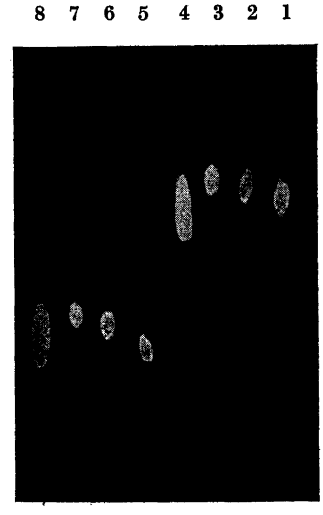

Fig. 2. One-dimensional chromatogram (Whatman No. 1 paper) in saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2), 8 hours. (1), (2), (3), (4), (5), (6), (7) and (8) same as in Fig. 1.

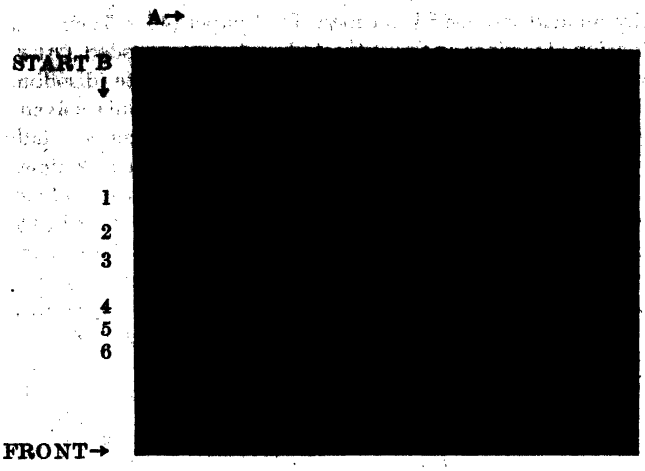

Fig. 3. Two-dimensional chromatogram (Whatman No. 1 paper) of a mixture of ATP, ADP, AMP, ITP, IDP and IMP, approx. 50 µg of each compound. (A) first solvent: n-propanol-ammonia-water (60 : 30 : 10), 40 hours; (B) second solvent: saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2), 8 hours; (1) AMP; (2) ADP; (3) ATP; (4) IMP; (5) IDP; (6) ITP.

direction, with saturated ammonium sulphate solution-water-isopropanol. This order of development gives the better results.

Application of the two-dimensional method to the separation of mixtures containing inorganic orthophosphate and pyrophosphate in addition to adenosine and inosine phosphates results in a clear separation of all components. Whereas after development in the first solvent the orthophosphate spot overlaps partly with that of ADP and IMP, and the pyrophosphate spot with that of ITP, in the second solvent both orthophosphate and pyrophosphate travel faster than any of the other components. By running the chromatogram in the ammonium sulphate system, the adenosine, inosine and inorganic phosphates appear as three sharply separated series of spots.

The results obtained by application of the method to the quantitative analysis of different adenosine and inosine phosphate preparations and the products of the reaction between ATP and actomyosin will be reported in a separate communication.

EXPERIMENTAL

ATP and ADP were prepared as the barium salts from rabbit muscle [15] and purified by ion-exchange chromatography [1]. AMP was a commercial preparation, free from ultraviolet-absorbing and phosphorus-containing contaminants. ITP, IDP and IMP were prepared from ATP, ADP and AMP by application of the method of Kleinzeller, described for ITP [16], and purified by ion-exchange chromatography [17]. From the appropriate fractions of the eluates, each compound was isolated as the barium salt and its identity confirmed by analysis (purine: acid labile P:total P). The barium salts were converted to the free acids by addition of an equivalent amount of 0.1 N sulphuric acid and the pH adjusted to 7 with ammonia. 5—10 µl of each solution, containing 10—50 µg nucleotide, were applied to the paper, the size of the starting spot not exceeding 0.5 cm in diameter. After each application, the spot was dried in a current of cold air.

Descending chromatography on acid-washed [2] Whatman No. 1 paper (46 × 53 cm) was used in all cases where determination of purines on the extracted spots was intended. Otherwise, the washing with acid was omitted and instead 0.1 % disodium versenate (disodium etylenediamine-tetraacetate dihydrate) was added [18] to the propanol-ammonia solvent. For such assays, Munktell No. OB paper has the advantage of faster running, specially in the propanol-ammonia system, whereby good resolution is obtained in shorter times. Developing times of 40 hours (20 hours with Munktell No. OB paper) in the first solvent were used without previous equilibration, by which time the solvent had overrun the paper. After previous equilibration overnight, the second solvent was run for 6—8 hours. The spots on the finished chromatogram were located by ultraviolet photography [19] or by spraying with the molybdate reagent with subsequent heating and reduction according to Hanes and Isherwood [2]. No consistent R_F-values are obtained without elaborate precautions. The relative positions of the substances, however, are reasonably constant and the spots can easily be identified by inspection. If necessary, marker substances can be run in each dimension, according to the procedure of Markham and Smith [19].

SUMMARY

A method for the separation of mixtures containing adenosine and inosine phosphates, inorganic orthophosphate and pyrophosphate by two-dimensional paper chromatography is described.

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Incorporation of Cytidylic Acids *a* and *b* Into Liver Pentose Nucleic Acid

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The existence of two isomers of cytidylic acid, both obtained from an alkaline hydrolysate of ribonucleic aid (PNA), has been demonstrated by Cohn [1] and by Loring *et al.*[2] The structure of these isomers, called cytidylic acids *a* and *b* respectively, has not been definitely established, but the evidence indicates that both isomers are derivatives of cytidine, one being the 2′-phosphate and the other the 3′-phosphate [3,4].

The biological significance of this type of isomerism, which occurs in all mononucleotides obtained from an alkaline hydrolysate of PNA, is not fully understood. Brown and Todd [4] have suggested that for the most part only one of the isomers exists in the polynucleotide and that a mixture of both is obtained through the action of alkaline hydrolysis. Studies with labelled formate [5] and adenine [6] showed no difference in the incorporation of these precursors into the *a* and *b* isomers of adenylic acids obtained from liver PNA. Differences in incorporation were found, however, when labelled adenylic acids *a* and *b* were added to growing *Lactobacillus casei* [7]. The *b* nucleotide was utilized for the synthesis of polynucleotides to an appreciable extent, while the utilization of the *a* isomer was very small and could have been due to isomerization to the *b* nucleotide during the course of the experiment.

The present experiments were carried out to investigate the metabolic pathways of the two N^{15}-labelled isomers of cytidylic acid in the rat. It was thought that the time period during which the animals received the isotopic substances should be as short as possible. In order to obtain a signifi-

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Table 1. Administration of N^{15}-cytidylic acids a *and* b *to partially hepatectomized rats. Each animal received a total of 210 mg of mononucleotide per kg of body weight divided among three equal doses given 20, 22, and 24 hours after the operation.*

		Cytidylic acid *a*		Cytidylic acid *b*	
	Isolated	N^{15} atom per cent excess	Per cent N^{15} calculated on basis of 100 per cent N^{15} in cytidylic acid injected	N^{15} atom per cent excess	Per cent N^{15} calculated on basis of 100 per cent N^{15} in cytidylic acid injected
		Injected cytidylic acid			
		4.20	100	4.20	100
PNA	Cytidine	0.097	2.3	0.123	2.9
		0.081	2.0	0.093	2.2
	Uridine	0.080	2.0	0.091	2.2
		0.071	1.7	0.079	1.9

cant incorporation of the isotope into the PNA the experiments were carried out in a rapidly growing tissue, namely regenerating rat liver, at the height of nucleic acid synthesis [8].

N^{15}-cytidylic acids *a* and *b* were obtained from biologically marked PNA and were injected subcutaneously into two groups of rats 20, 22 and 24 hours after the animals had been subjected to partial hepatectomy. Cytidine and uridine were isolated from the liver PNA in each group and analyzed for N^{15}. The results which are summarized in Table 1 indicate no significant difference in the utilization of the two isomers.

EXPERIMENTAL

Preparation of N^{15}-cytidylic acids. The starting material was biologically marked PNA, isolated from *E. Coli*, grown on an $N^{15}H_4Cl$ containing medium. The cultivation of the bacteria has been described earlier [9]. The extraction and preparation of PNA from the bacteria was carried out according to Hammarsten [10]. The free mononucleotides were prepared via the mercury salts [11] and chromatographically separated on a Dowex 50 (H^+) column using 0.1 *N* acetic acid as eluting agent [12]. The cytidylic acid fractions were localized by their characteristic light absorption in the ultra violet, pooled and evaporated to dryness *in vacuo*. Mixed cytidylic acids, totaling 950 mg, were obtained.

Separation of cytidylic acid isomers. Resolution of the isomers was carried out as described by Loring *et al* [13]. Ion exchange chromatography on Dowex 2 (formate) was

used. A solution of 800 mg of mixed cytidylic acids in 20 ml of water was made with the aid of sodium hydroxide (final pH 8) and adsorbed on a Dowex 2 (formate) column, length 20 cm, diameter 4 cm. Elution was carried out with 0.05 *N* formic acid, which was run through the column at a rate of 0.5 ml/min. In contrast to the experiments of Loring *et al*[13]. complete separation of the two isomers was obtained, probably because of the much smaller load on the column in the present experiment.

The combined fractions for each isomer were neutralized to pH 7 with *N* NaOH, and each solution was adsorbed on a Dowex 2 (formate) column, length 3 cm, diameter 1 cm. On elution with 0.5 *N* formic acid each isomer was eluted in a small volume and could be crystallized directly in good yield by the addition of two volumes of alcohol. Yields of 210 mg of cytidylic acid *a* ($[\alpha]_D = +48°$, *c* 0.5 per cent, at pH 11) and 290 mg of cytidylic acid *b* ($[\alpha]_D = -8°$, *c* 0.5 per cent, at pH 11) were obtained.

Administration of isotope — Eight rats, each weighing about 200 g were subjected to partial hepatectomy by a previously described technique [14]. The rats were divided into four groups each consisting of two animals. Twenty hours after the operation groups 1 and 2 received a subcutaneous injection of cytidylic acid *a*, groups 3 and 4 of cytidylic acid *b*. The injections were repeated 22 and again 24 hours after the operation. Each rat received a total of 210 mg of mononucleotide per kg of body weight equally divided among the three injections. The rats were killed 26 hours after the operation, and the livers of each group were pooled.

Preparation of pyrimidine nucleosides. Polynucleotides were prepared from the livers of each group according to Hammarsten [10]. The pyrimidine nucleosides from PNA were prepared as described earlier [15].

DISCUSSION

The present results do not show any significant difference in the incorporation of the two isomers of cytidylic acid into pyrimidines of PNA. They differ in this respect from the results obtained by Balis *et al.*[7] with *Lactobacillus casei*, in which the *b* nucleotide of adenylic acid was utilized to a far greater extent than was the *a* isomer *.

It has been shown earlier [16] that N^{15}-cytidine is an effective precursor of the pyrimidines of PNA. The present findings can therefore be expalined either by the assumption that both isomers of cytidylic acid are utilized by the rat for the synthesis of PNA to about the same extent, or that each of them is first dephosphorylated to cytidine and then utilized.

SUMMARY

N^{15}-cytidylic acids *a* and *b* were prepared from biologically marked PNA. After subcutaneous administration of each isomer to partially hepatectomized rats it was found that both mononucleotides were utilized to about the same extent for the synthesis of ribonucleic acid pyrimidines in regenerating rat liver.

* Brown and collaborators have found, however, that a difference in utilization of the two adenylic acids does not exist in the rat (personal communication from Dr. Brown).

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Short Communications

Platinum Compounds of Alkadienes (Diolefins)

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Zeise [1] in 1830 described some very interesting platinum compounds containing ethylene, *viz.* $K[PtCl_3C_2H_4]$ and $PtCl_2, C_2H_4$ *. Anderson [2] in 1934 found the latter compound to be dimeric. Other alkenes have yielded analogous compounds (see the review by Keller [3]), but until recently no compound containing more than one molecule of the alkene to one atom of platinum had been described. In 1950, however, Chatt and Wilkins [4] succeeded in preparing the compound $[PtCl_2(C_2H_4)_2]$ which is stable below −10°. Their recent publication [5] has prompted me to mention that in 1937 I prepared some platinum compounds of alkadienes in which one molecule appears to occupy two co-ordination places. This observation was not published because I intended to investigate these compounds further but pressure of other work prevented this.

When an excess of biallyl (1,5-hexadiene), $CH_2=CH-CH_2-CH_2-CH=CH_2$ is added to an aqueous solution of K_2PtCl_4, a precipitate slowly separates. This precipitate recrystallizes from chloroform to yield a pale yellow crystalline compound of composition $PtCl_2 \cdot C_6H_{10}$ and m.p. 173° (dec.). The corresponding iodide $PtI_2 \cdot C_6H_{10}$ is obtained in a similar way. The isomeric hexadiene, 2,4-hexadiene, behaves very differently: with K_2PtCl_4 only free platinum is obtained and with K_2PtI_4 a black precipitate consisting mainly of PtI_2. Compounds derived from the lower homologue butadiene have been prepared both by Gelman [6] and by Chatt and Wilkins [5], but the butadiene molecule seems only to occupy one co-ordination place in these compounds.

Diallylether gives a compound, $PtCl_2(C_6H_{10}O)$, which closely resembles the diallyl compound. Divinylether, on the other hand, reduces K_2PtCl_4 to free platinum. On addition of an excess of divinylether to K_2PtI_4, a dark red precipitate (resembling CrO_3) immediately separates, but this compound is very unstable and rapidly turns brown. Analysed as soon after formation as possible, it has the composition corresponding to platinum iodide with 1/2 molecule of divinylether, but the product smells of divinylether and may

* It has not been generally recognised that Zeise prepared this compound. Anderson writes: "Zeise considered that he had obtained the basic compound of the series, $PtCl_2 \cdot C_2H_4 \cdots$ His product consisted probably of the more or less decomposed acid of the series". As a matter of fact, however, Zeise's analysis of his *"platinum chloridæ inflammabile"* (*l.c.* § 39) corresponds excellently with the composition he proposed: Found (Zeise): Pt 66.53; Cl 23.88; C 8.25; H 1.34. Calc. for $PtCl_2 \cdot C_2H_4$: Pt 66.34; Cl 24.14; C 8.16; H 1.36. Also the properties described by Zeise are in agreement with those of the compound $PtCl_2 \cdot C_2H_4$, with the exception that the colour is described by Anderson as orange and by Zeise as very slightly yellow (*"citrini coloris perquam pallidi"*). This may, however, simply be a question of crystal size (Anderson recrystallized his product from benzene).

have contained 1 molecule at the beginning.

The behaviour of biallyl and diallylether is thus very different from that of similar compounds with smaller distances between the double bonds. It thus seems probable that both double bonds in the longer diolefins are involved in complex formation giving chelated complexes. Other compounds of the type $PtCl_2 \cdot$ alkene are dimeric, but the compounds of biallyl and diallylether were found by cryoscopic measurements to be monomeric, in accordance with the view that these compounds are chelate compounds.

This conclusion is corroborated by measurement of the dipole moments of these compounds. Although the compounds are very slightly soluble in benzene it is possible to determine the order of magnitude of their dipole moments which was found to be 6 – 7 D. This high value is only compatible with the assumption that the compounds are *cis*-compounds, and this is the only possible configuration if the diolefins occupy two coordination places.

Biallyl also gives a palladium compound with the composition $PdCl_2 \cdot C_6H_{10}$ by reaction with the benzonitrile complex $[PdCl_2(C_6H_5CN)_2]$.

An attempt to determine the structures of the biallyl compounds using X-rays was frustrated by the decomposition of the compounds. They blackened in the X-ray beam and the only identifiable reflections were due to metallic platinum and palladium.

EXPERIMENTAL

Biallyl-dichloroplatinum(II). Biallyl (1 ml) was added to a solution of K_2PtCl_4 (2 g) in water (10 ml) and the mixture shaken vigorously for a few minutes. On standing a precipitate slowly separated, and after 2 days it was filtered off (yield 1.3 – 1.5 g). Further addition of biallyl caused the separation of a little more of the same compound and the yield was almost quantitative (calc. 1.7 g) the filtrate from the last precipitate was almost colourless. At 50° the reaction occurred more rapidly but the result was essentially the same. The compound was recrystallized from chloroform and obtained in pale yellow crystals, m.p. 172 – 73° (benzene gives a better recovery but more recrystallizations are necessary to obtain the pure compound); it was rather soluble in chloroform, less so in benzene and almost insoluble in ether. It dissolved somewhat in boiling water but soon decomposed with the separation of a brown precipitate. The melting point was sharp, and under the microscope the melting was seen to occur without decomposition, but the melt soon turned black. Found: C 20.51; H 3.05; Pt 56.1; Cl 20.17. Calc. for $[PtCl_2(C_6H_{10})]$, (348.3): C 20.69; H 2.89; Pt 56.0; Cl 20.36. Molecular weight (cryoscopically in bromoform solution): 369, 382, 364. Dipole moment, $\mu = 6.1$ ($c = 0.00385$ molar in benzene, $\Delta\varepsilon = 0.0180$).

According to Gelman [7] biallyl can replace ethylene from $K[PtCl_3C_2H_4]$, probably with the formation of the compound $K_2[Cl_3PtC_6H_{10}PtCl_3]$. This compound was also formed as an intermediate in the preparation of the compound $[PtCl_2C_6H_{10}]$, especially when the reaction tookplace in acid solution or when no excess of biallyl was used.

Biallyl (1 ml) and 4 *N* hydrochloric acid (0.5 ml) were added to a solution of K_2PtCl_4 (2 g) in water (10 ml). A crystalline mustard-yellow precipitate soon began to separate. After two hours it was filtered off (yield 0.7 g). It was almost completely soluble in water at room temperature; on longer standing the yield was larger but more of the insoluble compound $[PtCl_2C_6H_{10}]$ was formed. The water-soluble substance could not be crystallized from water because it was partly decomposed in boiling water or by evaporation of an aqueous solution. The product was purified by extraction with boiling chloroform which dissolves $[PtCl_2C_6H_{10}]$. The yellow crystals have the composition $K_2[Pt_2Cl_6C_6H_{10}]$: Found: C 9.73; H 1.42. Calc. C 9.43; H 1.31. By addition of $[Pt(NH_3)_4]$ to an aqueous solution of this compound an insoluble cream-coloured precipitate, consisting of $[Pt(NH_3)_4]$ $[Pt_2Cl_6C_6H_{10}]$, was formed. Found: C 7.59; H 2.32; N 5.90; Pt 61.73. Calc. C 7.72; H 2.49; N 6.04; Pt 62.75.

Biallyl-di-iodoplatinum(II). Biallyl (0.5 ml) was added to a solution of K_2PtCl_4 (1 g) and KI (1.5 g) in tepid water (10 ml). After 1 hour the precipitate was filtered off (yield 0.8 g), washed with water, dried and recrystallized from chloroform. The compound forms orange-red crystals; on heating it turns black without melting. Found: C 13.24; H 2.06;

Pt 35.75. Calc. for $[PtI_2C_6H_{10}]$ (547.2): C 13.17; H 1.84; Pt 35.67.

Biallyl-dichloropalladium(II). Biallyl (0.17 ml) was added to a solution of the benzonitrile complex[8] $[PdCl_2(C_6H_5CN)_2]$ (0.5 g) in benzene (10 ml). A brownishyellow, crystalline precipitate separated. The quantity was increased by addition of petroleum ether and cooling in ice (yield 0.30 g). Found: C 28.58; H 4.12. Calc. for $[PdCl_2(C_6H_{10})]$: C 27.74; H 3.88.

Diallylether-dichloroplatinum(II). Diallylether (0.80 g) was added to a solution of K_2PtCl_4 (2 g) in water (10 ml). An almost white precipitate soon began to separate. After 3 days the precipitate was filtered off (yield 1.00 g). The dry product was dissolved in warm chloroform (100 ml) and carbon tetrachloride (50 ml) added to until crystallization just started. As the solution cooled the compound separated as a fine, white crystalline powder, m.p. 180° (dec.). Found: C 20.05; H 2.94; Pt 53.71. Calc. for $[PtCl_2(C_6H_{10}O)]$ (364.3): C 19.78; H 2.77; Pt 53.57. Molecular weight (cryoscopically in bromoform): 344.

The assistance of Mr. Chr. Petersen in checking and completing my old observations is gratefully acknowledged. I also wish to thank Dr. J. Chatt, Welwyn, for reading the manuscript.

1. Zeise, W. C. *De chlorido platinæ et alcohole vini sese invicem permutantibus nec non de novis substantiis inde oriundis.* Anniversary Volume of the University of Copenhagen, 1830. Complete German translations of this paper appeared in: *J. Physik und Chemie (Schweigger-Seidel)* **62** (1831) 393; **63** (1831) 121; *Ann. Physik und Chemie (Poggendorf)* **21** (1831) 497. A short summary of the last translation appeared in: *Magazin für Pharmazie* **35** (1831) 105. (In Keller's review this article is erroneously cited as Zeise's original paper).
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Platinum Compounds of *Cyclo*-octatetraene *

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In the preceding article platinous complexes of some alkadienes were described. I have prepared similar derivatives of *cyclo*octatetraene. A compound of composition close to $PtCl_2$, C_8H_8 separates as an orange-yellow precipitate when *cyclo*octatetraene is added to an aqueous solution of K_2PtCl_4. It is insoluble in water and organic solvents and therefore could not be purified. However, the corresponding iodide could be recrystallised from chloroform and was obtained as a beautiful orange-red (dichromate-coloured) crystalline powder of the exact composition PtI_2, C_8H_8.

The platinum atom in these compounds is certainly co-ordinated to one or more double bonds. The unlikely possibility that the platinum atom be bound to all 4 double bonds is definitely ruled out because compounds of the type $[Pta_4]Cl_2$ are generally colourless, easily soluble in water, and contain ionised chlorine. This is not the case with the *cyclo*octatetraene compound. Neither can the platinum atom be bound to only one double bond because the compound would then be dimeric, as is generally true of platinum compounds of the type $PtCl_2a$ (a = amine, phosphine, alkyl sulphide, etc.). A cryoscopic determination of the molecular weight in bromoform solution shows that PtI_2,C_8H_8 is monomeric; thus it seems that *cyclo*octatetraene occupies two co-ordination places, in the same way as biallyl. According to the preceding paper a certain distance between the double bonds is necessary for the formation of chelate compounds by

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In the preceding article platinous complexes of some alkadienes were described. I have prepared similar derivatives of *cyclo*octatetraene. A compound of composition close to $PtCl_2$, C_8H_8 separates as an orange-yellow precipitate when *cyclo*octatetraene is added to an aqueous solution of K_2PtCl_4. It is insoluble in water and organic solvents and therefore could not be purified. However, the corresponding iodide could be recrystallised from chloroform and was obtained as a beautiful orange-red (dichromate-coloured) crystalline powder of the exact composition PtI_2, C_8H_8.

The platinum atom in these compounds is certainly co-ordinated to one or more double bonds. The unlikely possibility that the platinum atom be bound to all 4 double bonds is definitely ruled out because compounds of the type $[Pta_4]Cl_2$ are generally colourless, easily soluble in water, and contain ionised chlorine. This is not the case with the *cyclo*octatetraene compound. Neither can the platinum atom be bound to only one double bond because the compound would then be dimeric, as is generally true of platinum compounds of the type $PtCl_2a$ (a = amine, phosphine, alkyl sulphide, etc.). A cryoscopic determination of the molecular weight in bromoform solution shows that PtI_2,C_8H_8 is monomeric; thus it seems that *cyclo*octatetraene occupies two co-ordination places, in the same way as biallyl. According to the preceding paper a certain distance between the double bonds is necessary for the formation of chelate compounds by

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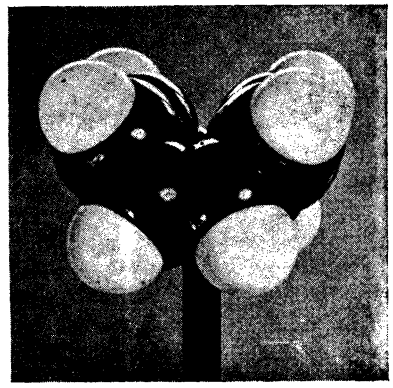
diolefins, thus if the platinum atom is bound to two of the double bonds in *cyclo*-octatetraene these cannot be neighbouring double bonds. Actually a very plausible formula for the complex can be constructed if it is supposed that $PtCl_2$ or PtI_2 is bound to two double bonds in the tub form of *cyclo*octatetraene (see the figure); a platinum atom with radius *ca.* 1.3 Å can form two bonds with valence angle 90° perpendicular to two of the double bonds. Most investigations of the molecular structure of *cyclo*octatetraene have been inconclusive, but a recent electron diffraction investigation [1] is strongly in favour of the tub configuration (D_{2d}).



The structure proposed for the platinum compounds is corroborated by the dipole moment of the iodide, which shows the compound to have the *cis*-configuration.

*Cyclo*octatetraene is easily transformed into other compounds and can, for instance, be isomerised to form styrene. The platinous compounds prepared from *cyclo*-octatetraene are, however, quite different from the platinous compounds of styrene, prepared by Anderson [2]. Furthermore, *cyclo*octatetraene — easily recognised by its odour — is liberated by boiling a suspension of the platinum compounds in water to which some pyridine or potassium cyanide has been added. When pyridine is added to a chloroform solution of $[PtI_2(C_8H_8)]$ *cyclo*octatetraene is liberated and a yellow crystalline precipitate of $[PtI_2py_2]$ is formed (no compound containing both *cyclo*octatetraene and pyridine could be prepared).

The filtrate from $[PtCl_2(C_8H_8)]$ still contains platinum and was tested to see whether it contained a soluble *cyclo*octatetraene platinous complex, *e.g.* corresponding to Zeise's acid or the biallyl complex described in the preceding paper. The warm (50°) filtrate, by addition of $[Pt(NH_3)_4]Cl_2$, yielded a pink precipitate; at room temperature a silver-grey precipitate was obtained and from the icecold filtrate a green precipitate. Although the grey and pink precipitates are formed at higher temperature than the green one they are both transformed into the latter by boiling with water. These precipitates contained only traces of C and H and had compositions close to that of Magnus' green salt, $[Pt(NH_3)_4]$ $[PtCl_4]$, which is known to exist also in a red form [3]; the silvery precipitate is a mixture of both forms. The tendency to yield the red form appears to be rather greater than usual, and it may be that the dimensions of the unit cell are modified by the presence of a small amount of a compound $[Pt(NH_2)_4]$ $[PtCl_3$ $C_8H_8PtCl_3]$

EXPERIMENTAL.

Cyclo-octatetraene-dichloroplatinum (II). *Cyclo*octatetraene (1 g) was added to a solution of K_2PtCl_4 (2 g) in water at 60° (10 ml) and the solution shaken vigorously. An orange-coloured precipitate soon began to separate and, after the mixture had stood overnight, the precipitate was filtered off and washed with water. Found: C 26.18; H 2.07; Pt 52.21. Calc. for $[PtCl_2 \cdot C_8H_8]$, (370.2): C 25.95; H 2.18; Pt 52.72.

Cyclooctatetraene-di-iodoplatinum (II). K_2PtCl_4 (2 g) was dissolved in water (10 ml) at 60°, KI (3 g) was added and the solution filtered and cooled. *Cyclo*octatetraene

(1 g) was then added with vigorous shaking. A precipitate immediately separated. After standing for 24 hours, the yellow precipitate was filtered off, washed and dried. Yield: 2.6 g (calc. 2.8 g). The compound was dissolved in 100 ml boiling chloroform, the solution filtered and cooled in ice. Orange-red (dichromate-coloured) crystals separated. The compound was recrystallised once more from chloroform. It does not melt on heating, but gradually turns black without change of the crystal form. Found: C 17.59; H 1.59; Pt 34.97. Calc. for $[PtI_2(C_8H_8)]$, (553.2) : C 17.37; H 1.46; Pt 35.38.

Molecular weight (cryoscopically in bromoform solution): 492, 610. Dipole moment:

c = 0.00198 molar, $\Delta\varepsilon$ = 0.0130, μ = 7.3D;
c = 0.00406 molar, $\Delta\varepsilon$ = 0.0240, μ = 7.0D.

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Release of Hydantoins from Proteins *

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In a note by the author [1], the procedure of Fraenkel-Conrat and Fraenkel-Conrat [2] for the release of hydantoins from proteins was erroneously quoted, the temperature for the reaction being given at 75° C instead of 36° C (*cf.* Fraenkel-Conrat and Fraenkel-Conrat [3]).

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The Occurrence in Lichens of the Folic Acid-, Folinic Acid-, and Vitamin B_{12}-Group of Factors

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In connection with investigations on the folic acid-, folinic acid-, and vitamin B_{12}-group of factors in algae it was considered of interest to study the occurrence of these factors in some lichens, a group of plants known to represent a symbiosis between fungi and algae. The following lichens have been studied using microbiological and bioautographic methods: *Cladonia silvatica, Umbilicaria pustulata, Parmelia physodes, Parmelia furfuracea, Cetraria islandica, Evernia prunastri, Alectoria jubata,* and *Usnea comosa.* The lichens were collected at Grönvik, Nämdö.

Escherichia coli 113-3[1] served as a test organism for the vitamin B_{12}-factors, *Leuconostoc citrovorum* ATCC 8 081[2] for the folinic acid and *Streptococcus faecalis* ATCC 8 043 for the folic acid tests. The organisms were utilized in the agar cup plate method. For *Streptococcus faecalis* the Difco folic acid assay medium with 1.6 % Bacto agar was used [3]. The solvent for the chromatographic separation was sec. butanol saturated with water, and containing 3 % acetic acid and 25 mg KCN/l.

The lichens were carefully cleaned and dried at room temperature. Two grammes of finely ground material were suspended in 25 ml of water or buffer solution. Three different methods of freeing the active substances were tried, namely, extraction with water at 37° C for 24 hrs, autoclaving for 20 min. at 120° C in water containing small amounts of KCN, and enzymatic treatment with a chicken pancreas homogenate at 37° C for 24 hrs at pH 7.5. Autoclaving gave the highest

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Table 1. Total activity expressed in μg standard per g dry weight of lichens.

Lichenes *	*E. coli* Vit. B_{12} std.	*L. citrovorum* Leucovorin std	*S. faecalis* Folvite std.
Cladonia silvatica	0.035	0.68	0.26
Umbilicaria pustulata	0.02	0.19	0.24
Parmelia furfuracea	< 0.005	0.09	0.43
Parmelia physodes	0.02	2.35	1.16
Cetraria islandica	< 0.005	1.80	1.16
Evernia prunastri	0.008	0.06	0.26
Alectoria jubata	< 0.005	0.01	0.41
Usnea comosa	0.015	0.28	0.41

* Symbiosis of fungi belonging to *Discomycetes*, order *Lecanorales* and algae belonging to *Chlorophyceae*, order *Chlorococcales*.

values for *E. coli*, enzymatic treatment the highest values for *L. citrovorum* and *S. faecalis*.

The results are given in Table 1.

Table 2 shows the various types of growth factors for *S. faecalis* and *L. citrovorum* as revealed after chromatographic separation of the lichen extracts.

The experiments with *E. coli* showed the presence in the lichens of substances having vitamin B_{12}-like activity in a concentration of from less than 0.005 to 0.035 μg B_{12}/g dry weight. This comparatively low activity was found to be due to three different vitamin B_{12}-factors having the same R_F-values as vitamin B_{12},

Table 2. L. Citrovorum and S. faecalis factors in lichens.

Factors	R_F-values	*L. citrovorum* activity in samples A	ET	*S. faecalis* activity in samples A	ET
Unidentified	0.07			+	+
Pteroic acid	0.10				
Unidentified	0.18		++++	+	+++
Pteroyltriglutamic acid	0.22			+	++
Unidentified	0.24	+	+		
Folic acid	0.27			(+)	(+)
Unidentified	0.37			+	+
Formylpteroylglutamic acid	0.45			+++	
Folinic acid	0.55	++	+	++	+
Formylpteroic acid	0.65			+	++
Thymidine	0.70	+	+	+	+

The number of plus signs indicates the intensity of the growth caused by the different factors as observed after chromatographic separation. A = autoclaved, ET = enzyme treated.

vitamin B_{12s}[4] and factor C[5,6]*, respectively. No evidence was found for the presence of factors having the same R_F-values as pseudovitamin B_{12}[7], vitamin B_{12f}[8], vitamin B_{12m}[9], factor A[5], or factor B[5]. The presence of vitamin B_{12s} is of interest because to date its only other source has been marine algae[4].

For *L. citrovorum* four growth factors were found. Two of these have been identified as the natural citrovorum factor[10,11] (folinic acid) (R_F 0.55) and thymidine (R_F 0.70). The other two factors had R_F-values of 0.18 and 0.24. It seems likely that these factors are identical with two of the citrovorum factors other than folinic acid found in a red marine algae *Furcellaria fastigiata*[12].

Altogether nine substances stimulating the growth of *S. faecalis* were observed. Folinic acid and thymidine, which stimulate the growth of both *S. faecalis* and *L. citrovorum*, and factors with the same R_F-values as pteroyltriglutamic acid (Teropterin, Lederle), N_{10}-formylfolic acid and N_{10}-formylpteroic acid (Rhizopterin) were found. Only traces of folic acid (pteroylglutamic acid) could be detected. The other factors observed had approximately the R_F-values 0.07, 0.18, and 0.37 respectively in this solvent system.

Enzymatic treatment of the extracts did not lead to release of folic acid (pteroylglutamic acid) or to folinic acid (N_5-formyl-tetrahydrofolic acid), but substances with lower R_F-values. It was also observed that the enzyme complex of the chicken pancreas homogenate did not seem to release folic acid from a solution of crystalline pteroyltriglutamic acid (Teropterine, Lederle). This is in agreement with previous findings[14,15]. Instead it converted the pteroyltriglutamic acid to a growth factor for *L. citrovorum* which is not folinic acid. The value of the total activity for *S. faecalis* and *L. citrovorum* in Table 1 do not therefore represent the true folic acid or folinic acid content of the lichens.

Most lichens investigated contained substances having an antibiotic effect on *E. coli*, *S. faecalis* and *L. citrovorum*, possibly due to usnic acid.

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We are also grateful to Dr. M. Waern, Institute of Plant Ecology, Upsala, Sweden, for botanical classification of the lichens.

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* A factor found in sewage sludge[6] and showing the properties of factor C has been used for comparison.

(1 g) was then added with vigorous shaking. A precipitate immediately separated. After standing for 24 hours, the yellow precipitate was filtered off, washed and dried. Yield: 2.6 g (calc. 2.8 g). The compound was dissolved in 100 ml boiling chloroform, the solution filtered and cooled in ice. Orange-red (dichromate-coloured) crystals separated. The compound was recrystallised once more from chloroform. It does not melt on heating, but gradually turns black without change of the crystal form. Found: C 17.59; H 1.59; Pt 34.97. Calc. for $[PtI_2(C_8H_8)]$, (553.2) : C 17.37; H 1.46; Pt 35.38.

Molecular weight (cryoscopically in bromoform solution): 492, 610. Dipole moment:

c = 0.00198 molar, $\Delta\varepsilon$ = 0.0130, μ = 7.3D;
c = 0.00406 molar, $\Delta\varepsilon$ = 0.0240, μ = 7.0D.

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Release of Hydantoins from Proteins *

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In a note by the author [1], the procedure of Fraenkel-Conrat and Fraenkel-Conrat [2] for the release of hydantoins from proteins was erroneously quoted, the temperature for the reaction being given at 75° C instead of 36° C (*cf.* Fraenkel-Conrat and Fraenkel-Conrat [3]).

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* *Correction to* Attempted Successive Applications of the Edman Degradation to Insulin [1].

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The Occurrence in Lichens of the Folic Acid-, Folinic Acid-, and Vitamin B_{12}-Group of Factors

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In connection with investigations on the folic acid-, folinic acid-, and vitamin B_{12}-group of factors in algae it was considered of interest to study the occurrence of these factors in some lichens, a group of plants known to represent a symbiosis between fungi and algae. The following lichens have been studied using microbiological and bioautographic methods: *Cladonia silvatica, Umbilicaria pustulata, Parmelia physodes, Parmelia furfuracea, Cetraria islandica, Evernia prunastri, Alectoria jubata,* and *Usnea comosa.* The lichens were collected at Grönvik, Nämdö.

Escherichia coli 113-3[1] served as a test organism for the vitamin B_{12}-factors, *Leuconostoc citrovorum* ATCC 8 081[2] for the folinic acid and *Streptococcus faecalis* ATCC 8 043 for the folic acid tests. The organisms were utilized in the agar cup plate method. For *Streptococcus faecalis* the Difco folic acid assay medium with 1.6 % Bacto agar was used [3]. The solvent for the chromatographic separation was sec. butanol saturated with water, and containing 3 % acetic acid and 25 mg KCN/l.

The lichens were carefully cleaned and dried at room temperature. Two grammes of finely ground material were suspended in 25 ml of water or buffer solution. Three different methods of freeing the active substances were tried, namely, extraction with water at 37° C for 24 hrs, autoclaving for 20 min. at 120° C in water containing small amounts of KCN, and enzymatic treatment with a chicken pancreas homogenate at 37° C for 24 hrs at pH 7.5. Autoclaving gave the highest

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10-Aminoacylphenothiazines

III. Quaternary salts

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Quaternization of pharmacologically active tertiary amines usually leads to compounds which differ from the parent amines in their pharmacological properties. In general, quaternization enhances the neurotropic antispasmodic effect while the musculotropic effect decreases [1]; the antihistamine activity may remain unchanged but is generally diminished [2]; the local anesthetic effect is almost completely destroyed [3]. Numerous exceptions from these generalisations are of course known.

The 10-aminoacylphenothiazines described in the previous papers in this series [4,5] have an unusually great range of action, as most of them possessing antihistaminic, antispasmodic, local anesthetic, and nicotinolytic action to a varying extent. It was considered by the present author therefore, that quaternization of these tertiary amines would afford an excellent opportunity of studying the general effect of this procedure on different pharmacological properties.

Most of the quaternary salts were prepared by the alkylation of a 10-dialkylaminoacylphenothiazine with a methyl or ethyl halide. The aminoacetyl and β-aminopropionyl derivatives were most reactive; α-Diethylaminopropionyl-, and β- and γ-diethylaminobutyrylphenothiazine could not be quaternized. When for instance the α-diethylaminopropionyl compound was heated with ethyl bromide, the hydrobromide of the tertiary amine starting material was formed.

A few quaternary salts were prepared from a halogenoacylphenothiazine and a tertiary amine. Only halogenoacetylphenothiazines reacted smoothly with amines in this way. When other halogenoacyl derivatives were treated with a tertiary amine, hydrogen halide was usually split off from the halogenoacyl group, and the hydrohalide of the amine was obtained as the only product. Pyridine, however, yielded quaternary pyridinium salts.

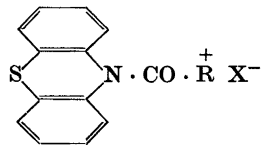
Most of the salts and especially the iodides were slightly soluble in water (usually < 1 %). Their aqueous solutions were rather unstable and became opalescent in a couple of days. In weakly alkaline solutions the salts were decomposed instantaneously into phenothiazine and the corresponding betaine.

The quaternary salts have been tested for local anesthetic, antispasmodic and antihistaminic activity by the same technique as the tertiary amines in the previous communications in this series *, and also for nicotinolytic and ganglion-blocking effect.

Local anesthetic effect. All compounds tested were inactive on rabbit *cornea.* Thus the quaternization had destroyed the strong effect of the parent amines.

Antispasmodic effect. The results of the tests on isolated guinea pig *ileum* for cholinolytic activity are shown in Table 1. The quaternary salts with one exception (compound XII) were more active than the parent tertiary amines. No consistent relationship between cholinolytic activity and chemical structure could be discerned.

Table 1. Cholinolytic and antihistaminic properties of quaternary salts of 10-aminoacyl-phenothiazines.



	R	X	Effect * in reducing the spasm produced by	
			Acetyl-choline	Histamine
I	$-CH_2 \cdot N(CH_3)_3$	Br	2.7	0.02
III	$-CH_2 \cdot N(CH_3)_2C_2H_5$	I	13	
IV	$-CH_2 \cdot N(C_2H_5)_2CH_3$	I	19	
V	$-CH_2 \cdot N(C_2H_5)_3$	Cl	36	0.03
VII	$-CH_2 \cdot NC_5H_5$ **	Cl	28	0.04
VIII	$-CH(CH_3) \cdot N(CH_3)_3$	I	16	
IX	$-CH(CH_3) \cdot NC_5H_5$ **	I	2.7	0.025
X	$-CH_2 \cdot CH_2 \cdot N(CH_3)_3$	Br	14	0.1
XII	$-CH_2 \cdot CH_2 \cdot N(CH_3)_2C_2H_5$	Br	4	0.02
XIV	$-CH_2 \cdot CH_2 \cdot N(C_2H_5)_2CH_3$	Br	12	0.04
XVI	$-CH_2 \cdot CH_2 \cdot NC_5H_5$ **	Cl	37	
XVII	$-CH_2 \cdot CH(CH_3) \cdot N(CH_3)_3$	Br	36	0.1
XVIII	$-CH_2 \cdot CH(CH_3) \cdot N(CH_3)_2C_2H_5$	Br	28	0.3
XIX	$-CH(CH_3) \cdot CH_2 \cdot N(CH_3)_3$	Br	3.5	
XX	$-(CH_2)_3 \cdot N(CH_3)_3$	I	22	0.07
	Atropine sulphate		30	
	Diphenhydramine-HCl		1.0	1.0

* The activity figures refer to the cationic part of the salts.
** Pyridinium.

* Acknowledgement is made to Dr. S. Wiedling of Astra's Biological Department for performing these tests. Details will be published elsewhere.

The quaternary salts have also been tested for musculotropic spasmolytic activity on spasm of guinea pig small intestine caused by barium chloride. The results were, however, rather confusing. This may be due to the recent finding, that barium ions do not produce a purely musculotropic spasm on guinea pig small intestine [6,7], but also stimulate the ganglion cells, thereby causing the release of the peripheric mediator at the nerve endings. Thus the spasm produced by barium is of "mixed" nature and can be abolished or reduced not only by musculotropic antispasmodics but also by anticholinergic or ganglion-blocking agents. It is evident, that a test may easily give confusing results especially with compounds having a broad range of action. Edlund and Lohi [7] found, however, that if rat *ileum* was used for the tests, the contractions caused by barium were unaffected by anticholinergic and ganglion-blocking drugs and are apparently of purely musculotropic origin. The quaternary salts described in this paper have been tested by this method together with the parent amines. The results will be published elsewhere [8], but it may be mentioned, that quaternization strongly decreases the high activity of the tertiary amines.

Antihistaminic effect. The results from the tests are shown in Table 1. Quaternization usually lowered the activity, but the two quaternary β-butyryl derivatives were more active than the parent amines.

Nicotinolytic effect. Some of the compounds have been tested for their ability to inhibit nicotine-induced tremors in rabbits *. It was found, that quaternization destroyed the nicotinolytic effect of the tertiary amines [9].

Ganglion-blocking effect. The salts showed in general a very strong ganglion-blocking activity when tested on the peristaltic reflex. The results will be published elsewhere [10].

EXPERIMENTAL

Halogenoacylphenothiazines

In addition to the halogenoacylphenothiazines previously reported [4,5] the following compounds of this class were prepared.

10-Bromoacetylphenothiazine. A solution of phenothiazine (10 g, 0.05 mole), and bromoacetyl bromide [11] (15.2 g, 0.075 mole) in benzene (50 ml) was refluxed for two hours. On cooling, the reaction product separated (8.5 g, 53 %) and was recrystallised twice from ethanol – light petroleum (1 : 1); m.p. 121–122°. (Found: C 52.5; H 3.26; Br 25.1. $C_{14}H_{10}BrNOS$ (320.2) requires C 52.5; H 3.15; Br 25.0 %.)

10-Iodoacetylphenothiazine. A mixture of 10-chloroacetylphenothiazine (5.5 g, 0.02 mole), potassium iodide (6.6 g, 0.04 mole), and acetone (50 ml) was refluxed for two hours. After filtration, the solvent was evaporated, and the crude product (5.6 g, 76 %) was recrystallised from ethanol yielding yellow crystals melting at 129–131°. (Found: C 45.6; H 2.84; I 34.6. $C_{14}H_{10}INOS$ (367.2) requires C 45.8; H 2.75; I 34.6 %.)

10-(β-Bromopropionyl)-phenothiazine. This compound was prepared in 82 % yield from β-bromopropionyl chloride ** and phenothiazine as described for the corresponding chloro compound; m.p. 144–145° (from ethanol). (Found: C 54.5; H 3.62; Br 24.0. $C_{15}H_{12}BrNOS$ (334.2) requires C 53.9; H 3.62; Br 23.9 %.)

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* Acknowledgement is made to Dr. T. Edlund, Uppsala, for performing these tests.

** β-Bromopropionyl chloride was prepared according to Rajagopalan [12]. The boiling point of the product was 65–67°/25 mm in agreement with the b.p. recorded by Hamilton and Simpson [13] but at variance with the value reported by Rajagopalan (115–117°/30 mm).

10-(γ-Chlorobutyryl)-phenothiazine. Prepared in 86 % yield by refluxing γ-chlorobutyryl chloride [14] and phenothiazine in toluene for six hours; m.p. 95—96° (from methanol). (Found: C 63.1; H 4.49; Cl 12.1. $C_{16}H_{14}ClNOS$ (303.8) requires C 63.3; H 4.64; Cl 11.7 %.)

Quaternary salts

The quaternary salts were prepared in essentially the same way. The appropriate dimethylamino- or diethylaminoacylphenothiazine was dissolved in acetone or nitrobenzene and an excess of the methyl or ethyl halide was added. In most cases the quaternary salt began to crystallise immediately. The mixture was allowed to stand over night at room temperature, and the product was collected, washed with acetone, and dried. In a few cases it was necessary to heat the reactants. The salts usually separated in a quite pure state. Most of them were recrystallised, but this procedure was omitted with some compounds, as the melting points and analyses seemed to indicate that they tended to decompose. A few of the quaternary salts were prepared by the reaction of haloacylphenothiazines with tertiary amines.

10-(Dimethylaminoacetyl)-phenothiazine methobromide (I). 10-(Dimethylaminoacetyl)-phenothiazine (2.0 g) was dissolved in acetone (20 ml), and a solution of methyl bromide (10 ml) in acetone (10 ml) was added. The salt began to separate instantly, and was collected next day and washed with acetone. The methobromide (2.65 g, 99 %) melted at 236—237° (dec.). (Found: C 53.5; H 5.33; Br 21.0. $C_{17}H_{19}BrN_2OS$ (379.3) requires C 53.8; H 5.05; Br 21.1 %.) The *methiodide* (II) was prepared similarly in 98 % yield; m.p. 234—235° (dec., from methanol). (Found: C 48.0; H 4.54. $C_{17}H_{19}IN_2OS$ (426.3) requires C 47.9; H 4.49 %.)

10-(Dimethylaminoacetyl)-phenothiazine ethiodide (III). The reactants were dissolved in nitrobenzene and heated on the water bath for 1.5 hours. Yield 91 %; m.p. 216—218° (dec.) after recrystallisation from methanol. (Found: C 49.2; H 4.74. $C_{18}H_{21}IN_2OS$ (440.4) requires C 49.1; H 4.81 %.)

10-(Diethylaminoacetyl)-phenothiazine methiodide (IV). The phenothiazine base was heated with methyl iodide in nitrobenzene at 100° for 24 hours. The salt separated on standing at room temperature for two months. Yield 24 %; m.p. 198—200° (dec., from methanol). (Found: C 50.1; H 5.11. $C_{19}H_{23}IN_2OS$ (454.4) requires C 50.2; H 5.10 %.)

10-(Diethylaminoacetyl)-phenothiazine ethochloride (V). 10-Chloroacetylphenothiazine (2.8 g, 0.01 mole) and triethylamine (5.0 g, 0.05 mole) were dissolved in nitrobenzene (25 ml) and heated at 100° for 5 hours. After cooling, the crystalline precipitate (2.3 g) was collected; m.p. 185—186° (dec.). Recrystallisation from acetone or ethanol-ether (1 : 1) did not change the m.p. Analysis indicated, that the reaction product was an *addition compound* containing approximately one mole of the expected quaternary salt and one mole of triethylamine hydrochloride, the latter obviously formed as a by-product. (Found: C 60.0; H 8.04; Cl 13.3; N 8.32. $C_{20}H_{25}ClN_2OS + (C_2H_5)_3N \cdot HCl$ (514.6) requires C 60.7; H 8.03; Cl 13.8; N 8.17 %.)

The above product (2.0 g) was heated in a sublimation apparatus at 0.01 mm at 100° for 12 hours. A part of the product sublimed; m.p. 253—254° undepressed on admixture with an authentic specimen of triethylamine hydrochloride. The residue (1.50 g, calc. 1.46 g) had m.p. 194—195° (dec.). Recrystallisation from ethanol-ether did not change the m.p. (Found: C 63.0; H 6.56; Cl 9.13; N 7.30. $C_{20}H_{25}ClN_2OS$ (376.9) requires C 63.7; H 6.69; Cl 9.41; N 7.43 %.)

The corresponding *ethiodide* (VI) was prepared in 77 % yield by heating the tertiary amine with ethyl iodide in nitrobenzene at 100° over night; m.p. 198—199° (dec., from methanol). (Found: C 50.8; H 5.26; N 6.19. $C_{20}H_{25}IN_2OS$ (468.4) requires C 51.3; H 5.38; N 5.98 %.)

10-(Pyridiniumacetyl)-phenothiazine chloride (VII). 10-Chloroacetylphenothiazine (2.8 g) was heated in pyridine (25 ml) on the water bath for one hour. The quaternary salt crystallised on cooling (3.4 g, 96 %); m.p. 252—253° (dec., from ethanol). (Found: C 63.9; H 4.24; Cl 9.23. $C_{19}H_{15}ClN_2OS$ (354.9) requires C 64.3; H 4.26; Cl 9.99 %.)

10-(α-Dimethylaminopropionyl)-phenothiazine methiodide (VIII). Prepared in 64 % yield in the same way as the corresponding acetyl derivative; m.p. 230—231° (dec., from methanol). (Found: C 48.8; H 4.77; N 6.30. $C_{18}H_{21}IN_2OS$ (440.4) requires C 49.1; H 4.81; N 6.36 %.)

10-(α-Pyridiniumpropionyl)-phenothiazine iodide (IX). 10-(α-Bromopropionyl)-phenothiazine (10.0 g) was heated in pyridine (75 ml) on the water bath for two hours. The resulting *bromo salt* (11.6 g, 84 %) melted at 218—220° (dec.) but analysis indicated that it was not quite pure. Addition of a saturated potassium iodide solution to the aqueous solution of the bromide yielded a faint yellow iodide; m.p. 218—219° (dec., from ethanol). (Found: C 51.8; H 3.78; N 5.98. $C_{20}H_{17}IN_2OS$ (460.3) requires C 52.2; H 3.72; N 6.09 %.)

10-(β-Dimethylaminopropionyl)-phenothiazine methobromide (X). The tertiary base was alkylated with methyl bromide in acetone at room temperature. Yield 88 %; m.p. 234—235°. (Found: C 54.8; H 5.34; Br 20.4. $C_{18}H_{21}BrN_2OS$ (393.4) requires C 55.0; H 5.38; Br 20.3 %.)

The *methiodide* (XI) was prepared similarly in 99 % yield; m.p. 198—200°. (Found: C 49.0; H 4.86. $C_{18}H_{21}IN_2OS$ (440.4) requires C 49.1; H 4.81 %.)

10-(β-Dimethylaminopropionyl)-phenothiazine ethobromide (XII). Prepared in the same manner as X in 84 % yield; m.p. 209—210°. (Found: C 56.2; H 5.42; Br 19.7. $C_{19}H_{23}BrN_2OS$ (407.4) requires C 56.0; H 5.69; Br 19.6 %.)

The *ethiodide* (XIII) was obtained similarly in 89 % yield; m.p. 167—168° (from methanol). (Found: C 50.0; H 5.00; N 6.33. $C_{19}H_{23}IN_2OS$ (454.4) requires C 50.2; H 5.10; N 6.17 %.)

10-(β-Diethylaminopropionyl)-phenothiazine methobromide (XIV). Prepared by the same method in 86 % yield; m.p. 202—203°. (Found: C 56.8; H 6.18. $C_{20}H_{25}BrN_2OS$ (421.4) requires C 57.0; H 5.98 %.)

The *methiodide* (XV) was obtained similarly in 96 % yield; m.p. 183—184° (from methanol). (Found: C 50.8; H 5.19; N 5.98. $C_{20}H_{25}IN_2OS$ (468.4) requires C 51.3; H 5.38; N 5.98 %.)

10-(β-Pyridiniumpropionyl)-phenothiazine chloride (XVI). Prepared in 60 % yield by heating 10-(β-chloropropionyl)-phenothiazine with pyridine on the water bath for 3 hours; m.p. 216—217° (dec., from ethanol-light petroleum 1 : 1). (Found: C 64.4; H 4.70; N 7.94. $C_{20}H_{17}ClN_2OS$ (368.9) requires C 65.1; H 4.65; N 7.60 %.)

10-(β-Dimethylaminobutyryl)-phenothiazine methobromide (XVII). The tertiary base was alkylated with methyl bromide in acetone at room temperature. Yield 88 %; m.p. 226—228° (dec.). (Found: C 55.8; H 5.73; Br 19.7. $C_{19}H_{23}BrN_2OS$ (407.4) requires C 56.0; H 5.69; Br 19.6 %.)

10-(β-Dimethylaminobutyryl)-phenothiazine ethobromide (XVIII). Prepared similarly to XVII in 73 % yield; m.p. 205—206° (dec.). (Found: C 56.7; H 6.10; Br 19.1. $C_{20}H_{25}BrN_2OS$ (421.4) requires C 57.0; H 5.98; Br 19.0 %.)

10-(β-Dimethylaminoisobutyryl)-phenothiazine methobromide (XIX). Prepared in the same way as XVII in 95 % yield; m.p. 243—246° (dec., from ethanol). (Found: C 56.0; 5.71; Br 20.0. $C_{19}H_{23}BrN_2OS$ (407.4) requires C 56.0; H 5.69; Br 19.6 %.)

10-(γ-Dimethylaminobutyryl)-phenothiazine methiodide (XX). Prepared similarly to XVII in 47 % yield; m.p. 185—187°. (Found: C 50.6; H 5.35. $C_{19}H_{23}IN_2OS$ (454.4) requires C 50.2; H 5.10 %.)

SUMMARY

A series of quaternary salts of 10-aminoacylphenothiazines has been prepared and tested for pharmacological activity. Most of the new compounds are powerful cholinolytic and ganglion-blocking agents.

The author wishes to thank Mr. L.-E. Österberg for skilful experimental assistance.

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Basically Substituted Derivatives of Phenothiazine-10-carboxylic Acid

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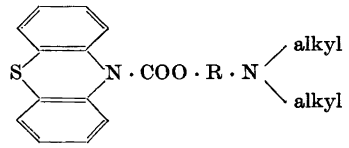
Previous work on 10-aminoacylphenothiazines has shown that several members of this class possess strong spasmolytic activity [1–3]. However, most synthetic antispasmodic agents hitherto known are in their chemical structure carboxylic acid esters of aminoalcohols, and it was therefore considered to be of interest to study the pharmacological properties of some amino-esters containing the phenothiazine nucleus. A number of esters of phenothiazine-10-carboxylic acid were therefore synthesised and tested.



R = $-CH_2 \cdot CH_2-$ or $-CH(CH_3) \cdot CH_2-$

The new esters were smoothly obtained by the reaction of phenothiazine-10-carbonyl chloride with the appropriate aminoalcohol, or by the reaction of the acid chloride with a halohydrin to form a halogenoalkylester, which was then treated with a secondary amine. Attempts to obtain aminoesters by transesterification of methyl phenothiazine-10-carboxylate were unsuccessful.

In addition to the amino esters one thiolester and some amides of phenothiazine-10-carboxylic acid were prepared, and some of the aminoesters were converted into quaternary salts by means of alkyl halogenides.

Absorption spectra. In a previous paper [1] the ultra-violet absorption spectra of some 10-alkyl and 10-acyl derivatives of phenothiazine were measured. As a comparison the absorption of two esters (methyl and β-diethylamino-ethyl phenothiazine-10-carboxylate) and two amides [N-(phenothiazine-10-carbonyl)-piperidine and -pyrrolidine] were determined. The measurements were made with a Beckman Model DU spectrophotometer using ethanol as solvent. The two types of spectra are shown in Fig. 1. The esters, which had almost identical spectra, had maxima at 231 mμ and 256 mμ and a point of

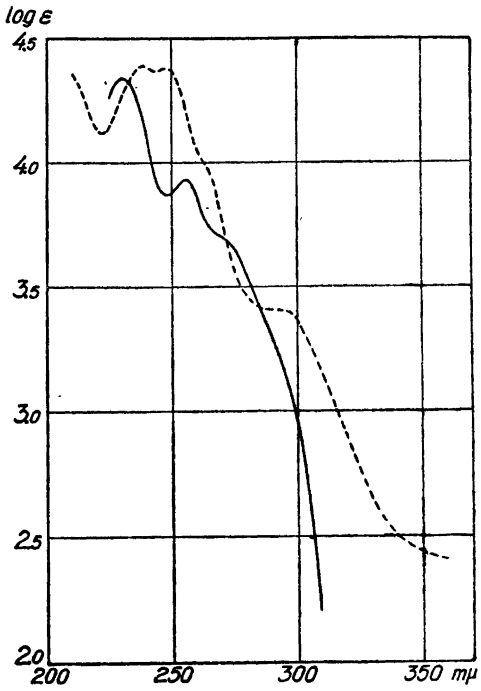


Fig. 1. The absorption spectra of β-diethylaminoethyl phenothiazine-10-carboxylate (————) and N-(phenothiazine-10-carbonyl)-piperidine (— — — —) in abs. ethanol (concentrations ca. 3 × 10⁻⁵ M).

inflection at 270 mμ. The amides had also very similar spectra with maxima at 238 mμ and 248 mμ and a point of inflection at 290 mμ.

PHARMACOLOGY

The new compounds have been tested for local anesthetic, antispasmodic, antihistaminic, nicotinolytic, and ganglionic blocking activity *.

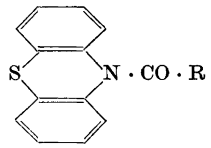
Local anesthetic effect. The tertiary amines were strong anesthetics (1—6 times the activity of Xylocaine, when tested on the rabbit *cornea*). They were, however, rather irritating and had a longer time of onset. The quaternary salts were inactive.

Antispasmodic effect. The results of the tests for cholinolytic activity on isolated guinea pig intestine are shown in Table 1. Several compounds possess considerable cholinolytic activity. Quaternization of the tertiary amines seems to have little influence on the activity.

The new compounds have also been tested for musculotropic spasmolytic activity on spasm of rat *ileum* caused by barium chloride. The results will be

* Acknowledgement is made to Dr. S. Wiedling of Astra's Biological Department for performing the tests for local anesthetic, cholinolytic and antihistaminic effect.

Table 1. Cholinolytic and antihistaminic properties of derivatives of phenothiazine-10-carboxylic acid.



	R	Salt tested	Effect * in reducing the spasm produced by	
			Acetyl-choline	Histamine
IV	$-O \cdot CH_2 \cdot CH_2 \cdot N(CH_3)_2$	HCl	5.5	0.4
V	$-O \cdot CH_2 \cdot CH_2 \cdot N(C_2H_5)_2$	HCl	13	0.13
VI	$-O \cdot CH_2 \cdot CH_2 \cdot NC_4H_8O$	$H_2C_2O_4$	0.15	
VII	$-O \cdot CH_2 \cdot CH_2 \cdot NC_4H_8$	HCl	1.2	0.2
VIII	$-O \cdot CH(CH_3) \cdot CH_2 \cdot N(CH_3)_2$	$H_2C_2O_4$	2.6	0.02
IX	$-O \cdot CH(CH_3) \cdot CH_2 \cdot NC_5H_{10}$	$H_2C_2O_4$	0.15	
X	$-S \cdot CH_2 \cdot CH_2 \cdot N(C_2H_5)_2$	$H_2C_2O_4$	19	0.04
XI	$-O \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}(CH_3)_3$	Br^-	3.3	0.2
XII	$-O \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}(CH_3)_2C_2H_5$	Br^-	6	
XIII	$-O \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}(C_2H_5)_2CH_3$	Br^-	15	0.12
XIV	$-O \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}(C_2H_5)_3$	Br^-	12	0.07
XVIII	$-NH \cdot CH_2 \cdot CH_2 \cdot N(C_2H_5)_2$	HCl	18	0.1
	Atropine sulphate		30	
	Diphenhydramine-HCl		1	1

* The activity figures refer to the base. The activities of the quaternary salts refer to the cationic part of the molecule.

published and discussed elsewhere [4]. Great activity was shown by β-diethylaminoethyl phenothiazine-10-carboxylate (V) especially. The quaternary compounds were less active than the parent tertiary amines.

Antihistaminic effect. The results are shown in Table 1. None of the compounds were as active as diphenhydramine. Quaternization seems to lower the activity a little.

Nicotinolytic effect. Some of the compounds have been tested by the method of Bovet and Longo [5]. Outstanding nicotinolytic properties were revealed by β-diethylaminoethyl phenothiazine-10-carboxylate (V) [6,7], which seems to be one of the most active nicotinolytic agents known at present. One of the quaternary compounds (XIII) was tested but was ineffective.

Ganglionic blocking effect. The quaternary salts (XI—XIV) showed a strong ganglionic blocking activity when tested on the peristaltic reflex. The results will be published elsewhere [8].

EXPERIMENTAL

Esters of phenothiazine-10-carboxylic acid

Phenothiazine-10-carbonyl chloride (I) used as starting material was obtained by the method of Paschkowezky [9] by heating phenothiazine with a small excess of phosgene in toluene in a sealed vessel at 95—100°, for two hours. Yield 85—90 %; m.p. 172—173°.

Methyl phenothiazine-10-carboxylate (II). A solution of phenothiazine (10.0 g) and methyl chloroformate (6.0 g) in toluene (15 ml), was heated in a sealed vessel at 100° for 20 hours, and then at 120° for a further 4 hours. After cooling, the dark mixture was filtered giving 6.5 g (51 %) of the crude crystalline ester. Two recrystallisations from methanol afforded colourless crystals, m.p. 118—120°. (Found: C 65.5; H 4.45; N 5.62. $C_{14}H_{11}NO_2S$ (257.3) requires C 65.3; H 4.31; N 5.45 %.)

β-Chloroethyl phenothiazine-10-carboxylate (III). A mixture of I (2.6 g) and ethylene chlorohydrin (10 ml), was refluxed until hydrogen chloride was no longer evolved (12 hours). On cooling, white crystals separated (2.0 g, 65 %); m.p. 146—148° after two recrystallisations from acetone. (Found: C 59.2; H 3.80; Cl 12.0; N 4.59. $C_{15}H_{12}ClNO_2S$ (305.8) requires C 58.9; H 3.96; Cl 11.6; N 4.58 %.)

β-Dimethylaminoethyl phenothiazine-10-carboxylate hydrochloride (IV). A solution of I (2.6 g, 0.01 mole) and β-dimethylaminoethanol (2.2 g, 0.025 mole) in toluene (10 ml) was refluxed for two hours. After cooling the resultant β-dimethylaminoethanol hydrochloride was removed by filtration. The toluene solution was washed with water and dried over calcium chloride, and the aminoester isolated by the addition of ethereal hydrogen chloride. The crude product (2.9 g, 83 %) was recrystallised from ethanol; m.p. 212—213° (dec.). (Found: C 57.8; H 5.48; N 7.99. $C_{17}H_{18}N_2O_2S \cdot HCl$ (350.9) requires C 58.2; H 5.46; N 7.99 %.)

β-Diethylaminoethyl phenothiazine-10-carboxylate (V). The *hydrochloride* of this compound was prepared by the method used for IV. Yield 95 %; m.p. 163—164° after recrystallisation from ethanol-light petroleum (2 : 1). (Found: C 59.8; H 6.25; N 7.20. $C_{19}H_{22}N_2O_2S \cdot HCl$ (378.9) requires C 60.2; H 6.12; N 7.40 %.) From the hydrochloride the free base was obtained in solid form; m.p. 54—56° (from ethanol). (Found: C 66.7; H 6.59; N 8.15. $C_{19}H_{22}N_2O_2S$ (342.5) requires C 66.6; H 6.47; N 8.18 %.)

β-Morpholinoethyl phenothiazine-10-carboxylate oxalate (VI). Prepared from I and β-morpholinoethanol [10]. Yield, 34 %; m.p. 111—114° (dec.), from acetone. (Found: C 56.5; H 5.16; N 5.96. $C_{19}H_{20}N_2O_3S \cdot H_2C_2O_4$ (446.5) requires C 56.5; H 4.97; N 6.28 %.)

β-Pyrrolidinoethyl phenothiazine-10-carboxylate hydrochloride (VII).

Method A. I and β-pyrrolidinoethanol [11] afforded VII by the usual method. Yield, 82 %; m.p. 215—217° (dec.) after recrystallisation from ethanol. (Found: C 60.8; H 6.00; N 7.35. $C_{19}H_{20}N_2O_2S \cdot HCl$ (376.9) requires C 60.5; H 5.62; N 7.43 %.)

Method B. A solution of β-chloroethyl phenothiazine-10-carboxylate (1.8 g) and pyrrolidine (1.05 g) in toluene (10 ml), was refluxed for two hours. The reaction mixture was filtered, washed with water and dried over calcium chloride. Addition of ethereal hydrogen chloride gave the hydrochloride of the β-pyrrolidinoethyl ester. Yield, 0.60 g, 27 %; m.p. 214—216° (dec.), from ethanol, undepressed on admixture with the product prepared by method A above.

β-Dimethylaminoisopropyl phenothiazine-10-carboxylate oxalate (VIII). Obtained from I and β-dimethylamino*iso*propanol. Yield, 29 %; m.p. 181—182° (dec.) after recrystallisation from acetone. (Found: C 57.5; H 5.16; N 6.58. $C_{18}H_{20}N_2O_2S \cdot H_2C_2O_4$ (418.5) requires C 57.4; H 5.30; N 6.70 %.)

β-Piperidinoisopropyl phenothiazine-10-carboxylate oxalate (IX). Prepared from I and β-piperidino*iso*propanol [12]. Yield, 68 %; m.p. 170—171° (dec.), from ethanol. (Found: C 59.6; H 5.81; N 5.88. $C_{21}H_{24}N_2O_2S \cdot H_2C_2O_4$ (458.5) requires C 60.2; H 5.72; N 6.11 %.)

β-Diethylaminoethyl phenothiazine-10-thiocarboxylate oxalate (X). Prepared by the same method as the preceding esters from I and β-diethylaminoethyl mercaptan [13]. Yield 56 %; m.p. 158—160° (dec.) after two recrystallisations from acetone. (Found: C 56.3; H 5.42; N 6.12. $C_{19}H_{22}N_2OS_2 \cdot H_2C_2O_4$ (448.5) requires C 56.2; H 5.39; N 6.25 %.)

Quaternary derivatives

β-Dimethylaminoethyl phenothiazine-10-carboxylate methobromide (XI). β-Dimethylaminoethyl phenothiazine-10-carboxylate hydrochloride (0.63 g) was dissolved in water and the solution was made alkaline. The oily base was extracted with ether and the extract was dried and evaporated. The residue was dissolved in acetone (5 ml) and methyl bromide (2 ml) added. The quaternary salt began to crystallise immediately. The mixture was allowed to stand over night at room temperature, and the product was collected and washed with acetone. Yield, 0.63 g, 78 %; m.p. 248—249° (dec.) after recrystallisation from acetone-ethanol (1 : 1). (Found: C 53.1; H 5.20; N 6.75. $C_{18}H_{21}BrN_2O_2S$ (409.4) requires C 52.8; H 5.17; N 6.84 %.)

The *methiodide* was prepared similarly in 71 % yield; m.p. 235—236° (dec.), from acetone-ethanol. (Found: C 46.9; H 4.74; N 6.27. $C_{18}H_{21}IN_2O_2S$ (456.4) requires C 47.4; H 4.64; N 6.14 %.)

β-Dimethylaminoethyl phenothiazine-10-carboxylate ethobromide (XII). Prepared in the same manner as XI in 78 % yield; m.p. 233—234° (dec.) after recrystallisation from methanol. (Found: C 53.2; H 5.53; N 6.71. $C_{19}H_{23}BrN_2O_2S$ (423.4) requires C 53.9; H 5.48; N 6.62 %.)

β-Diethylaminoethyl phenothiazine-10-carboxylate methobromide (XIII). Obtained in 68 % yield; m.p. 220—221° (dec.), from acetone-ethanol. (Found: C 55.7; H 5.87; N 6.43. $C_{20}H_{25}BrN_2O_2S$ (437.4) requires C 54.9; H 5.76; N 6.41 %.)

β-Diethylaminoethyl phenothiazine-10-carboxylate ethobromide (XIV). The reactants were kept at 40° for 48 hours. Yield 55 %; m.p. 213—215° (dec.), from acetone-ethanol. (Found: C 55.4; H 5.74; N 6.35. $C_{21}H_{27}BrN_2O_2S$ (451.4) requires C 55.9; H 6.03; N 6.21 %.)

Amides of phenothiazine-10-carboxylic acid

N-(Phenothiazine-10-carbonyl)-diethylamine (XV). Phenothiazine-10-carbonyl chloride (I, 2.6 g) was refluxed with diethylamine (2.2 g) in toluene (10 ml) for two hours. The mixture was filtered in order to remove diethylamine hydrochloride, washed with water and evaporated to dryness *in vacuo*. The residue (2.9 g, 97 %) was recrystallised from ethanol; m.p. 91—93°. (Found: C 68.3; H 6.00; N 9.43. $C_{17}H_{18}N_2OS$ (298.4) requires C 68.4; H 6.08; N 9.39 %.)

N-(Phenothiazine-10-carbonyl)-piperidine (XVI). Obtained by the method for XV above, from I and piperidine. Yield, 73 %; m.p. 118—120°, from ethanol. (Found: C 69.2; H 5.61; N 9.33. $C_{18}H_{18}N_2OS$ (310.4) requires C 69.6; H 5.84; N 9.03 %.)

N-(Phenothiazine-10-carbonyl)-pyrrolidine (XVII). Prepared from I and pyrrolidine. Yield 68 %; m.p. 137—139°, from ethanol. (Found: C 68.8; H 5.75; N 9.52. $C_{17}H_{16}N_2OS$ (296.4) requires C 68.9; H 5.44; N 9.45 %.)

N-(Phenothiazine-10-carbonyl)-N',N'-diethylethylenediamine hydrochloride (XVIII). A solution of I (5.0 g) and N,N-diethylethylenediamine [14] (5.5 g) in toluene (10 ml) was refluxed for two hours. After cooling the clear solution was washed with water. On extraction of the solution with 2 *N* hydrochloric acid the reaction product separated as the hydrochloride (7.0 g, 98 %); m.p. 180—181° (dec.). Recrystallisation of this product from methanol yielded crystals of m.p. 142—144°, apparently containing two moles of methanol of crystallisation. (Found: C 57.5; H 6.97; N 9.52. $C_{19}H_{23}N_3OS \cdot HCl + 2\ CH_3OH$ (442.0) requires C 57.1; H 7.30; N 9.51 %.) On drying at 105° the weight was constant after a loss of 13.8 % (calc. for 2 CH_3OH: 14.5 %) and the m.p. was 184—186° (dec.). (Found: C 59.9; H 6.39; N 11.0. $C_{19}H_{23}N_3OS \cdot HCl$ (377.9) requires C 60.4; H 6.40; N 11.1 %.)

SUMMARY

A series of basically substituted derivatives of phenothiazine-10-carboxylic acid has been prepared. Some of the compounds possess strong spasmolytic and nicotinolytic activity.

The author wishes to thank Mr. L.-E. Österberg for skilful experimental assistance.

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Dialkylaminoalkylthiazoles

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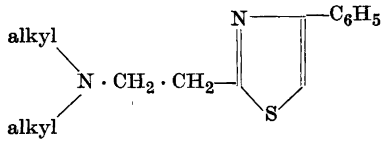
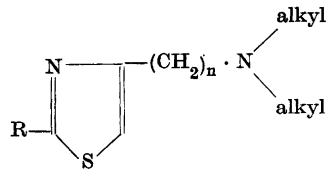
Attempts to prepare dialkylaminoalkylthiazoles of type I met with unexpected difficulties owing to the peculiar reaction of β-dialkylamino-substituted propio- and butyronitriles with hydrogen sulphide [1]. It was therefore decided to interchange the substituents in the thiazole nucleus and prepare a series of thiazoles of the general structure II.



I.



R = CH_3–, C_6H_5– or $C_6H_5 \cdot CH_2$–

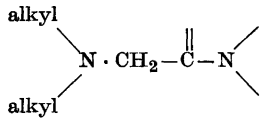
n = 1 or 2

II.

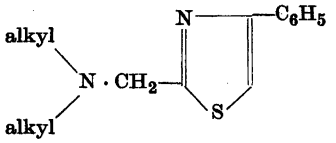
Compounds of type II were easily obtained by the reaction of 4-chloromethylthiazoles with secondary amines (n = 1) or by the reaction of thioamides with 1-bromo-4-dialkylamino-2-butanones (n = 2).

The new compounds were tested pharmacologically. None of them possessed antihistaminic or spasmolytic properties. When the substituent R was phenyl or benzyl the compounds were strong local anesthetics superior, in some cases, to Xylocaine, when tested on rabbit *cornea*. They were, however, irritating to the rabbit eye and had a longer time of onset than Xylocaine.

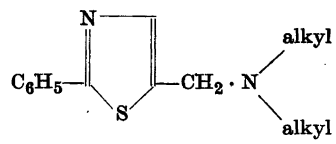
The local anesthetic effect of these thiazoles was somewhat unexpected, as the related aminoalkyl derivatives of 2-benzylthiazole [2] had no such properties. In order to investigate if the anesthetic effect was due to the group



which is also present in Xylocaine [3] (and *iso*gramine [4]) some isomeric 2-dialkylaminomethyl-4-phenylthiazoles (III) and 5-dialkylaminomethyl-2-phenylthiazoles (IV) were synthesized.

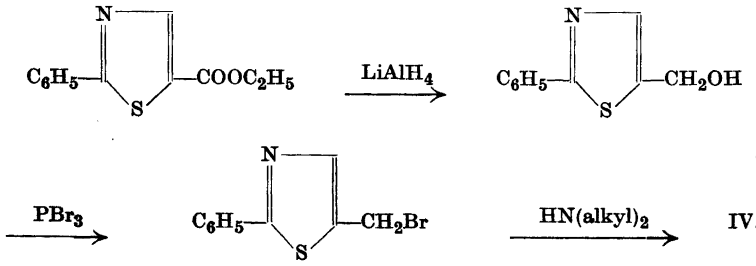


III.



IV.

The compounds of type III were prepared by the reaction of 2-bromomethyl-4-phenylthiazole with secondary amines. The compounds of type IV were obtained by the following reaction sequence:



The compounds of both types possessed considerable anesthetic activity, but like their isomers of type II they were eye irritants and had a longer time of onset than Xylocaine. No noticeable antihistaminic or antispasmodic effects were found.

EXPERIMENTAL

4-Dialkylaminomethylthiazoles

4-Diethylaminomethyl-2-methylthiazole. A solution of 4-chloromethyl-2-methylthiazole [5] (4.5 g, 0.03 mole) and diethylamine (5.9 g, 0.08 mole) in nitrobenzene (25 ml) was heated on the water bath for one hour. The mixture was cooled, washed with water and extracted with 2 *N* hydrochloric acid. On addition of an aqueous solution of picric acid to the extract the reaction product separated as the *picrate* (2.9 g, 24 %); m.p. 98—99° on recrystallisation from methanol. (Found: N 17.5. $C_9H_{16}N_2S \cdot C_6H_3N_3O_7$ (413.4) requires N 16.9 %.)

The free base was obtained as an oil, from the picrate, in the usual way. B.p. 150° (bath temperature) at 0.01 mm. (Found: C 58.1; H 8.60; N 14.9. $C_9H_{16}N_2S$ (184.3) requires C 58.6; H 8.75; N 15.2 %.)

4-Dimethylaminomethyl-2-phenylthiazole dihydrochloride. 4-Chloromethyl-2-phenylthiazole [6] (2.1 g) and dimethylamine (4 ml) were heated in ethanol (25 ml), in a sealed bottle, at 70° overnight. The reaction mixture was cooled, poured into water and the resulting oil was extracted with ether. The ethereal solution was dried and the dihydrochloride of the thiazole base was precipitated by the addition of ethereal hydrogen chloride. The crude salt (2.0 g, 69 %) was recrystallised from methanol; m.p. 199—200°. (Found: C 50.2; H 5.42; N 10.1. $C_{12}H_{14}N_2S \cdot 2\ HCl$ (291.2) requires C 49.5; H 5.54; N 9.62 %.)

4-Diethylaminomethyl-2-phenylthiazole. 4-Chloromethyl-2-phenylthiazole (9.0 g) and diethylamine (11.2 g) were heated in nitrobenzene (40 ml) on the water bath for 3 hours. The mixture was cooled, washed with water and extracted with 2 *N* hydrochloric acid. The extract was made alkaline, and the resulting oil was extracted with ether. The ethereal solution was dried, the solvent was evaporated and the residue distilled *in vacuo* giving an almost colourless oil (7.6 g, 68 %), b.p. 170—180/0.05 mm. (Found: C 68.1; H 7.36. $C_{14}H_{18}N_2S$ (246.4) requires C 68.2; H 7.36 %.)

The thiazole base was further characterized by the preparation of the *picrate* m.p. 103—104° (from methanol), (Found: C 50.3; H 4.24; N 14.9. $C_{14}H_{18}N_2S \cdot C_6H_3N_3O_7$ (475.5) requires C 50.5; H 4.45; N 14.7 %), and the oxalate m.p. 129—131° (from acetone), (Found: C 57.5; H 6.20. $C_{14}H_{18}N_2S \cdot H_2C_2O_4$ (336.4) requires C 57.1; H 5.99 %).

4-Pyrrolidinomethyl-2-phenylthiazole. This compound was prepared in the usual way by refluxing 4-chloromethyl-2-phenylthiazole and pyrrolidine in ethanol for 10 hours. The reaction product was isolated in 23 % yield as the *picrate*, m.p. 134—136° (from ethanol). (Found: N 14.9. $C_{14}H_{16}N_2S \cdot C_6H_3N_3O_7$ (473.5) requires N 14.8 %.)

The free base obtained from the picrate distilled at 250° (bath temperature) at 0.01 mm. (Found: C 68.9; H 6.67; N 11.3. $C_{14}H_{16}N_2S$ (244.4) requires C 68.8; H 6.60; N 11.5 %.)

4-(β-Dialkylaminoethyl)-thiazoles

These compounds were all prepared in the same way. Equivalent amounts of the appropriate thioamide (thioacetamide, thiobenzamide or phenylthioacetamide) and a 1-bromo-4-dialkylamino-2-butanone hydrobromide (dialkylamino = dimethylamino-, diethylamino-, and piperidino-) were dissolved in ethanol and refluxed on the water bath for one hour. The solution was filtered and cooled, giving the crystalline dihydrobromide (in one case the monohydrobromide) of the 4-(β-dialkylaminoethyl)-thiazole in almost pure form. The salt was recrystallised from ethanol. The 1-bromo-4-dialkylamino-2-butanone hydrobromides required as starting materials were prepared by the method of Djerassi, Mizzoni and Scholz [7].

4-(β-Dimethylaminoethyl)-2-phenylthiazole dihydrobromide. M.p. 240—242° (dec.); yield 49 %. (Found: C 39.9; H 4.81; Br 39.4. $C_{13}H_{16}N_2S \cdot 2$ HBr (394.2) requires C 39.6; H 4.60; Br 40.5 %).

4-(β-Dimethylaminoethyl)-2-benzylthiazole dihydrobromide. M.p. 228—230°; yield 64 %. (Found: C 41.0; H 4.88; Br 37.8. $C_{14}H_{18}N_2S \cdot 2$ HBr (408.2) requires C 41.2; H 4.94; Br 39.2 %).

4-(β-Diethylaminoethyl)-2-methylthiazole dihydrobromide. M.p. 222—224°; yield 72 %. (Found: C 33.0; H 5.27; Br 43.9. $C_{10}H_{18}N_2S \cdot 2$ HBr (360.2) requires C 33.3; H 5.60; Br 44.4 %).

4-(β-Diethylaminoethyl)-2-phenylthiazole dihydrobromide. This compound was rather soluble in cold ethanol. It was therefore precipitated from the reaction mixture with ether, and recrystallised from methanol. M.p. 195—197°; yield 57 %. (Found: C 42.5; H 5.20; Br 38.0. $C_{15}H_{20}N_2S \cdot 2$ HBr (422.2) requires C 42.7; H 5.25; Br 37.9 %).

4-(β-Diethylaminoethyl)-2-benzylthiazole dihydrobromide. Precipitated with ether and recrystallised from methanol. M.p. 192—193°; yield 63 %. (Found: C 44.0; H 5.36; Br 36.6. $C_{16}H_{22}N_2S \cdot 2$ HBr (436.3) requires C 44.0; H 5.54; Br 36.6 %.)

4-(β-Piperidinoethyl)-2-methylthiazole dihydrobromide. M.p. 229—230°; yield 44 %. (Found: C 35.3; H 5.60; Br 42.0. $C_{11}H_{18}N_2S \cdot 2$ HBr (372.2) requires C 35.5; H 5.42; Br 42.9 %).

4-(β-Piperidinoethyl)-2-phenylthiazole monohydrobromide. M.p. 230—232° (dec.); yield 42 %. (Found: C 53.9; H. 5.97; Br 23.8. $C_{16}H_{20}N_2S \cdot$ HBr (353.3) requires C 54.4; H 5.99; Br 22.6 %).

4-(β-Piperidinoethyl)-2-benzylthiazole dihydrobromide. M.p. 275—277°; yield 47 %. (Found: C 44.6; H 5.36; Br 34.2. $C_{17}H_{22}N_2S \cdot 2$ HBr (448.3) requires C 45.5; H 5.40; Br 35.7 %).

2-Dialkylaminomethylthiazoles

2-Bromomethyl-4-phenylthiazole. The method of Olin and Johnson [8] could not be repeated, and the compound was therefore prepared in the following way. A mixture of 2-hydroxymethyl-4-phenylthiazole (4.3 g), phosphorus tribromide (10.0 g) and chloroform (30 ml) was refluxed for one hour. The reaction mixture was cooled, washed with cold sodium bicarbonate solution, and the chloroform was separated and dried over calcium chloride. The solvent was evaporated and the residue distilled *in vacuo* giving a colourless oil (3.3 g, 58 %), b.p. 140—145/0.05 mm (Olin and Johnson [8] record b.p. 195°/15 mm). The distillate soon solidified to white crystals; m.p. 45—46° on recrystallisation from light petroleum. (Found: C 47.3; H 3.30; Br 31.2. Calc. for $C_{10}H_8BrNS$ (254.2): C 47.3; H 3.17; Br 31.5 %).

2-Dimethylaminomethyl-4-phenylthiazole oxalate. 2-Bromomethyl-4-phenylthiazole (5.1 g) reacted readily at room temperature with a solution of dimethylamine (3.5 ml) in benzene (40 ml). The mixture was filtered, washed with water and extracted with 2 *N* hydrochloric acid. The extract was made alkaline with sodium carbonate solution and the oily base was extracted with ether. By addition of ethereal oxalic acid to the extract, the oxalate of the base was isolated (4.55 g, 74 %); m.p. 179—180° (dec.) on recrystallisation from acetone. (Found: C 54.6; H 5.20; N 9.35. $C_{12}H_{14}N_2S \cdot H_2C_2O_4$ (308.4) requires C 54.5; H 5.23; N 9.09 %).

2-Diethylaminomethyl-4-phenylthiazole oxalate. This compound was prepared in the same way in 60 % yield by the above method. M.p. 146—147° (from acetone). (Found: C 57.6; H 6.10; N 8.36. $C_{14}H_{18}N_2S \cdot H_2C_2O_4$ (336.4) requires C 57.1; H 5.99; N 8.33 %).

2-Piperidinomethyl-4-phenylthiazole. The solid base was obtained in 89 % yield. M.p. 67—68° on recrystallisation from 50 % ethanol. (Found: C 69.3; H 6.80; N 10.5. $C_{15}H_{18}N_2S$ (258.4) requires C 69.7; H 7.02; N 10.8 %).

5-Dialkylaminomethylthiazoles

5-Hydroxymethyl-2-phenylthiazole. A solution of ethyl 2-phenylthiazole-5-carboxylate [9] (6.0 g) in ether (50 ml) was added, with mechanical stirring, to a suspension of lithium aluminium hydride (1.0 g) in ether (30 ml), at such a rate that gentle reflux was obtained. The mixture was stirred under reflux for a further half hour. Excess hydride was destroyed with ethyl acetate (5 ml), and the mixture was made strongly alkaline with 5 *N* sodium hydroxide. The ether layer was separated, the aqueous solution extracted thoroughly with ether, and the combined ether solutions were dried over calcium chloride. The solvent was evaporated and the residue distilled *in vacuo* giving a colourless oil (4.2 g, 85 %). The distillate soon solidified to white crystals; m.p. 76—77° on recrystallisation from light petroleum-ethanol (6 : 1). (Found C 62.4; H 4.70; S 16.5. $C_{10}H_9NOS$ (191.2) requires C 62.8; H 4.74; S 16.8 %.)

5-Bromomethyl-2-phenylthiazole. The above hydroxy compound (1.4 g) was refluxed with phosphorus tribromide (4.3 g) in chloroform for one hour. After cooling, the mixture was washed with sodium bicarbonate solution, the chloroform was separated, and dried over calcium chloride. The solvent was evaporated and the solid residue (1.6 g, 86 %) crystallised from methanol; m.p. 72—73°. (Found: C 48.0; H 3.09; Br 31.9. $C_{10}H_8BrNS$ (254.2) requires C 47.3; H 3.17; Br 31.5 %.)

5-Dimethylaminomethyl-2-phenylthiazole hydrochloride. The above bromo compound reacted readily at room temperature with dimethyl amine in toluene solution. The reaction product was isolated as the hydrochloride. Yield 81 %; m.p. 236—237°, from ethanol. (Found: C 56.6; H 5.81; Cl 14.3. $C_{12}H_{14}N_2S \cdot HCl$ (254.8) requires C 56.6; H 5.93; Cl 13.9 %.)

5-Diethylaminomethyl-2-phenylthiazole hydrochloride. The reactants were refluxed in benzene for one hour. Yield 60 %; m.p. 173—175°, from ethanol. (Found: C 59.7; H 6.41; N 9.57. $C_{14}H_{18}N_2S \cdot HCl$ (282.8) requires C 59.4; H 6.77; N 9.91 %.)

SUMMARY

A series of new dialkylaminoalkyl derivatives of substituted thiazoles has been prepared for pharmacological study.

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Cyclodehydration of Acylated α-Amino Acid Amides

I. Saturated Amides

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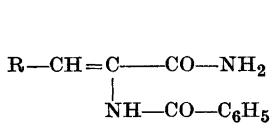
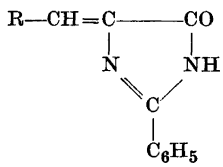
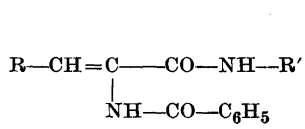
In 1900 Erlenmeyer [1] observed that α-benzamidocinnamamide (I, R = C_6H_5) underwent intramolecular dehydration to 2-phenyl-4-benzylidene-5-imidazolone (II, R = C_6H_5) on treatment with hot aqueous alkali.



I



II

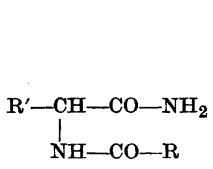


III

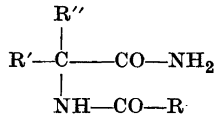
Additional examples in further communications [2-4] from the same laboratory demonstrated the general nature of this ring-closure reaction of unsaturated α-benzamido acid amides (I). Substituents in the amide-grouping, however, precluded ring-formation under the usual reaction conditions as evidenced from the stability of the corresponding anilides (III, R′ = C_6H_5). It remained for Gränacher *et al.*[5,6] to extend the reaction to N-substituted amides (III), although with certain limitations as to the nature of R′. They showed that heating of the amides *in vacuo* above the melting points would bring about the cyclisations desired. Narang and Rây [7] later reported that heating with phosphoryl chloride transformed various substituted α-benzamidocinnamanilides into the corresponding imidazolones. Still another, although more special modification, has recently been introduced through the observation by Shaw and McDowell [8] that certain unsaturated hydroxamic acids (III, R = C_6H_5, R′ = OH or $OCH_2C_6H_5$) undergo cyclisation in hot mineral acid.

Little attention has been given to studies of the analogous cyclisation of *saturated* α-acylamido amides (IV), a reaction which may be of interest for the discussion of the structure of alkali-treated proteins. Mohr [9] found that α-benzamido*iso*butyramide (V, R = C_6H_5, R′ = R″ = CH_3) readily suffered dehydration to the imidazolone (VI, R = C_6H_5, R′ = R″ = CH_3) in alkaline

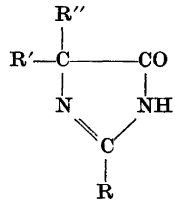
solution. Again, Gränacher and Mahler [5] reported two cases in which heating *in vacuo* of properly substituted α-benzamido*iso*butyramides yielded the corresponding imidazolones.



IV



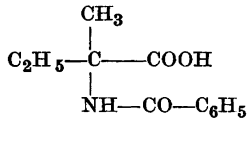
V



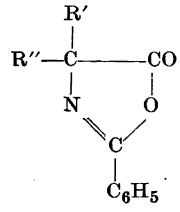
VI

As part of a broader investigation in the imidazolone series it became desirable to study more closely this dehydration reaction in order to determine its scope, synthetic potentialities and possible bearing on protein chemistry. Because Mohr [9] failed to achieve cyclisation of N-benzoylphenylalaninamide (IV, R = C_6H_5, R′ = $C_6H_5CH_2$) the behaviour of some amides of tertiary α-amino acids was first investigated in order to ascertain that the α-benzamido*iso*butyramides do no represent unique cases.

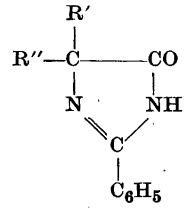
DL-*iso*Valine was benzoylated in 76 per cent yield following the general procedure of Steiger [10], a significant improvement over the 20—30 per cent yield previously reported by Slimmer [11]. Treatment of benzoyl*iso*valine (VII) with acetic anhydride gave the crystalline 2-phenyl-4-methyl-4-ethyl-5-oxazolone (VIII, R′ = CH_3, R″ = C_2H_5). The azlactone was characterised by its reactions with water, ammonia and aniline to give benzoyl*iso*valine, its amide and anilide, respectively.



VII



VIII

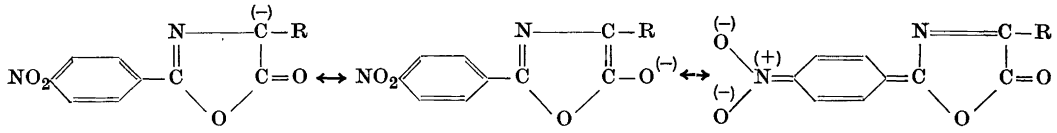


IX

Upon treatment with aqueous alkali at ordinary or slightly higher temperature, the amide lost the elements of water yielding a compound with all the characteristics of an imidazolone (IX, R′ = CH_3 R″ = C_2H_5). An example of an imidazolone, carrying an aromatic substituent in 4-position, was furnished by starting from α-benzamidohydratropic acid which yielded an oily azlactone (VIII, R′ = CH_3, R″ = C_6H_5). Ammonolysis of the latter gave α-benzamidohydratropamide, smoothly converted into 2,4-diphenyl-4-methyl-5-imidazolone (IX, R′ = CH_3, R″ = C_6H_5) under the usual conditions. The same sequence of reactions, carried out with α,α-diethylhippuric acid as the starting material, yielded the imidazolone (IX, R′ = R″ = C_2H_5).

That the nature of the acyl-grouping in the tertiary α-amino acid amides is no limiting factor in the cyclisations to imidazolones was demonstrated by the following examples. α-*p*-Nitrobenzamido*iso*butyric acid was prepared and transformed into its azlactone (X, R′ = R″ = CH_3, R = *p*-$NO_2C_6H_4$) as usual.

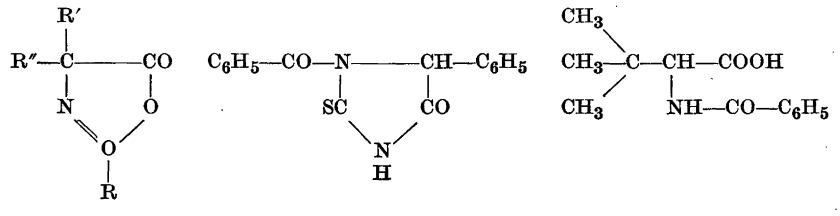
No colour was produced on treating this azlactone with alkali or pyridine, in contrast to the violet or blue colours observed when the ordinary, non-tertiary α-amino acids are treated with *p*-nitrobenzoyl chloride and aqueous alkali or pyridine (the *Waser*-reaction). Karrer and Keller [12] ascribed the formation of colour in this reaction to the presence of a resonance-stabilised anion of the oxazolone:



The failure of the nitrophenyl-oxazolone to produce the blue colour supports this explanation, because in the present case the requisite hydrogen-atom in 4-position is missing.

Ammonolysis of the oxazolone proceeded smoothly to give α-*p*-nitrobenzamido*iso*butyramide which readily afforded the imidazolone (VI, R = *p*-$NO_2C_6H_4$, R′ = R″ = CH_3) in alkali. Spectrophotometrically it could be shown that the introduction of the nitro-grouping in the molecule resulted in an increased rate of cyclisation (Fig. 1). A similar experiment with α-benzamido*iso*butyramide demonstrated that about 220 hours were required for the cyclisation to go to completion under similar conditions.

When α-phenacetamido*iso*butyric acid was treated with acetic anhydride, 2-benzyl-4,4-dimethyl-5-oxazolone resulted. Ammonolysis yielded the amide which easily cyclised in alkali to 2-benzyl-4,4-dimethyl-5-imidazolone (VI, R = $C_6H_5CH_2$, R′ = R″ = CH_3), crystallising as a monohydrate isomeric with the open amide. Removal of the water of crystallisation upon heating was accompanied by simultaneous destruction of the compound, but the imidazolone-structure could be definitely established on spectroscopical evidence (Fig. 2). Similarly, phenacetylation of DL-α-phenylalanine yielded the acylated, tertiary amino acid. Azlactonisation gave an oily oxazolone (X, R = $C_6H_5CH_2$, R′ = CH_3, R″ = C_6H_5) which could easily be opened with ammonia to α-phenacetamidohydratropamide. The facile ring-closure in alkaline solution to the imidazolone (VI, R = $C_6H_5CH_2$, R′ = CH_3, R″ = C_6H_5) points to the generality of this reaction of acylated, tertiary α-amino acid amides.



X XI XII

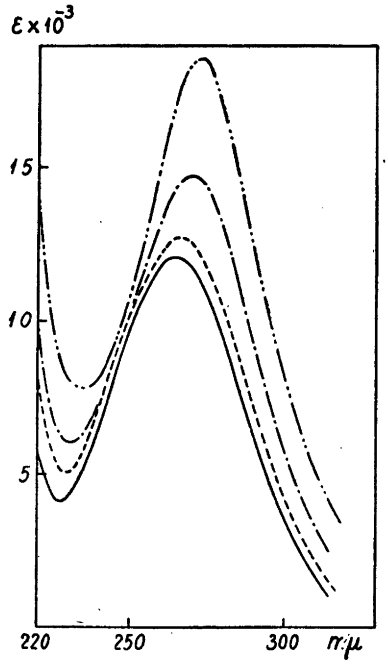


Fig. 1. UV-absorption spectra of a 0.08 M *solution of α-(*p*-nitrobenzamido)-isobutyramide in 0.01* N *methanolic KOH, measured immediately, and after standing at 23°. ——— Immediately after dissolution, — — — after 8 hours, — · — after 44 hours and — · · — after 140 hours. The latter curve is identical with the one obtained from authentic imidazolone in methanolic KOH.*

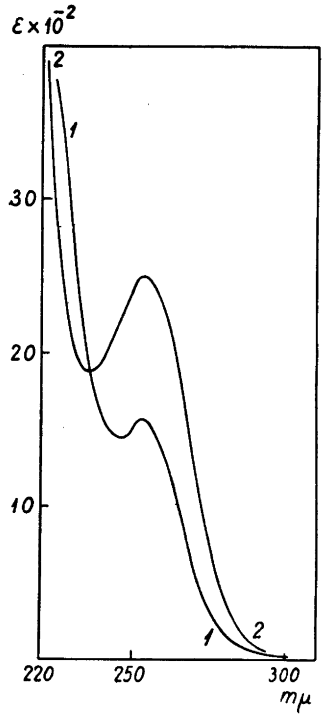


Fig. 2. UV-absorption spectra of: 1. 2-Benzyl-4,4-dimethyl-5-imidazolone in methanol; 2. The same in 0.1 N *methanolic KOH.*

Despite numerous attempts, no crystalline imidazolone could be obtained from ring-closure reactions of DL-α-acetamidohydratropamide (V, R = R′ = CH_3, R″ = C_6H_5). Undoubtedly, however, the oily product represented essentially pure imidazolone (VI, R = R′ = CH_3, R″ = C_6H_5) as inferred from analysis and general chemical properties. This result is consistent with the observation by Steiger [13] who previously described the oily imidazolone, although without analytical data. Both α-amino*iso*butyric acid and α-phenylalanine were formylated, but the further processing to the corresponding amides and imidazolones was abolished owing to the rather labile nature of the formamido-grouping of these substances.

The saturated imidazolones reported above are all colourless compounds with relatively high melting points. They are readily soluble in dilute alkali, less so in acid, rather soluble in alcohol, but sparingly soluble in ether, benzene and chloroform. In addition to the spectroscopical evidence, discussed in

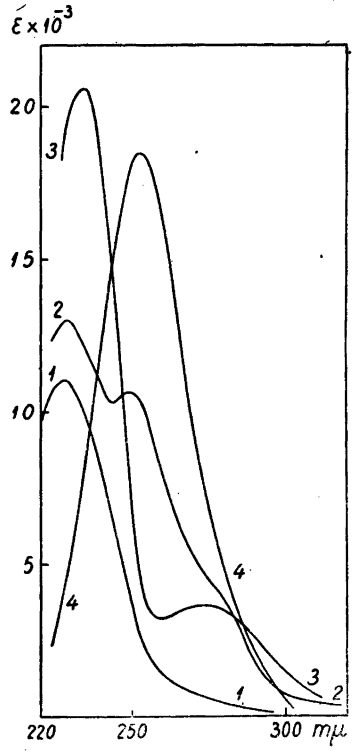
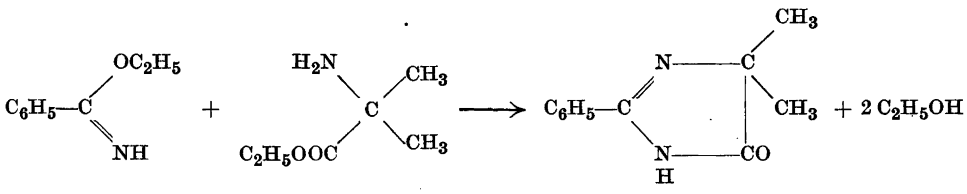
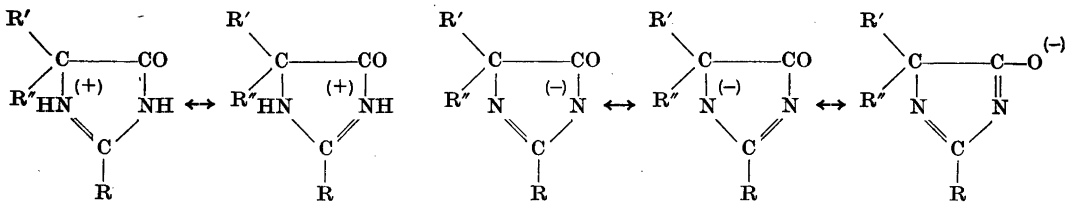


Fig. 3. UV-absorption spectra of: 1. α-Benzamidoisobutyramide in methanol; 2. 2-Phenyl-4,4-dimethyl-5-imidazolone in methanol; 3. The same in 0.1 N *methanolic KOH; 4. The same in 0.08* N *methanolic HCl.*

the following, an independent synthesis of a typical representative, *viz.* 2-phenyl-4,4-dimethyl-5-imidazolone, served to confirm the structure. Upon heating in xylene of ethyl iminobenzoate and ethyl α-amino*iso*butyrate a crystalline substance formed, which proved identical with Mohr's compound, resulting from the intramolecular dehydration of α-benzamido*iso*butyramide.



The protolytic nature of the imidazolones is clearly reflected in their UV-spectra. In Fig. 3 the spectra of 2-phenyl-4,4-dimethyl-5-imidazolone in neutral, alkaline and acidic solution are reproduced. They can be regarded as typical for this class of compounds. For comparison, the spectrum in methanol of the corresponding α-benzamido*iso*butyramide is presented also. The amide is devoid of notable acidic or basic properties, while the pronounced and reversible shift of the absorption curves for the imidazolones with changes in pH, reflects the important contribution of resonance-structures such as



in acidic and alkaline solutions, respectively. Other charged structures may of course be implied also, but their contributions will most likely be of minor importance.

Attention was next turned to acylated α-amino acid amides containing a hydrogen atom in the α-position (IV). In a previous communication [14] we demonstrated the report by Karrer and Gränacher [15] on the cyclisation of hippuramide to 2-phenyl-5-imidazolone to be incorrect, owing to a misinterpretation of the structure of the reaction product. No positive results attended numerous experiments to cyclise N-benzoylphenylalaninamide and N-benzoylalaninamide under widely varied reaction conditions, the straightforward hydrolysis to acid and ammonia being in most cases the predominant reaction. It was thought that an aromatic substituent on the α-carbon atom might facilitate the cyclisation. DL-α-Benzamidophenylacetic acid was transformed into its yellow azlactone (VIII, R′ = H, R″ = C_6H_5) which was characterised through its reactions with water, aniline and ammonia to give the acid, its anilide and amide (IV, R = R′ = C_6H_5), respectively. The Komatsu-Johnson reaction of the azlactone with ammonium thiocyanate and acetic anhydride led to the unknown 1-benzoyl-5-phenyl-2-thiohydantoin (XI). All attempts to cyclise α-benzamidophenylacetamide proved abortive, regardless of the conditions selected (alkali, acid, heat, dehydrating agents).

Next it was reasoned that steric factors might be implied. In order to test this possibility the preparation of the unknown N-benzoyl-DL-*pseudo*leucinamide was undertaken from *pseudo*leucine *via* its N-benzoyl-derivative (XII) and the corresponding azlactone. The latter was characterised by its reactions with methylamine, morpholine and aniline. Again, no indications of ring-closure were observed despite numerous efforts, demonstrating that the presence of a bulky substituent on the α-carbon atom is not *per se* sufficient to bring about cyclisation.

The findings above suggest a rather fundamental difference in the chemical behaviour of α-acylamido amides of non-tertiary and tertiary acids, the latter resembling in many respects the amides of unsaturated acids. These have been further studied in the hope of gaining more insight in the structural requirements for ring-closure. The results are presented in the following paper.

EXPERIMENTAL *

N-Benzoyl-DL-iso*valine*. DL-*iso*Valine (13.7 g) was treated with benzoyl chloride (13.6 ml) and a total amount of 120 ml of 2 *N* NaOH, according to the directions given by

* All melting points are uncorrected and determined in capillary tubes in an electrically heated block, those below 80° in a water-bath.

Steiger[10]. The yield was 19.8 g (76 %) of the colourless benzoyl-derivative. M.p. 192—193°. An analytical sample was obtained as platelets from aqueous acetone, containing some ethanol. M.p. 196°.

$C_{12}H_{15}O_3N$ (221.3) Calc. N 6.33
Found » 6.48

2-Phenyl-4-ethyl-4-methyl-5-oxazolone. Finely pulverised benzoyl*iso*valine (5.0 g) was suspended in 40 ml of freshly distilled acetic anhydride and shaken into solution at 70—80° within 12 minutes. Removal *in vacuo* of volatile material gave a slightly yellow oil, rapidly crystallising in the ice-box. Recrystallisation from *n*-hexane yielded the azlactone (3.65 g) as colourless rhombic plates, m.p. 48°.

$C_{12}H_{13}O_2N$ (203.2) Calc. C 70.93 H 6.45 N 6.90
Found » 71.03 » 6.41 » 6.98

On reaction with water containing a trace of alkali the azlactone afforded benzoyl-*iso*valine, identified by mixed melting point determination.

N-Benzoyl-iso*valinanilide.* This substance was readily isolated after treatment of the oxazolone with aniline in dry benzene. It separated from ethanol in thin needles. M.p. 199—200°.

$C_{18}H_{20}O_2N_2$ (296.4) Calc. C 72.93 H 6.80 N 9.45
Found » 73.02 » 6.78 » 9.40

N-Benzoyl-iso*valinamide.* When the azlactone above was treated for 5 minutes at 60° with 15 % aqueous ammonia, a clear solution remained which on cooling deposited the crystalline amide. Colourless prisms were obtained from water, m.p. 161—162°.

$C_{12}H_{16}O_2N_2$ (220.3) Calc. C 65.41 H 7.32 N 12.72
Found » 65.54 » 7.22 » 12.75

2-Phenyl-4-ethyl-4-methyl-5-imidazolone. The amide (161 mg) was dissolved within about 5 minutes in 2.5 ml of 1 *N* NaOH at *ca.* 85°. On addition of a few drops of glacial acetic acid a colourless oil separated which rapidly solidified. Recrystallisation of the product from water yielded 115 mg (78 %) of the imidazolone as colourless needles, m.p. 146—147°.

$C_{12}H_{14}ON_2$ (202.3) Calc. C 71.23 H 6.98 N 13.85
Found » 70.99 » 6.68 » 13.87

The UV-absorption spectrum in methanol showed three maxima at 230 mμ, 236 mμ and 250 mμ with molecular extinction values of 12 000, 11 900 and 11 000, respectively. Minima were found at 234 mμ and 242 mμ with ε-values of 11 750 and 10 600. An inflection point around 280 mμ (ε *ca.* 4 200) was also apparent.

DL-*α-Benzamidohydratropic acid.* Six grams of DL-α-amino-α-phenylpropionic acid[16] were benzoylated as described above for *iso*valine. The reaction product (5.5 g) tended to form an oil upon recrystallisation. By carefully adding petroleum ether to a solution in ethyl acetate, colourless rhombic platelets were obtained. M.p. 146—147°.

$C_{16}H_{15}O_3N$ (269.3) Calc. C 71.35 H 5.61 N 5.20
Found » 71.32 » 5.55 » 5.21

α-Benzamidohydratropamide. When the above acid was treated with acetic anhydride as usual, a pale yellow, oily azlactone remained after removal of excess reagents *in vacuo*. The oil did not crystallise on prolonged keeping in the ice-box and no attempts were made to purify it further.

Treatment of the product with ethanolic ammonia resulted in an 85 % yield of the crystalline amide, separating from aqueous ethanol as clusters of fine needles. M.p. 129—130°.

$C_{16}H_{16}O_2N_2$ (268.3) Calc. C 71.63 H 6.01 N 10.44
Found » 71.76 » 6.08 » 10.31

2,4-Diphenyl-4-methyl-5-imidazolone. Within 15 minutes 572 mg of the above amide went into solution in 10 ml of 1 *N* NaOH at 75—85°. No formation of ammonia was noticeable. After cooling and acidification, a crystalline mass was obtained and recrystallised from aqueous ethanol, giving 385 mg of fine needles. M.p. 151—152°.

$C_{16}H_{14}ON_2$ (250.3) Calc. C 76.79 H 5.64 N 11.20
Found » 76.96 » 5.60 » 11.23

α,α-Diethylhippuric acid. α-Aminodiethylacetic acid[17] (4.6 g) was benzoylated according to the general method. A sample for analysis was recrystallised twice from 30 % ethanol, m.p. 210°.

$C_{13}H_{17}O_3N$ (235.3) Calc. C 66.34 H 7.28 N 5.95
Found » 66.12 » 7.47 » 6.07

α,α-Diethylhippuramide. The acid was transformed into its azlactone as usual. This showed no signs of crystallisation and was submitted to ammonolysis without further purification. The amide was recrystallised from 50 % ethanol and melted at 198—200°.

$C_{13}H_{18}O_2N_2$ (234.3)	Calc.	C	66.63	H	7.74	N	11.96
	Found	»	66.47	»	7.50	»	11.92

2-Phenyl-4,4-diethyl-5-imidazolone. Upon treatment of the amide with alkali at moderate temperature, the imidazolone was obtained as a crystalline substance after scratching and cooling. M.p. 189—190°, after recrystallisation from aqueous ethanol.

$C_{13}H_{16}ON_2$ (216.3)	Calc.	C	72.18	H	7.46	H	12.95
	Found	»	72.41	»	7.10	»	12.90

The UV-absorption spectrum in methanol showed two maxima at 235 mμ and 250 mμ with the extinction values 12 250 and 12 600. A minimum was noticed at 245 mμ (ε 11 900). The usual inflection at 280 mμ was quite prominent (*cf.* Fig. 3).

N-(p-Nitrobenzoyl)-isobutyric acid. α-Amino*iso*butyric acid (10.3 g) was nitrobenzoylated, following the general procedure by Wright *et al.*[18]. After one recrystallisation from dilute ethanol the yield amounted to 17.1 g (68 %). An analytical sample was produced by two additional recrystallisations and appeared as small, pale yellow needles, m.p. 183.5°.

$C_{11}H_{12}O_5N_2$ (252.2)	Calc.	C	52.38	H	4.80	N	11.11
	Found	»	52.10	»	4.76	»	11.04

2-(p-Nitrophenyl)-4,4-dimethyl-5-oxazolone. When treated with acetic anhydride as usual, the acid above readily yielded the yellowish, crystalline azlactone. This recrystallised from anhydrous acetone as thin needles, m.p. 201—202°.

$C_{11}H_{10}O_4N_2$ (234.2)	Calc.	C	56.41	H	4.30	N	11.96
	Found	»	56.52	»	4.20	»	11.81

The UV-spectrum in methanol was characteristic of an aromatic nitro-compound with a broad maximum at 270 mμ (ε 12 100) and minimum at 230 mμ (ε 3 300), agreeing with the spectroscopical data previously reported [12] for similar compounds.

α-(p-Nitrobenzamido)-isobutyramide. This was obtained in 95 % yield from the oxazolone with ethanolic ammonia at room temperature as colourless needles with m.p. 223—225°.

$C_{11}H_{13}O_4N_3$ (251.2)	Calc.	C	52.58	H	5.22	N	16.73
	Found	»	52.42	»	4.79	»	16.70

In Fig. 1 the UV-spectrum in 0.01 *N* methanolic KOH is presented. When measured immediately after the preparation of the solution, this spectrum differs only slightly from that in pure methanol.

2-(p-Nitrophenyl)-4,4-dimethyl-5-imidazolone. The amide above was readily transformed in alkaline solution into the imidazolone, which crystallised from ethanol in colourless prisms with m.p. 216—217°.

$C_{11}H_{11}O_3N_3$ (233.2)	Calc.	C	56.65	H	4.75	N	18.02
	Found	»	56.72	»	4.54	»	18.07

In Fig. 1 the spectrum in 0.01 *N* methanolic KOH is reproduced together with some intermediate curves determined at different times in order to follow the cyclisation. The spectrum in pure methanol is quite different from the one here reported and reminiscent of the general imidazolone-spectrum in Fig. 3.

α-Phenacetamidoisobutyramide. α-Phenacetamido*iso*butyric acid [19] was transformed into its azlactone [19] as usual. Ammonolysis of the latter yielded the amide as long, colourless needles which were recrystallised from water. M.p. 184—185°.

$C_{12}H_{16}O_2N_2$ (220.3)	Calc.	C	65.45	H	7.32	N	12.72
	Found	»	65.43	»	7.24	»	12.65

2-Benzyl-4,4-dimethyl-5-imidazolone. The amide readily dissolved in 2 *N* NaOH. After acidification with glacial acetic acid, an oil separated which solidified on keeping in the ice-box overnight. On recrystallisation from water beautiful crystals were obtained which sintered from about 80° and gave analytical figures indicating a monohydrate.

$C_{12}H_{14}ON_2$, H_2O (220.3)	Calc.	C	65.45	H	7.32	N	12.72
	Found	»	65.24	»	7.25	»	12.75

Attempts to dehydrate the compound over P_2O_5 *in vacuo* at 56° resulted in slow sublimation and inconclusive analytical results on the residue. The imidazolone-structure was confirmed by the UV-spectra in methanol and methanolic KOH (Fig. 2).

DL-*α-Phenacetamidohydratropic acid.* Phenacetylation of 5.0 g of DL-α-amino-α-

phenylpropionic acid [16] by the Schotten-Baumann method gave 5.2 g (61 %) of the acylamido acid. Recrystallisation from aqueous ethanol yielded colourless needles. M.p. 184—185°.

$C_{17}H_{17}O_3N$ (283.3)	Calc.	C	72.06	H	6.05	N	4.94
	Found	»	71.96	»	5.96	»	4.93

α-Phenacetamidohydratropamide. Upon treatment of the above acid with acetic anhydride, the azlactone was obtained as a viscous oil which did not crystallise. Without further purification it was ammonolysed in the ordinary way to give the amide which crystallised from dilute ethanol as dense, colourless crystals, m.p. 130—132°.

$C_{17}H_{18}O_2N_2$ (282.3)	Calc.	C	72.33	H	6.43	N	9.93
	Found	»	72.30	»	6.52	»	10.06

2-Benzyl-4-methyl-4-phenyl-5-imidazolone. Alkali-treatment of the above amide brought about the cyclisation, and an 83 % yield of the crystalline imidazolone was obtained. It formed colourless, spear-formed prisms on recrystallisation from dilute ethanol. M.p. 163—165°.

$C_{17}H_{16}ON_2$ (264.3)	Calc.	C	77.25	H	6.10	N	10.60
	Found	»	77.38	»	6.21	»	10.64

2,4-Dimethyl-4-phenyl-5-imidazolone. When α-acetamidohydratropamide [20] was carefully heated in 1 *N* NaOH at 75—85° a clear solution was obtained within 8 minutes with no detectable evolution of ammonia. The mixture was acidified with glacial acetic acid and concentrated *in vacuo* to half its volume. On keeping at 0° an oil separated which was washed with cold water and thoroughly dried before analysis.

$C_{11}H_{12}ON_2$ (188.2)	Calc.	C	70.28	H	6.43	N	14.88
	Found	»	69.91	»	6.20	»	14.98

α-Formamidoisobutyric acid. Finely pulverised α-amino*iso*butyric acid (8.1 g) was dissolved in 10 ml of anhydrous formic acid on the steam bath. 17 g of the mixed anhydride, prepared from equimolecular amounts of formic acid and acetic anhydride, were added. A slightly exothermic reaction with evolution of gas (CO), was noticed. Next day the volatile contents were removed *in vacuo* at low temperature and the residue recrystallised from water. Clusters of flat prisms were obtained, (5.2 g, 51 %). M.p. 143—144°.

$C_5H_9O_3N$ (131.1)	Calc.	C	45.80	H	6.92	N	10.68
	Found	»	46.06	»	6.94	»	10.69

DL-*α-Formamidohydratropic acid.* Following the procedure above, an 84 % yield was obtained from DL-α-amino-α-phenylpropionic acid [16]. An analytical sample, recrystallised from hot water, melted at 178°.

$C_{10}H_{11}O_3N$ (193.2)	Calc.	C	62.16	H	5.74	N	7.25
	Found	»	62.20	»	5.90	»	7.22

DL-*α-Phenylhippuramide.* DL-α-Aminophenylacetic acid was benzoylated according to Steiger [10], and the α-phenylhippuric acid transformed into its slightly yellow azlactone, crystallising in thin needles [21]. The latter reacted with water to give α-phenylhippuric acid and with aniline to give the corresponding anilide, m.p. 214—215° (Ref. [21] 208—210° and 210—212°).

Reaction with ammonia gave α-phenylhippuramide in 88 % yield. It separated as colourless crystals from ethanol, m.p. 197°.

$C_{15}H_{14}O_2N_2$ (254.3)	Calc.	C	70.85	H	5.55	N	11.02
	Found	»	71.02	»	5.53	»	11.04

Numerous attempts were made to effect cyclisation of the amide, but all unsuccessful. Upon heating *in vacuo* the amide sublimed unchanged. Trituration with hot alkali or acid resulted in hydrolysis to the acid, whereas treatment with acetic anhydride or phosphorus pentachloride gave unchanged material contaminated with gummy products which could not be brought into a tractable form.

1-Benzoyl-5-phenyl-2-thiohydantoin. By following the general procedure of Johnson and Nicolet [22] for the reaction between azlactones and ammonium thiocyanate, an 87 % yield of crystalline thiohydantoin was obtained from the crude 2,4-diphenyl-5-oxazolone above. Very pale yellow needles separated from ethanol, m.p. 200—201°.

$C_{16}H_{12}O_2N_2S$ (296.3)	Calc.	C	64.86	H	4.08	N	9.46
	Found	»	64.68	»	4.40	»	9.24

DL-pseudo*Leucine (α-amino-β,β-dimethylbutyric acid).* Pinacolone (157.1 g) was oxidised in three portions with alkaline potassium permanganate. The method was that of

Richard [23] with minor modifications. Acidification, followed by ether extraction and distillation *in vacuo*, gave a total of 199.2 g (97 %) of analytically pure trimethylpyruvic acid as a viscous, colourless oil, b.p. 73.5—75° at 10 mm. (Ref.[23] 80 %).

When 53.4 g of trimethylpyruvic acid, 42.0 g of hydroxylamine hydrochloride and 50 g of anhydrous potassium carbonate in 140 ml of water were kept at room temperature for 20 hours, a thick suspension of crystalline material resulted. Further amounts of the same product separated upon addition of conc. hydrochloric acid. The crystals were taken up in ether and the aqueous phase repeatedly extracted with portions of fresh ether. Concentration *in vacuo*, followed by repeated evaporations with benzene in order to remove the last traces of water, gave a crystalline residue (55.0 g, 92 %) of the oxime, m.p. 112—115° (dec.). The anhydrous substance separated in colourless prisms from toluene. M.p. 116—117° (dec.). (Ref.[23] gives m.p. 85° for the monohydrate.)

After only discouraging results were obtained with various catalytic hydrogenations of the oxime, recourse was taken to the reduction with aluminium amalgam, previously mentioned in the literature [24]. A 40 % yield of pure *pseudo*leucine was obtained after repeated recrystallisations from aqueous acetone. The amino acid gave a deep-blue colour with ninhydrin, but only after heating for some time on the steam bath.

N-Benzoyl-DL-pseudo*leucine*. This was obtained in 70 % yield by the usual Schotten-Baumann benzoylation. An analytical sample separated from dilute ethanol in colourless plates, m.p. 164—166°.

$C_{13}H_{17}O_3N$ (235.3)	Calc.	C	66.34	H	7.28	N	5.95
	Found	»	66.37	»	7.53	»	5.71

*2-Phenyl-4-*tert.*-butyl-5-oxazolone*. This azlactone was readily produced from benzoyl-*pseudo*leucine and acetic anhydride as usual. Recrystallisation from hexane afforded colourless prisms, m.p. 73—74°.

$C_{13}H_{15}O_2N$ (217.3)	Calc.	C	71.85	H	6.96	N	6.45
	Found	»	72.04	»	7.33	»	6.21

*N-Benzoyl*pseudo*leucinmethylamide*. Produced from the azlactone and aqueous methylamine at room temperature. It separated as colourless prisms from aqueous ethanol, m.p. 202—203°.

$C_{14}H_{20}O_2N_2$ (248.3)	Calc.	C	67.71	H	8.12	N	11.29
	Found	»	67.42	»	7.87	»	11.45

*N-Benzoyl*pseudo*leucinmorpholide*. A solution of the azlactone above and morpholine in dry benzene was kept at room temperature overnight. Addition of hexane precipitated an oil which rapidly crystallised. Slender, colourless needles were obtained from aqueous ethanol. M.p. 181°.

$C_{17}H_{24}O_3N_2$ (304.3)	Calc.	C	67.06	H	7.95	N	9.20
	Found	»	66.91	»	7.99	»	9.40

*N-Benzoyl*pseudo*leucinanilide*. The azlactone was readily ring-opened on standing at room temperature in benzene solution containing a slight excess of aniline. When hexane was added, the anilide separated and was recrystallised from ethanol as colourless prisms, m.p. 223—225° after gradual sintering from *ca.* 170°.

$C_{19}H_{22}O_2N_2$ (310.4)	Calc.	C	73.50	H	7.15	N	9.03
	Found	»	73.58	»	7.20	»	9.12

*N-Benzoyl*pseudo*leucinamide*. One gram of the azlactone was added to 5 ml of ethanol, saturated at 0° with ammonia. A clear solution resulted from which the separation of prisms started in less than one minute. After standing, 0.97 g (89 %) of the amide could be collected. An analytical sample was obtained from ethanol. M.p. 202° (dec.).

$C_{13}H_{18}O_2N_2$ (234.3)	Calc.	C	66.63	H	7.57	N	11.96
	Found	»	66.40	»	7.68	»	12.19

Neither heating *in vacuo* nor treatment with the usual reagents afforded any indication of imidazolone-formation.

2-Phenyl-4,4-dimethyl-5-imidazolone. A solution of ethyl iminobenzoate (4.2 g) and ethyl α-amino*iso*butyrate (3.8 g) in 10 ml of anhydrous xylene was refluxed for 3 hours. After standing at room temperature for a few days, a crop of beautiful crystals (0.6 g) separated. The substance was recrystallised from water, m.p. 201—202°, alone or in admixture with a specimen prepared by the alkali-induced cyclodehydration of α-benzamido*iso*butyramide.

Ultraviolet absorption spectra. The UV-absorption spectra reported in this paper were determined in 1 cm cells on a model DU Beckman quartz spectrophotometer.

SUMMARY

A series of amides of acylated, tertiary α-amino acids has been prepared and their facile cyclisation to imidazolones demonstrated. The nature of the acyl-grouping and of the substituents on the α-carbon atom seems to play no decisive rôle, although considerable variation in the rate of the cyclisation-reaction has been observed.

Despite numerous attempts under widely varying conditions, no example has been found of a similar cyclisation within the class of non-tertiary α-acylamido acid amides.

An improved preparation of DL-*pseudo*leucine is reported and numerous new compounds described.

Microanalyses were carried out in this laboratory by Mr. A. Grossmann.

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Cyclodehydration of Acylated α-Amino Acid Amides

II. Unsaturated Amides

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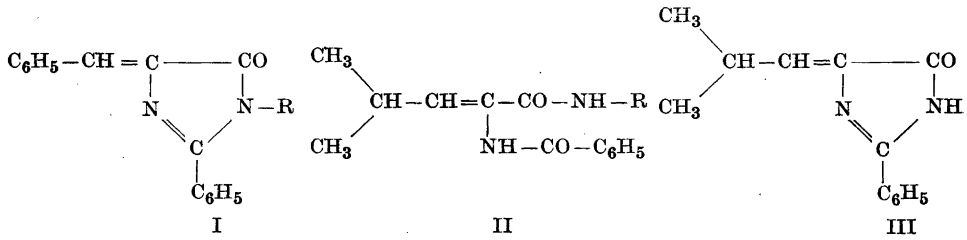
The preceding paper described the cyclisation of various tertiary α-acylamido acid amides to saturated 5-imidazolones and reviewed briefly the relevant literature on former applications of the reaction to unsaturated α-acylamido amides. The present communication is concerned with attempts to extend the scope of the reaction within the latter group.

While the cyclisation of α-benzamidocinnamamides to 4-benzylidene-5-imidazolones (I) is a well-established reaction, no case seems to be on record of an unsaturated, *aliphatic* amide being exposed to the usual ring-closure conditions. We have found that α-benzamido-β-*iso*propylacrylamide (II, R = H), easily prepared by ammonolysis of the unsaturated oxazolone, can be smoothly converted into 2-phenyl-4-*iso*butylidene-5-imidazolone (III) in alkali. Contrary to expectations, however, neither alkali-treatment, nor heating *in vacuo* above the melting point, resulted in cyclisation of the corresponding methylamide (II, R = CH_3), which could be recovered in high yield

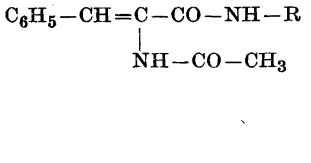


I II III

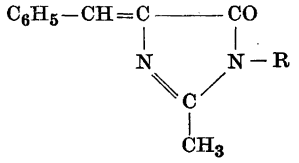
after the latter treatment. For comparison, the analogous N-methylcinnamamide was tested under similar conditions and found to yield the imidazolone (I, R = CH_3) upon melting *in vacuo*. Treatment with aqueous alkali, however, resulted in hydrolysis of the amide-grouping without detectable ring-formation.

Attention was next directed to unsaturated amides carrying an aliphatic acyl-grouping. 2-Methyl-4-benzylidene-5-oxazolone was ammonolysed to α-acetamidocinnamamide (IV, R = H) which could be smoothly converted

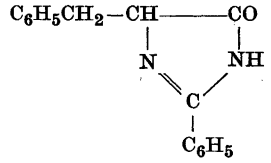
into 2-methyl-4-benzylidene-5-imidazolone (V, R = H) upon treatment with aqueous alkali. It was previously reported [1] that the analogous methylamide (IV, R = CH_3) cyclised on heating to give the imidazolone (V, R = CH_3).



IV



V

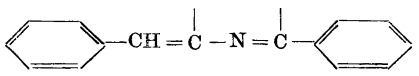


VI

The unsaturated imidazolones appear as high-melting crystalline compounds with yellow colours of varying intensity. Those having no substituent on the nitrogen-atom readily dissolve in alkali while all are only sparingly soluble in acid. The protolytic nature of the compounds were reflected in their UV-absorption spectra discussed below.

The easy preparative access of numerous unsaturated imidazolones by the Erlenmeyer-synthesis suggested a useful route to 4-substituted, saturated imidazolones by hydrogenation of the *exo*cyclic double bond of the former. Some explorative experiments proved this to be a feasible preparative method. For example, when 2-phenyl-4-benzylidene-5-imidazolone in ethanol was shaken with a palladium catalyst and hydrogen, the theoretical amount of hydrogen was rapidly consumed with simultaneous disappearance of the yellow colour. The filtrate afforded a good yield of 2-phenyl-4-benzyl-5-imidazolone (VI). This result is in marked contrast to that of Gränacher and Mahler [1]. They found that catalytic hydrogenation of unsaturated imidazolones resulted in complete reduction, including the C = N-grouping of the ring. Our attempts to hydrogenate the unsaturated imidazolone exhaustively, resulted in a very slow uptake of the second molecule of hydrogen, followed by isolation of a mixture of partially and completely hydrogenated material. The latter was conveniently prepared by reduction with sodium amalgam as previously described [1,3].

The ultraviolet absorption curves of the unsaturated imidazolones differ markedly from those reproduced in the preceding paper, belonging to compounds with no *exo*cyclic double-bond. Ekeley and Ronzio [2] previously reported absorption data in dioxane and aqueous alkali for a product, formed upon reaction between benzamidine, glyoxal and benzaldehyde in alkaline solution. The spectra of this compound, later proved to be 2-phenyl-4-benzylidene-5-imidazolone [3], are qualitatively similar to those reported in Fig. 1. There is a striking similarity between this spectrum and that of 2-phenyl-4-benzylidene-5-oxazolone [4], suggesting that the main absorption is due to the same chromophoric system, *viz.*



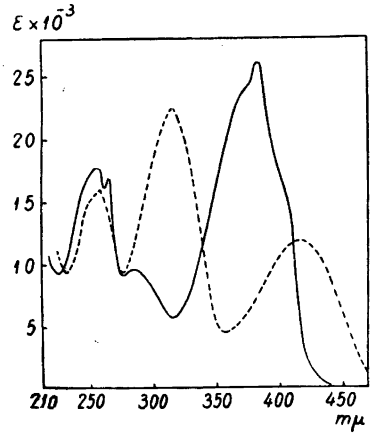


Fig. 1. Ultra-violet absorption spectrum of 2-phenyl-4-benzylidene-5-imidazolone in 96% ethanol: ——— and in 0.04 N ethanolic KOH: — — —.

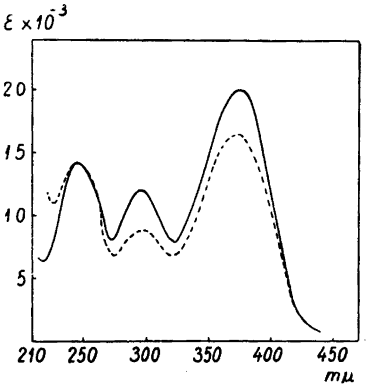
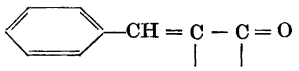


Fig. 2. Ultra-violet absorption spectrum of 1-methyl-2-phenyl-4-benzylidene-5-imidazolone in 96 % ethanol: ——— and in 0.04 N ethanolic KOH: — — —.

In Fig. 2 the absorption spectrum of the N-methylated imidazolone (I, R = CH_3) is shown. Although its general shape is unaltered, several small peaks and shoulders have disappeared; noteworthy is furthermore the minor influence of alkalisation in this case, clearly demonstrating the acidic character of the imide-hydrogen of (I, R = H).

α-Benzamido- and α-acetamidocinnamamide (IV, R = H) have similar absorption curves in methanol * (Fig. 3), indicating the principal influence of the common grouping



on the resonating system involved in the absorption of light. In the imidazolone-series the exchange of a methyl substituent with a phenyl-group has a pronounced effect on the absorption curves.

UV-absorption spectra have further been provided for α-benzamido-β-*iso*propylacrylamide (II, R = H) and the corresponding imidazolone (Fig. 4). The former represents the characteristic benzamide-absorption, unchanged on addition of acid or alkali. The ring-closed product, however, has absorption data reminiscent of those of the benzylidene-compound (*cf.* Fig. 1); again, addition of alkali changes the spectrum profoundly.

When a freshly prepared solution of α-acetamidocinnamamide in 0.01 *N* methanolic potassium hydroxide is kept at room temperature the imidazolone-formation can be followed spectrophotometrically (Fig. 5). The final curve agrees well with that obtained from the authentic imidazolone under similar

* The spectra of α-benzamidocinnamic acid and its hydrazide have been previously reported [4,5]. They differ only slightly from the curve of the corresponding amide shown in Fig. 3.

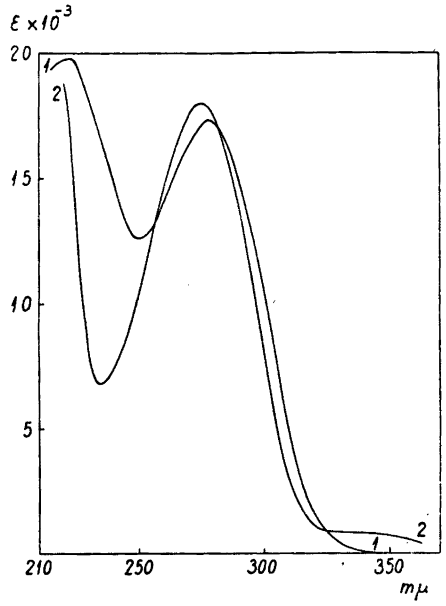


Fig. 3. Ultra-violet absorption spectra of 1 : α-benzamidocinnamamide and 2: α-acetamidocinnamamide, both in methanol solution.

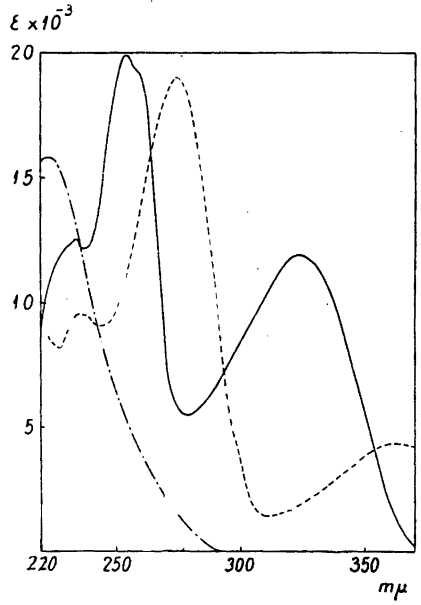


Fig. 4. Ultra-violet absorption spectra of α-benzamido-β-isopropylacrylamide in 96 % ethanol: – – ——— and of 2-phenyl-4-isobutylidene-5-imidazolone in 96 % ethanol: ———. The curve: — — — represents the latter dissolved in 0.05 N ethanolic KOH.

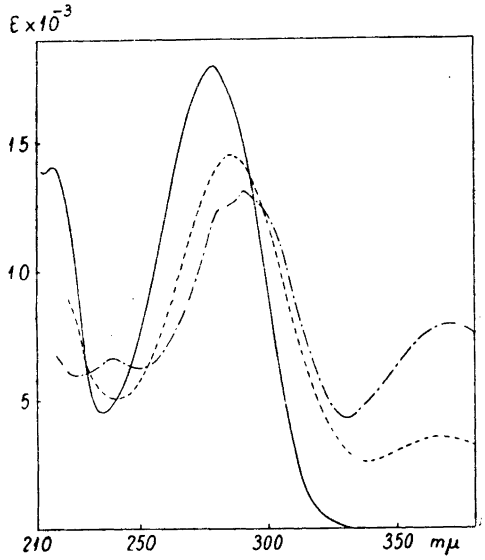


Fig. 5. Ultra-violet absorption spectra of a 0.08 M solution of α-acetamidocinnamamide in 0.01 N methanolic KOH, measured immediately, and after standing at 23°. Immediately after dissolution: ———; after 4 hours: — — — and after 21 hours: – – ——.

conditions. The rate of cyclisation in the present case is considerably higher than that reported in the preceding paper for analogous saturated amides. While more than 100 hours were required to cyclise the most favorable cases of the latter, the ring-closure of the present amide is virtually complete within *ca.* 20 hours.

EXPERIMENTAL *

α-Benzamido-β-isopropylacrylamide. 2-Phenyl-4-*iso*butylidene-5-oxazolone [6] was dissolved in ethanolic ammonia and kept for 3 days at room temperature. After concentration *in vacuo* and addition of water the amide separated. It crystallised, as a monohydrate, in colourless, rhombic plates from aqueous ethanol. M.p. 79–81°.

$C_{13}H_{16}O_2N_2 + H_2O$ (250.3)	Calc.	C 62.37	H 7.25	N 11.19
	Found	» 62.22	» 7.38	» 11.19

2-Phenyl-4-isobutylidene-5-imidazolone. The amide was dissolved in 2 *N* NaOH at 40–50° and acidified with glacial acetic acid, when pink crystals separated. Recrystallisation from ethanol, with addition of a little charcoal, yielded the imidazolone as clusters of pale yellow needles. M.p. 182° (decomp.). The compound is soluble in dilute alkali with a strong blue fluorescence.

$C_{13}H_{14}ON_2$ (214.3)	Calc.	C 72.85	H 6.59	N 13.08
	Found	» 72.49	» 6.40	» 13.08

α-Benzamido-β-isopropylacrylmethylamide. This was prepared in 91 % yield from the azlactone and methylamine in aqueous dioxane. The amide separated from dilute ethanol in colourless, flat prisms, m.p. 197°.

$C_{14}H_{18}O_2N_2$ (246.3)	Calc.	C 68.26	H 7.37	N 11.38
	Found	» 68.64	» 7.22	» 11.12

The amide is insoluble in alkali, even on heating, and several attempts to cyclise the substance at higher temperature proved fruitless. The crystalline starting material, contaminated with varying amounts of a dark brown oil, was the only product noticed after the reaction.

α-Benzamidocinnammethylamide. The amide resulted in 94 % yield when 2-phenyl-4-benzylidene-5-oxazolone was treated with ethanolic methylamine on the steam bath. It separated in clusters of colourless needles from ethanol. M.p. 188–189°. The amide proved very difficultly combustible in microanalysis, and it was only after mixing with potassium dichromate that correct figures were obtained.

$C_{17}H_{16}O_2N_2$ (280.3)	Calc.	C 72.83	H 5.75	N 9.99
	Found	» 72.73	» 6.02	» 9.99

1-Methyl-2-phenyl-4-benzylidene-5-imidazolone. While the above amide is unaffected by aqueous alkali and acid, heating at 200° *in vacuo* for 1.5 hours transformed it into an amorphous mass, which readily crystallised upon trituration with ethanol. Two recrystallisations from ethanol afforded the imidazolone as pale yellow needles, m.p. 135°.

$C_{17}H_{14}ON_2$ (262.3)	Calc.	C 77.83	H 5.38	N 10.68
	Found	» 77.98	» 5.47	» 10.72

α-Acetamidocinnamamide. When 2-methyl-4-benzylidene-5-oxazolone [7] was dissolved in ethanolic ammonia, the separation of the amide started within a few minutes. It could be recrystallised from methanol as colourless needles. M.p. 198–200° (decomp.).

$C_{11}H_{12}O_2N_2$ (204.2)	Calc.	C 64.71	H 5.93	N 13.73
	Found	» 64.60	» 5.79	» 13.84

After the present work had been concluded, Rothstein [8] described this amide with m.p. 205° (decomp.).

2-Methyl-4-benzylidene-5-imidazolone. The above amide was treated with alkali in the usual fashion, yielding the imidazolone which separated from aqueous ethanol in yellow needles, m.p. 169–172° (decomp.).

$C_{11}H_{10}ON_2$ (186.2)	Calc.	C 70.94	H 5.41	N 15.05
	Found	» 70.62	» 5.60	» 15.15

* All melting points are uncorrected and determined in capillary tubes in an electrically heated block, those below 80° in a water-bath.

2-Phenyl-4-benzyl-5-imidazolone. A solution of 2-phenyl-4-benzylidene-5-imidazolone in ethanol was shaken with Adam's PdO-catalyst in a hydrogen atmosphere at 1 at pressure. Within 30 minutes the calculated amount of hydrogen was taken up, followed by a sudden decrease in hydrogen-consumption. Upon evaporation of the filtrate, crystalline material separated from which traces of yellow impurities were removed by washing with a little ether. The colourless, crystalline powder melted at 250° under destruction and proved to give correct analytical figures without further purification. On rapid heating the m.p. 165—167° was observed.

$C_{16}H_{14}ON_2$ (250.3)	Calc.	C	76.78	H	5.64	N	11.20
	Found	»	76.68	»	5.42	»	11.14

2-Phenyl-4-benzyl-5-imidazolidone. The preparation was accomplished as previously described [1] by reduction of the unsaturated imidazolone with sodium amalgam and acetic acid in ethanol solution. Our product, twice recrystallised from xylene, melted sharply at 137—138°. Previously, the melting points 145—146° (Ref. [1]) and 150—151° (Ref.[3]) have been reported.

$C_{16}H_{16}ON_2$ (252.3)	Calc.	C	76.17	H	6.39	N	11.11
	Found	»	76.00	»	6.31	»	10.87

Ultraviolet absorption spectra. The UV-spectra were determined in 1 cm cells with a model DU Beckman quartz spectrophotometer.

SUMMARY

The cyclisation of unsaturated, aliphatic α-benzamido acid amides and α-acetamidocinnamamides to imidazolones is described.

The catalytic hydrogenation of the *exo*cyclic double bond in unsaturated imidazolones has been demonstrated to occur. A new route to 4-substituted 5-imidazolones is suggested through this finding.

The characteristic UV-absorption spectra are presented and discussed.

Microanalyses were carried out in this laboratory by Mr. A. Grossmann.

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The Separation of Choline Esters by Paper Chromatography

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In connection with work on the identification of various choline esters in tissue extracts, a method was worked out in this laboratory for the separation of such compounds by means of filter-paper chromatography.

Only a few papers have been published on the application of paper chromatography to the separation of choline esters. In the course of our experiments, Whittaker and Wijesundera [1] reported a method employing *n*-propanol- and *n*-butanol-water mixtures. Special accounts have been given by these authors [2] of the separation of the hydrolysis products of succinyldicholine. At the same time, Malyoth and Stein [3] published their work on the separation of choline esters, sugars, and aneurine. These authors used mixtures of acetic ester and pyridine with certain amounts of water. The method described in the present communication gives good separation of the choline esters. In addition the quantitative determination of acetylcholine based on the elution technique with paper chromatograms is reported.

METHODS

Chromatographic technique. Munktell No. OB filter paper was used in all the experiments, unless otherwise stated. Whatman No. 4 was used in some cases, but it showed no appreciable advantage over the Munktell paper. The Whatman paper possesses the properties of slower ascension and gives the same R_F values as the Swedish paper. Aqueous solutions 0.2—1 % of the compounds to be chromatographed were placed, with micropipettes, at points marked on a pencil line. The volume of solution used was about 5 μl, corresponding to 10—50 μg of the compound; the diameter of the spot was about 10 mm. The spots were then air-dried. The papers were placed in an air-tight glass chamber in a room, the temperature of which was maintained at 20.0 ± 0.5° C. Both ascending and descending techniques were used; the former was found to be more advantageous. When the solvent had run a convenient distance (about 40 cm in 16 hours in the upward and in 9 hours in the downward irrigation), the paper was dried and developed.

Solvents. A great many solvents have been tried which will be discussed below. The following is the composition of the mixture which we have found most useful: *n*-butanol-ethanol-acetic acid-water (8 : 2 : 1 : 3).

Colour developing reagent. The compounds were made visible on the paper by spraying with a solution of dipicrylamine (hexanitro diphenylamine, $Mg[N(C_6H_2(NO_2)_3)_2I_2]$ containing 0.2 g in 50 ml acetone and 50 ml distilled water. On spraying dried sheet with this

solution, choline and its esters at once produce dark yellow spots on a light yellow ground. The colour is strongest immediately after spraying and fades on standing for a week. Dipicrylamine also produces colour with other compounds which are described below.

Other developing reagents have been tried, *e.g.*, the iodine method (immersion in an alcoholic solution of iodine), spraying with a solution of bromphenol blue in 1 *M* K_2HPO_4, and the carboxylic reagent (hydroxylamine-ferric chloride) applied by Hestrin [4] to the quantitative estimation of acetylcholine. All three methods have been found to be less convenient than that described above.

The modification of Hestrin's method recently described by Whittaker and Wijesundera [1], but published after the above method had been worked out, has been tried in this laboratory. Non-aqueous reagents are used in this modification in contradistinction to Hestrin's reagents. This method seems to have definite advantages over the older one; it produces distinct spots (with acetylcholine) which are visible for several weeks. It is applicable only for detection of esters on chromatograms, not for the quantitative assay in solutions or extracts.

Quantitative estimation of acetylcholine. The quantitative estimation of acetylcholine on chromatograms was carried out by using the elution technique of spots in combination with the original method of Hestrin [4]. A test strip was cut out and developed. This strip with the coloured spots was placed alongside the rest of the chromatogram, the corresponding spots marked with a pencil, and the areas cut out and eluted with 2.0 ml of 0.0001 *M* hydrochloric acid (acetylcholine has maximum stability at pH 4). The acetylcholine present was estimated by mixing 1.0 ml of this eluate with 2.0 ml of a mixture of equal volumes of 3.5 *M* sodium hydroxide and 2 *M*-hydroxylamine hydrochloride. After adjusting to pH 1.4 ± 0.2 with hydrochloric acid, 1.0 ml 0.37 *M* ferric chloride ($FeCl_3 \cdot 6H_2O$) in 0.1 *M* HCl was added and the red colour promptly determined colorimetrically. The extinction coefficient of the solution was measured at 5 400 Å with an EEL colorimeter (Evans Electroselenium LTD, Harlow, Essex).

Compounds tested. Choline and choline esters were used as the chlorides with the exception of succinyldicholine iodide * and the M compounds **.

(M 111, the iodide of dicholine adipic acid ester; M 114, the bromide of the ethyl derivative of M 111; M 116, the iodide of the dicholine sebacic acid ester.) Phosphorylcholine chloride was used as the calcium salt, and acetylaneurine as the chloride hydrochloride.

RESULTS

The R_F values of the compounds tested for the solvent *n*-butanol-ethanol-acetic acid-water are presented in Table 1. Both descending and ascending techniques were employed, but the latter were found to be more convenient. The values obtained are the same whether one or many compounds are run in a mixture; this is illustrated with some compounds in Fig. 1.

Several solvent mixtures have proved unsatisfactory. Alkaline solvents, for instance those containing ammonia, cannot be used due to hydrolysis of the esters. Phenol is not useful; buffering with sodium citrate, KH_2PO_4, and ascorbic acid gives no separation. *Iso*-butanol-acetic acid gives small spots and high R_F-values. We have found *n*-butanol-acetic acid to be more satisfactory, giving lower R_F values. Good resolving power has been exhibited by *n*-butanol-ethanol, but in some cases duplicate spots are obtained with one ester. The resolving power of ethanol-acetic acid is unsatisfactory. However, the addition of certain amounts of acetic acid to the *n*-butanol-ethanol-water

* Prepared and kindly supplied by assistant L.-E. Tammelin, Research Institute of National Defence, Sweden.

** These compounds were kindly placed at our disposal by Messrs. Österreichische Stickstoffwerke, Austria.

Table 1. The R_F values (mean values) in n-*butanol-ethanol-acetic acid-water (8 : 2 : 1 : 3) at 20° C. The chlorides were used, unless otherwise stated.*

Substance	R_F	
	Descending	Ascending
Choline	0.37	0.38
Acetylcholine	0.47	0.46
Acetylthiocholine iodide	—	0.58
Acetyl-β-methyl-choline (mecholyl)	0.55	0.55
Propionylcholine	0.57	0.57
Butyrylcholine	0.67	0.66
Benzoylcholine	0.70	0.71
Carbaminoylcholine	0.30	0.30
Succinyldicholine iodide	0.18	0.12
M 111	0.22	—
M 114	0.25	—
M 116	0.54	—
Salicylcholine	0.64	0.64
Acetylsalicylcholine	0.65	0.67
Phosphorylcholine	0.19	0.21
Acetylaneurine	0.31	0.33
Aneurine	0.25	0.26
Betaine	0.47	0.46
D-Tubocurarine	0.45	0.45
Physostigmine salicylate	0.72	0.75
Prostigmine bromide	0.63	0.63
Histamine	—	0.21

mixture gives the best results. Formic acid may replace acetic acid but gives somewhat higher R_F-values; hydrochloric acid can also be used, but the paper must be neutralized before development with dipicrylamine. With propionic acid or *iso*-butyric acid the spots are more elongated and the R_F values low.

The solvent mixture, ethylene chlorohydrin-*n*-butanol-acetic acid-water, in the proportions 2 : 10 : 1 : 3, has been found useful. The R_F values produced are about the same as those obtained with the *n*-butanol mixture used generally in these studies. Distinct spots are obtained, but duplicate spots are usually produced by acetylcholine. The solvent mixture may be found satisfactory in a second run for twodimensional chromatograms.

Small variations in the composition of solvent produce great changes in the results. The proportion of acetic acid to water has been found to be of especially great importance. The most suitable composition of the solvent mixture used, *n*-butanol-ethanol-acetic acid-water, is 8 : 2 : 1 : 3. This solvent produces good resolving power with most of the choline esters studied. The following esters are easily separated in one-dimensional chromatogram: acetyl-, acetyl-β-methyl- or propionyl-, butyryl- or benzoyl, phosphoryl-, and carbaminoylcholine, and also choline. The dicholine esters of succinic acid and adipic acid (M 111) respectively, produce "tailing" and are not separated satisfactorily. The diester of sebacic acid (M 116), however, is easily separated from the other dicholine esters studied; aqueous solutions of M 116 kept for 3 hours or more give two distinct spots. Salicylcholine and especially phosphorylcholine give

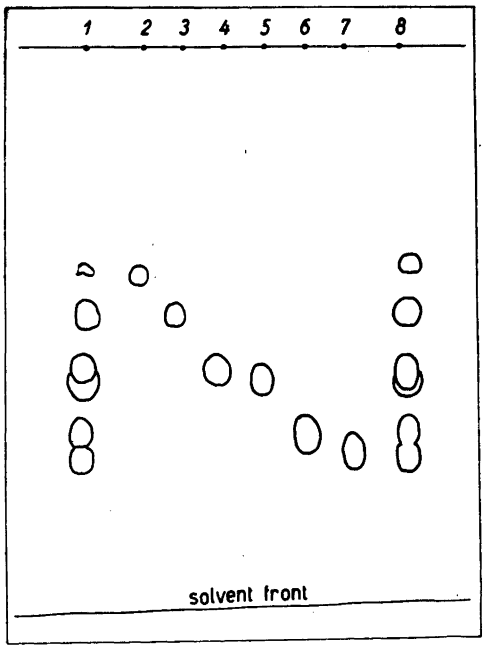

Fig. 1. Chromatogram of choline and various choline esters. Solvent: n-*butanol-ethanol-acetic acid-water (8 : 2 : 1 : 3). Upward irrigation at 20° C. Run of solvent front 42 cm in 16 hours. 1 and 8: mixtures of all compounds 2—7; 2: choline; 3: acetylcholine; 4: acetyl-β-methylcholine; 5: propionylcholine; 6: butyrylcholine; 7: benzoylcholine.*

spots which are more or less elongated. The spots produced by acetylsalicylcholine, on the other hand, show no "tailing".

In preliminary experiments some non-choline esters which may be of interest in connection with acetylcholine, and its occurrence and metabolism in tissues were tested. A mixture of physostigmine and prostigmine can be resolved satisfactorily; the "anticholinesterases" show R_F values which differ greatly from those of choline and acetylcholine. Tubocurarine produces "tailing". Acetylaneurine separates from acetylcholine and aneurine. The method may also be useful for the separation of histamine from acetylcholine and other choline esters. Betaine gives the same R_F value as acetylcholine but decolourises the dipicrylamine.

The dipicrylamine reagent is most useful for the development of colour on the chromatogram of choline esters. It was first used by Ackermann and Mauer [5] as a sensitive agent for the detection (precipitation) of acetylcholine and later used as developing reagent in paper chromatography [3]. Dipicrylamine gives yellow spots on a light yellow ground for choline and its esters. Sometimes choline appears as a light purple spot and may then be distinguished from the esters by this colour difference. Succinylcholine gives a colour which is darker than that produced by the other choline esters. The esters and choline itself may be detected in concentrations of about five micrograms or more. Phosphorylcholine must be used in higher concentrations in order to be visible after development. All spots must be marked soon after development.

The dipicrylamine reagent is not specific for choline and its esters. Among compounds which give positive reaction, acetylaneurine produces a yellow-red colour. Most other compounds, not mentioned in Table 1, appear in case of positive reaction as yellow spots, *e.g.* adrenaline and nor-adrenaline, creatine, hordenine, spermine, pilocarpine. The amino acids with the exception of arginine and tryptophane are not detected with this reagent. Negative results are also obtained with nucleic acids and their degradation products. Betaine produces decolourization.

Elution of spots of acetylcholine. The amount of acetylcholine (or any carboxylic acid ester of choline) on chromatograms can be estimated quantitatively with great accuracy by using the elution procedure. Acetylcholine can be eluted quantitatively with 10^{-4} *M* hydrochloric acid from a spot containing about 0.20 μmoles (38 μg acetylcholine chloride). The results obtained with 0.30 μmole of acetylcholine chromatographed together with the same amount of choline, are recorded in Fig. 2. The chromatogram is shown in the lower half of the figure. The numbered areas (one cm wide) were extracted with hydrochloric acid after cutting in small pieces and the amount of acetylcholine in each area was determined by the hydroxylamine-ferric chloride test (described by Hestrin [4]). It will be seen that good recovery and localization were obtained. It is unnecessary to mince the paper.

The extracts of the spots of acetylcholine have also been used for pharmacological assay. The guinea-pig ileum was used as test object in the usual way. It has thus been possible to estimate acetylcholine in parallel experiments both chemically and pharmacologically. These investigations are still in progress.

The enzymatic hydrolysis of dicholine esters. It has been proved previously in this laboratory that succinylcholine is split enzymatically by human plasma, although the rate of hydrolysis as measured with the Warburg technique is comparatively slow. This hydrolysis reaction has also been studied by Löw and Tammelin [6]. Moreover, it has been demonstrated by Whittaker [2] and by Ginzel *et al.*[7] that the monocholine ester of succinic acid is formed as an intermediate during the enzymatic hydrolysis of the dicholine ester.

The chromatographic technique described in the present paper is suitable for the analysis of reaction mixtures, such as those formed in enzymatic hydrolysis processes. Succinyldicholine (1 % solution) was incubated for three hours with horse plasma (the cholinesterase activity of which is about three times higher than that of human plasma). The reaction was carried out under optimum conditions for enzymatic hydrolysis, and the reaction mixture was chromatographed in the manner described above. A new spot with R_F 0.25 was found which is assumed to correspond to succinylmonocholine, and which was separated from choline (R_F 0.38) and the dicholine ester (R_F 0.12).

Similar preliminary studies have been performed with the dicholine esters of adipic acid and sebacic acid respectively. Both these compounds are destroyed comparatively rapidly by horse plasma. New spots are produced on the chromatograms by the reaction mixtures, but a detailed analysis of the compounds produced have not yet been made.

Experiments have also been performed with human erythrocytes in similar enzyme studies. Solutions of the dicholine esters were incubated for two hours

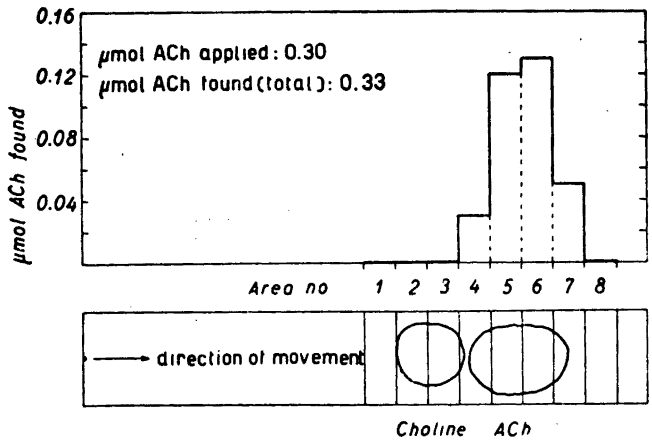

Fig. 2. Quantitative estimation of acetylcholine on a chromatogram by the hydroxylamine-ferric chloride test. Below a duplicate of the paper strip used for the elution of the choline and acetylcholine spots. Areas cut one cm wide.

or more at 25° C with intact washed red cells, in the presence of sodium chloride and magnesium chloride. The cells were then centrifuged and the supernatant liquid analysed by the paper chromatographic method. The original compounds, as found on the chromatograms, were unchanged, and new spots corresponding to degradation products of the esters could not be detected. It is difficult in such experiments to prevent hemolysis.

DISCUSSION

Choline, and most of its esters which have been studied can be separated by paper chromatography using a *n*-butanol-ethanol-acetic acid-water mixture. Dipicrylamine is a valuable reagent for the development of the chromatograms. This method is especially valuable for the separation and identification of mixtures of choline and acetylcholine. Partly purified extracts of various tissues have been examined, but difficulties arise when the method is applied to such material. It has been proved that acetylcholine and choline are in combination with proteins and that these combinations give rise to distorted chromatograms. The method is also valuable for the analysis of reaction mixtures containing acetylcholine and physostigmine or prostigmine. With most general techniques, acetylcholine and physostigmine precipitate together or stay in solution together, but the two compounds can be separated satisfactorally by chromatographic analysis. This technique also separates acetylcholine from histamine.

The method is not suitable for the analysis of choline esters, the acid portion of which is a dicarbonic acid. The method, however, has been used in preliminary experiments with hydrolysis mixtures of dicholine esters, showing that the adipic and sebacic acid esters, and to a lower degree the succinic acid ester, are destroyed by blood plasma and most probably not by erythrocytes.

SUMMARY

The separation of choline esters from each other and from choline by filterpaper chromatography is described. The solvent found to give the best separation is a *n*-butanol-ethanol-acetic acid-water mixture.

The esters on the paper were detected by spraying with a dipicrylamine solution in acetone. Acetylcholine was also estimated quantitatively by the elution technique in conjunction with the hydroxylamine-ferric chloride test.

Preliminary studies have been performed to separate the degradation products from the enzymatic hydrolysis of some dicholine esters (*e.g.* succinyldicholine), by paper chromatography.

This work has been supported by a grant from *Statens Naturvetenskapliga Forskningsråd* and *Magn. Bergvalls Stiftelse*. We wish to express our thanks to Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc., Nutley, New Jersey, for supplying the propionylcholine chloride, to Assistant L.-E. Tammelin, Research Institute of National Defence, Department I, Sundbyberg, Sweden, for succinylcholine, to Messrs. LKB-Produkter AB, Sweden, for acetylsalicylcholine chloride, to Messrs. Österreichische Stickstoffwerke, Linz, Austria, for the M compounds, and to Ing. H. Hasselqvist for the preparation of salicylcholine chloride.

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Synthetic High Molecular Weight Enzyme Inhibitors

I. Polymeric Phosphates of Phloretin and Related Compounds *

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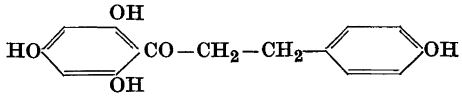
This investigation was started in order to throw some more light upon the mechanism of tubular reabsorption of glucose and the wellknown inhibitory action of phlorizin on this mechanism. According to results reported by Ellinger and Lambrechts [2] phlorizin appears to be absorbed from the tubular fluid by the epithelial cells of the proximal tubules, and the possibility was considered, that in this absorption phlorizin is chemically transformed into a specific agent, acting on glucose reabsorption. In view of the current hypotheses regarding the reabsorption of glucose, phosphorylation of phlorizin may be an obligatory step during its tubular absorption prior to its inhibitory action. Since alkaline phosphatase is generally assumed to be involved in the tubular reabsorption of glucose, some structural analogues of phlorizin were phosphorylated and their action on this enzyme was studied.

While the present study throws little light upon the possible occurrence of a renal phosphorylation of phlorizin, some interesting information concerning a new group of enzyme inhibitors has been obtained.

The phosphorylated substances, reported here, have been found to be the most potent inhibitors of alkaline phosphatase yet described. In addition it has been found, that these substances are very strong inhibitors of both hyaluronidase and urease. The first substance to be phosphorylated was phloretin, the aglucone of phlorizin. According to the structural formula



* A preliminary report [1] of some of these data was presented at the XVIII. Intern. Congress of Physiology, Copenhagen, 1950.

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this substance contains four hydroxy groups. During the phosphorylation of phloretin with phosphorus oxychloride in pyridine at low temperature it was assumed that a mixture of the mono-, di-, tri- and tetra-phosphates would result. However, during this phosphorylation the reaction mixture thickened within a few minutes and suddenly formed a gel, which contained products insoluble in all solvents. It was obvious that a polymerization reaction had occurred, leading to a presumably cross-linked high molecular weight polymer. When using an equimolecular amount of the phosphorylating agent and stopping the reaction just prior to this critical point a product could be isolated, which was soluble in water at a neutral or alkaline reaction, but insoluble in dilute mineral acid. This product did not dialyze through a cellophane membrane and has been shown by ultracentrifugation to have a molecular weight of about 15 000 [3]. If the reaction was stopped earlier, the degree of polymerization was lower. When investigating the polymer as to its effect on alkaline phosphatase and hyaluronidase, a very strong inhibitory effect was found. Therefore a systematic study was made under various experimental conditions of the phosphorylation of phloretin and other related substances. Among these other substances were phlorizin, flavone and flavanone glucosides and their aglucones, *viz.* rutin, quercetin, naringenin and naringin. From all these substances it was possible to obtain high molecular weight condensation polymers with a high antienzymic activity.

EXPERIMENTAL

A. Preparation of inhibitors

I. Polyphloretin phosphate. In a flask fitted with a thermometer, a calcium chloride drying tube and a dropping funnel 11 g of phloretin was dissolved in 50 ml of dry pyridine. The solution was cooled in an ice-salt bath to −10 to −12°. A solution of 4.5 ml of phosphorus oxychloride in 15 ml of dry pyridine was added with shaking at such a rate that the temperature remained at about −10° (about 10 minutes). When the flask was left for a further 3 minutes in the ice-salt bath, the solution gradually became viscous and was then hydrolyzed with crushed ice. The clear or almost clear solution was evaporated *in vacuo* and the thick oil thus obtained dissolved in about 100 ml of 2.5 *N* sodium hydroxide solution. After evaporating *in vacuo* until the smell of pyridine had disappeared, polyphloretin phosphate was precipitated as a viscous mass upon addition of dilute hydrochloric acid. The precipitate was washed with a small quantity of water and then dried *in vacuo* over phosphorus pentoxide and potassium hydroxide, resulting in a hard mass, which after pulverizing yielded a fine powder of a light green or light brown colour. If the moist precipitate was first treated with a mixture of equal parts of dry acetone and ether, a fine powder resulted; yield 10−12 g.

Analysis (from several runs): Moisture (sample dried at 100° *in vacuo*) 2−5 %; Pyridine (determined spectrophotometrically after alkalization and distillation) 5−10 %; Chlorine (combustion): 1−3 %; Phosphorus (calculated on a substance, free from moisture, pyridine and chlorine): 10−11 %.

During the phosphorylation other tertiary amines, *e.g.* quinoline, could be used instead of pyridine. The reaction proceeded more slowly in quinoline than in pyridine. If, for example, one mole of phosphorus oxychloride per mole of phloretin was used, the reaction mixture could be kept at room temperature for more than 24 hours without becoming thick or semi-solid. If a larger amount of phosphorus oxychloride was used (1.5−2 moles) the reaction mixture became semi-solid within one to two hours.

II. Polyquercetin phosphate. Six g of anhydrous quercetin was dissolved in 50 ml of dry pyridine. To this solution, cooled to −10°, a mixture of 2 ml of phosphorus oxychlor-

ide and 5 ml of dry pyridine was added over a period of five minutes. After a further 15—20 minutes at this temperature a thick, brown precipitate was formed. The mixture was then hydrolyzed and treated as above. The product was a yellow to brown powder; yield about 5 g. Phosphorus: 7—8 %. If the reaction time was prolonged a semi-solid product resulted which, however, slowly dissolved in water.

III. Polynaringenin phosphate. To a solution of 8.2 g of naringenin in 50 ml of dry pyridine, cooled to −10°, was added 3 ml of phosphorus oxychloride in 10 ml of dry pyridine over a period of five minutes. After a further 15 minutes at this temperature the solution became turbid. Three hours later the mixture was hydrolyzed and treated as in the previous examples. The product was obtained as a light brown powder; yield about 9 g. Phosphorus: 8.5 %.

IV. Polynaringenin chalcone phosphate. One g of naringenin chalcone (4,2′,4′,6′-tetrahydroxychalcone) was dissolved in 10 ml of dry pyridine. To this solution, cooled to −10°, 0.35 ml of phosphorus oxychloride in 5 ml of dry pyridine was added dropwise. As early as two minutes after the addition the solution became turbid. After a further 15 minutes at the same temperature a precipitate was formed. The mixture was then hydrolyzed and treated as in the previous examples. The product was a deep orange powder; yield about 0.7 g. Phosphorus: 8.7 %.

V. Polyphlorizin phosphate. A solution of 4.4 g of anhydrous phlorizin in 25 ml of dry pyridine was cooled to −10° and 1.5 ml of phosphorus oxychloride in 10 ml of dry pyridine was added with shaking over a period of 3 minutes. After a further 40 minutes the solution became turbid and after an additional 1 hour in the ice-salt bath the mixture was hydrolyzed, the resulting solution evaporated *in vacuo* and precipitated by the addition of dilute hydrochloric acid, saturated with sodium chloride. The semi-solid product was dissolved in a saturated sodium bicarbonate solution. The solution was filtered and the filtrate poured into an equal volume of dilute hydrochloric acid, saturated with sodium chloride. The semi-solid product was dried over phosphorus pentoxide and potassium hydroxide; yield 5.35 g. This product had a very high inhibitory effect on hyaluronidase. If the synthesis was carried out with 1.0 ml of phosphorus oxychloride, the inhibitory effect was similar. If 2 or 3 ml were used however, the inhibition was significantly less and with 5 ml the effect disappeared, presumably due to the formation of products with a relatively low molecular weight.

VI. Polyphlorizin sulphuric acid ester phosphate. To 15 ml of dry pyridine, cooled to −10°, was added 2.7 ml of chlorosulphonic acid. To this mixture a solution of 8.7 g of anhydrous phlorizin in 50 ml of dry pyridine was added slowly with cooling. The mixture was slowly heated on a water bath to 90° and was then left at room temperature for 15 hours. The reaction mixture was cooled to −10° and 2 ml of phosphorus oxychloride in 10 ml of dry pyridine was added and the mixture left at room temperature for 2 days and then hydrolyzed with crushed ice. The clear solution was alkalized with sodium carbonate and dialyzed against distilled water. The inner solution was evaporated *in vacuo* to dryness yielding a colourless powder; yield 11.4 g. Analysis: 4.4 % P; 8.9 % S. Thus the product contained about 2 atoms of sulphur per atom of phosphorus.

VII. Polyphloretin-4′-rhamnoglucoside phosphate. Phloretin-4′-rhamnoglucoside was synthesized by hydrogenation of naringin with Raney nickel catalyst in alkaline solution, m.p. 166—168°. Hydrolysis of this product by boiling with dilute sulphuric acid gave phloretin in good yield.

A solution of 2.2 g of anhydrous phloretin-4′-rhamnoglucoside was treated as above with 1.5 ml of phosphorus oxychloride. After 15 minutes in the ice-salt bath the solution became viscous and turbid. The mixture was hydrolyzed and treated as in V. A colourless precipitate formed with hydrochloric acid; yield 2.2 g. When using 1 ml of phosphorus oxychloride the inhibitory effect was unchanged. With 2.5 ml, however, the effect was significantly lowered.

VIII. Polyrutin phosphate. A solution of 1.2 g of rutin in 25 ml of dry pyridine was treated as above with 0.6 ml of phosphorus oxychloride in 5 ml of dry pyridine. The mixture was hydrolyzed and treated as in V. A precipitate was formed with hydrochloric acid. Yield 1.6 g. When 0.4 and 0.2 ml of phosphorus oxychloride were used in this experiment the antienzymic effect of the product gradually decreased. This decrease was even greater if a larger quantity (1.0 ml) was used.

IX. Polyphlorizin thiophosphate. To 1.1 g of anhydrous phlorizin in 15 ml of dry pyridine was added a solution of 0.6 ml of thiophosphoryl chloride in 10 ml of dry pyridine

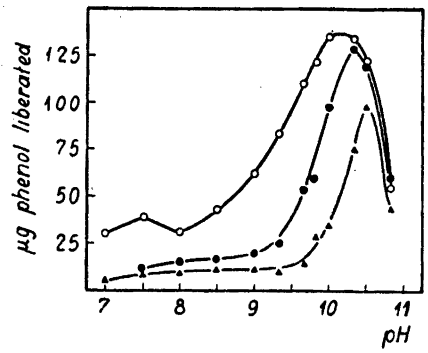


Fig. 1. Relationship between pH and inhibition of the enzymic hydrolysis of phenyl phosphate by polyphloretin phosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate concentration, 0.004 M. Enzyme-protein concentration, 1.2 µg per ml.

—○—○—, *without inhibitor*
—●—●—, *0.025 µg polyphloretin phosphate per ml.*
—▲—▲—, *0.25 µg polyphloretin phosphate per ml.*

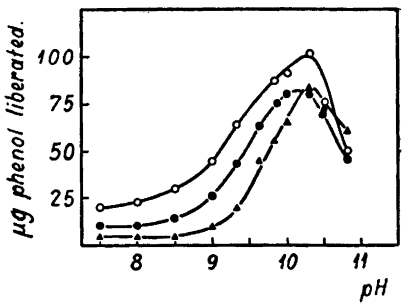


Fig. 2. Relationship between pH and inhibition of phosphatase by polyquercetin phosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate 0.004 M phenyl phosphate. Enzyme protein concentration, 1.2 µg per ml.

—○—○—, *without inhibitor*
—●—●—, *0.25 µg polyquercetin phosphate per ml.*
—▲—▲—, *2.5 µg polyquercetin phosphate per ml.*

with shaking over a period of 1—2 minutes at —10°. The mixture was left in the ice-salt bath for 3 hours and then at room temperature for 15 hours. After hydrolysis the solution was treated as in V; yield 1.25 g.

High molecular weight enzyme inhibitors were also prepared from hesperetin, phloretin-2'-methylether, phloretin-4-methylether, hesperidin and other similar compounds by analogous methods.

B. Enzyme experiments

Alkaline phosphatase.

The enzyme was prepared from the kidneys of young rabbits using the method described by van Thoai, Roche and Sartori [4]. In some experiments an enzyme preparation purified by electrophoresis was used.

Substrates: Phenyl phosphate, β-glycerophosphate and glucose-6-phosphate * were used.

Analytical methods: Phenol was determined with Folin's reagent as described by Buch and Buch [5]. Inorganic phosphate was estimated according to Martin and Doty [6]. The measurements were carried out in a Beckman spectrophotometer.

Conditions of enzyme experiments: Enzyme activity determinations were carried out within the pH range 7.0—11.0, using a 0.04 *M* borate-carbonate-acetate buffer without the addition of magnesium or manganese ions. Reaction volume, generally 2.5 ml; incubation time 10 or 16 min. at 37°. Enzyme concentration generally 1.2 µg of protein per ml.

Results: The inhibition of kidney alkaline phosphatase by polyphloretin phosphate, using phenyl phosphate as substrate is presented in Fig. 1. It appears from Fig. 1 that inhibition occurred on the acid side of the pH optimum only. A similar type of inhibition

* Glucose-6-phosphate was kindly supplied by Dr. O. Lindberg, Wennergren's Institute, Stockholm.

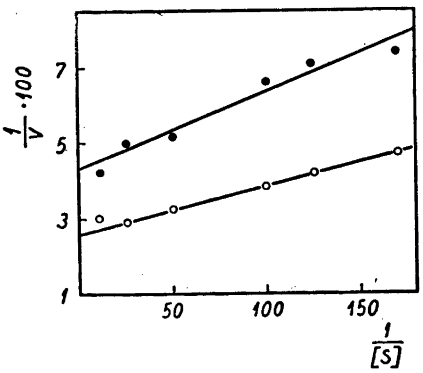


Fig. 3. Relationship between enzyme activity and substrate concentration in the presence and in the absence of polyphloretin phosphate. Activity is expressed as the reciprocal of the amount of P liberated in 10 min. at 37°. Enzyme protein concentration, 1.2 µg per ml. Substrate, phenyl phosphate. Acetate-carbonate-borate buffer pH 7.5.

—○—○—, *without inhibitor*
—●—●—, *0.025 µg polyphloretin phosphate per ml.*

was obtained by the use of other compounds of this group; in Fig. 2 the inhibition of alkaline phosphatase by polyquercetin phosphate is illustrated. As with polyphloretin phosphate, no inhibition could be demonstrated on the alkaline side of the pH optimum.

Substrate specificity: The inhibition of alkaline phosphatase is not substrate specific; using phenyl phosphate, β-glycero-phosphate or glucose-6-phosphate a similar inhibition was apparent.

Reversibility: It was found that the inhibition of alkaline phosphatase could not be reversed by magnesium or manganese ions in a concentration of 10^{-3} *M*. On the other hand, the inhibition could be quantitatively reversed by small amounts of basic proteins such as protamine sulfate or methyl gelatine in a concentration of 0.5 g/l. These two compounds did not activate the enzyme in the concentrations used.

Type of inhibition: To determine, whether the inhibition of alkaline phosphatase by polyphloretin phosphate was competitive or non-competitive, the concentration of phenyl phosphate was varied at a constant concentration of the enzyme in the presence and in the absence of the inhibitor. In Fig. 3 the reciprocal of the rate of enzymic hydrolysis (*v*, expressed as µg of P liberated after 10 min. incubation at 37° C) is plotted as ordinate against the reciprocal of the substrate concentration (*S*), following the procedure of Lineweaver and Burk [7].

It appears from Fig. 3 that the inhibition is not substrate competitive. A similar type of inhibition of kidney alkaline phosphatase by oestradiol-3,17-diphosphate has been reported previously [8].

Hyaluronidase.

Bovine hyaluronidase: The enzyme was prepared from bull testes and contained about 5 000 V.R.U. per mg N. The enzyme should be free from any stabilizer.

Hyaluronic acid: The substrate, hyaluronic acid, was prepared from umbilical cords by the method of Jeanloz and Forchielli [9] and a 0.2 % solution of the substrate in McIlvaine buffer, pH 7, was used for the test.

Inhibitors: The inhibitors studied were shaken with McIlvaine buffer, pH 7 ($Na_2HPO_4 \cdot 2H_2O$ 16.1 g; citric acid 2.02 g; NaCl 3.05 g; water to 1 000 ml) and then diluted with buffer to a concentration of 0.1 mg per ml.

Viscosity reducing test. Calibration of Ostwald viscosimeter: The readings were made in a bath at 37.5° C. 4 ml of the buffer solution was poured into an Ostwald tube and run through several times. The flow time was recorded.

Determination of viscosity of substrate solution: A mixture of 3 ml of the substrate solution and 1 ml of the buffer solution was run through as before. The flow time was recorded.

Determination of viscosity reduction by the enzyme: 0.1 ml of an enzyme solution, containing 2 V.R.U. (one V.R.U. is defined as the amount of enzyme, which will reduce the viscosity of the substrate used by one half in 10 minutes) and 0.9 ml of the buffer solution

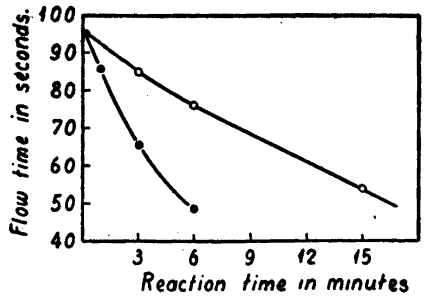


Fig. 4. Determination of anti-hyaluronidase activity. Flow time of buffer and substrate solution 11.5 and 95.5 sec. resp. Half time with and without inhibitor 15.5 and 5 min.

resp. % *inhibition* $\frac{15.5-5}{15.5} \times 100 = 68.$

—●—●—, *without inhibitor*
—○—○—, *with inhibitor.*

was mixed with 3 ml of the substrate solution. The stop watch was started immediately after the addition and the mixture was run several times through the viscosimeter with subsequent readings of the flow time.

Determination of the inhibition: 0.1 nl of an enzyme solution and 0.7 ml of the buffer solution were added to a mixture of 0.2 ml of the inhibitor solution and 3 ml of the substrate solution. The test was performed as above. In Fig. 4 a typical result is given.

Results. As a rule the inhibitory effect was measured at an inhibitor concentration of 5 μg per ml. The total amount of inhibitor used in each test was thus 20 μg.

Table 1. The effect of various inhibitors on hyaluronidase activity.

Polymer prepared from	Inhibitory effect on hyaluronidase, %
Phloretin	100
Phloretin-2′-methylether	85
Phloretin-4-methylether	85
4,2′,4′-trihydroxy-3-methoxy-chalcone	70
Naringenin	65
Naringenin chalcone	95
Hesperetin	20
Quercetin	55
Phlorizin	95
Phlorizin sulphuric acid ester	85
Phloretin-4′-rhamnoglucoside	95
Naringin	40
Hesperidin *	70
Rutin	90
Phlorizin and $PSCl_3$	100

Suramin showed no inhibitory effect in this test at a concentration of 10 μg per ml. With 40 μg the inhibitory effect was 42 %.

With 1 mg of protamine or methylated gelatine the inhibitory effect was reversed. With 0.1 mg the effect was decreased by about 50 %. Polyphloretin phosphate was also tested on a bacterial hyaluronidase *(Staphylococcus pyogenes)*. The effect was of the same order of magnitude, but it was necessary to perform the test at pH 6. At pH 7 the inhibitory action was insignificant at a concentration of 5 μg per ml.

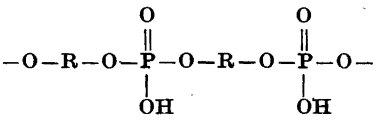
* Beiler and Martin [10] claim to have obtained a mixture of the tetra- and penta-phosphates of hesperidin by phosphorylating hesperidin with a large excess of phosphorus oxychloride.

Other enzymes.

A preliminary trial has been made on the inhibitory effect on several other enzymes. A very strong inhibition took place with prostate acid phosphatase, urease, β-amylase and brain hexokinase. With the last mentioned three enzymes a contact period between inhibitor and enzyme was, however, necessary. A detailed study on the inhibition of these and some other enzymes by the compounds described is in progress.

DISCUSSION

In the present paper, some properties and reactions of a new class of enzyme inhibitors are described and methods of their synthesis are reported. The substances in question are all polymeric and may be obtained by a condensation polymerization reaction between phloretin or other related aromatic polyphenols and a suitable phosphorylating agent. The degree of condensation depends on the particular conditions of synthesis. The polymers with a high degree of condensation are insoluble resinous substances. The members with a lower molecular weight are soluble at a neutral or alkaline reaction. The structure may contain elements of the type



Thus, the substances concerned are polyanions, and this structural property may be of significance as to their effect on enzymes. In this context, the inhibitory effect on enzymes of suramin [11], likewise a rather large anion, may be mentioned. The specificity of inhibition towards different enzymes seems quite similar, though the activity of the present substances surpasses that of suramin by several orders of magnitude. The inhibitory effect, however, does not seem to depend on electrical properties only. Combination between enzyme and inhibitor may also occur on the alkaline side of the isoelectric point of the enzyme, where both enzyme and inhibitor have negative charges. On the other hand, all inhibitions observed, could be reversed by the positively charged protamine, indicating the possibility of an electrostatic interaction. The ability of combining with alkaline proteins seems to be non-specific, since the enzyme inhibition is reversed besides protamine by various alkaline proteins among them gelatine, rendered basic by methylation.

With regard to the effect on enzymes, the difference between the various inhibitors described appears to be quantitative only, the ratio of the inhibitory effect on two enzymes being the same for two inhibitors of this class. Large differences exist, however, as to the effect on different enzymes. In the present work, experiments have been made with alkaline phosphatase, hyaluronidase, urease, brain hexokinase, β-amylase and prostate acid phosphatase. Very strong inhibitions were found with alkaline phosphatase, hyaluronidase and urease. The substances described here are the strongest inhibitors hitherto known of these enzymes.

As regards the mode of the inhibitory action, experiments with alkaline phosphatase revealed that the inhibition in this case was non-competitive, so that the site of combination with the inhibitor and with the substrate may be different. In the case of hyaluronidase the nature of the reaction and that of the substrate do not allow the performance of similar experiments. Since in this case both the substrate and the inhibitor are polyanions, the possibility of a competitive inhibition has to be considered.

SUMMARY

High molecular weight polyesters of phosphoric acid and phloretin and related polyphenols have been prepared. These polyesters have been shown to possess a very high inhibitory effect on several enzymes, above all alkaline phosphatase and hyaluronidase.

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Synthetic High Molecular Weight Enzyme Inhibitors

II. Polymeric Phosphates of Aromatic Hydroxy and Amino Compounds

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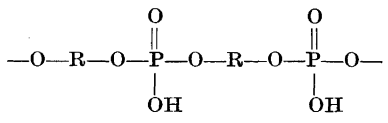
In a previous paper [1] it was shown, that by phosphorylating phloretin and other structurally related polyphenols it is possible to obtain high molecular weight polymers. These polymers are polyanions and they exert a strong inhibitory effect on various enzymes, particularly alkaline phosphatase, hyaluronidase and urease.

From these results it seems possible that the nature of the initial molecules is without significance and that it should be possible to prepare polymers of the same general type and with similar properties from compounds with a different structure, provided that they contain groups, capable of reacting with a phosphorylating agent. *A priori* it should be possible to perform a condensation polymerization between two compounds, if both of them contain at least two reactive groups. The commonest phosphorylating agent is phosphorus oxychloride. This compound has three reactive chlorine atoms, two of which, however, are more reactive than the third [2]. The chlorine atoms of phosphorus oxychloride may react with hydroxy, amino and sulphhydryl groups (hereafter termed reactive groups). The simplest organic aromatic hydroxy compounds, from which it should be possible to prepare polymers of this type are pyrocatechol, resorcinol and hydroquinone. While with pyrocatechol ring formation is known to result [3] it was possible with the other two compounds to prepare non-dialyzable polymers. These polymers may contain elements of the type



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A systematic synthetic study was undertaken with other aromatic compounds containing at least two reactive groups and in practically all cases it was possible to obtain soluble, non-dialyzable polymers of this general type. Some of the compounds from which it was possible to prepare polymers by phosphorylation with phosphorus oxychloride or thiophosphoryl chloride under suitable conditions are listed in Table 1.

Pyrocatechol, *o*-aminophenol and resacetophenone did not form high molecular weight polymers under various experimental conditions. In order to obtain a high degree of polymerization the reaction conditions are of great importance. In most cases the compounds to be phosphorylated were dissolved in pyridine or quinoline, and thereafter at a temperature of −10 to −15° a solution of phosphorus oxychloride was added with cooling and shaking. The reaction velocity depends upon the reactivity of the compound and the number of reactive groups available. In the case of compounds with three or more reactive groups, employing a pyridine solution, the reaction is usually terminated within a few minutes; if, however, the compound contains only two reactive groups, polymerization takes longer. In quinoline the reaction proceeds more slowly than in pyridine. The ratio of the reactants is also of great importance. If the molecules contain two reactive groups, 2/3 mole of the phosphorylating agent was usually employed; if three reactive groups, one mole and so on. If a large excess of phosphorylating agent is used, the degree of polymerization is lowered and an increasing amount of dialyzable material is obtained.

The polymers formed are all very active enzyme inhibitors. Hitherto they have been investigated only as to their effect on hyaluronidase *. It may be assumed, however, that their inhibitory effect on other enzymes is of the same order of magnitude as that of polyphloretin phosphate. As will be seen from the experimental part the degree of inhibition on hyaluronidase may vary to a rather large extent but usually a strong inhibition is observed in concentrations of 5 μg per ml and even less under the test conditions used. It should be pointed out, however, that conditions for the phosphorylation reaction are very specific and even small changes in the conditions of the reaction may give rise to polymers of different properties, *i.e.* either higher or lower antienzymic activities. As to the stability of the polymers in water solution, this varies within a rather wide range from relatively unstable to practically stable products.

From compounds with two reactive groups it might be assumed, that a linear condensation polymer is formed. Some of the third chlorine atoms may also react, however, thus forming a branched polymer. This matter has been studied with hydroquinone and resorcinol. If 2/3 mole of phosphorus oxychloride is used it may be assumed that at first a reaction takes place leading to compounds of the type $HOC_6H_4OP(O)(Cl)OC_6H_4OP(O)(Cl)OC_6H_4OH$. At a later stage of the polymerization also the remaining chlorine atoms may react. It is well known that in triesters of phosphoric acid one ester group is more easily hydrolyzed than the other two [4]. Polymers of this kind are

* Polyphloroglucinol phosphate has an inhibitory effect on alkaline phosphatase of the same order of magnitude as polyphloretin phosphate (E. Diczfalusy; personal communication).

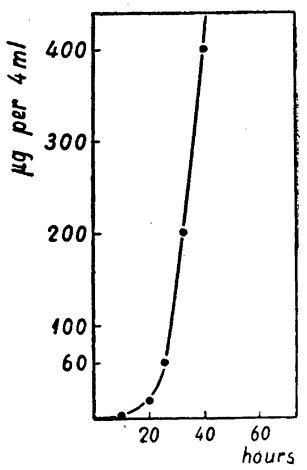

Fig. 1. Hydrolysis of polyphloroglucinol phosphate. Concentration necessary to effect 80 % inhibition is plotted against time in hours.

rather unstable in aqueous solution. As a criterion of stability we have chosen the inhibitory effect on hyaluronidase.

With three or more reactive groups the resulting polymer will be branched. As the degree of branching increases the resulting polymer will be less soluble. If the polymerization is allowed to proceed, an insoluble polymer is formed, as in the case of phloroglucinol. The positions of the reactive groups are, however, of importance. With gallic acid or pyrogallol this stage is not reached, presumably due to ring formation. With phloroglucinol and other similar compounds it is necessary to stop the phosphorylation process at a suitable stage in order to arrive at polymers of the desired degree of polymerization. It is also possible to allow the phosphorylation to proceed to completion and then hydrolyze the resulting insoluble polymer. This may be accomplished by heating or, more conveniently, by boiling with dilute hydrochloric acid. This latter method has been studied in the case of phloroglucinol.

An insoluble polyphloroglucinol phosphate (Example II) was boiled in 0.1 *N* hydrochloric acid until a clear solution was formed. This solution contained soluble polyphloroglucinol phosphates of varying molecular size. 6 % of the organically bound P ($P_{org.}$) dialyzed through a cellophane membrane and 13 % of the total P content was inorganic P.

According to the method of assay used 0.5 µg (corresponding to phloroglucinol) per 4 ml was necessary to effect an inhibition of 80 %. The solution was boiled and portions withdrawn after various length of time. According to Fig. 1 the quantity of material necessary to produce an inhibition of 80 % shows an accelerated increase.

After 20 hours' boiling 20 µg was required and after 100 hours 5 000 µg. During the same time % $P_{inorg.}$ of $P_{tot.}$ increased to 32 and 57 % respectively (Fig. 2). After 24 hours 44 % of $P_{org.}$ dialyzed (Fig. 2). When a solution was tested before and after dialysis all activity remained in the inner solution.

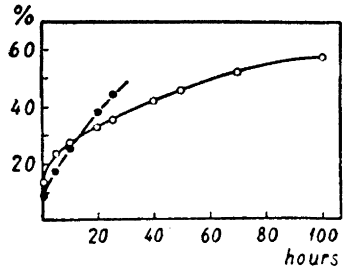

Fig. 2. Hydrolysis of polyphloroglucinol phosphate. Inorganic phosphorus in per cent of total phosphorus (open circles) and dialyzable in per cent of total organically bound phosphorus (filled circles) are plotted against time in hours.

From these and similar experiments it is obvious that the stability of a polymer may be studied by measuring its antienzyme effect and that the antienzymic activity is bound to the non-dialyzable material.

The reactions leading to this type of polymers may be interpreted in the following way. At first a condensation product between the phosphorylating agent and the aromatic compounds is formed and then this product, which, if the initial molecule is a phenol, may be written $ROP(=O)Cl_2$, begins to react with other molecules bearing free OH-groups and condensation polymers are formed. It is impossible, however, to distinguish between these two stages. As the reaction proceeds the phosphorylating agent may well condense with already polymerized molecules. This is especially the case if the phenol or amine in question contains more than two reactive groups. The study of this type of condensation polymerization from a kinetic point of view is thus complicated by the above factors.

EXPERIMENTAL

Preparation of inhibitors

I. *Polyphloroglucinol phosphate* (in quinoline). In a flask fitted with a thermometer, a calcium chloride drying tube and a dropping funnel 10.1 g of phloroglucinol was dissolved in 120 ml of dry quinoline. At −10° a solution of 4 ml of phosphorus oxychloride in 20 ml of dry quinoline was added with stirring (10 minutes). After an additional 10 minutes in the ice-salt bath the temperature was raised to 20° and held there for 30 minutes. After cooling to − 10° a solution of 8 ml of phosphorus oxychloride in 20 ml of dry quinoline was added. After 10 minutes the temperature was again raised to 20° and after 15 minutes the solution became turbid and viscous. The mixture was hydrolyzed with crushed ice, made alkaline with dilute sodium hydroxide solution, extracted several times with ether and then acidified with hydrochloric acid. The resulting precipitate was filtered by suction, washed with dilute hydrochloric acid and dried in a desiccator over phosphorus pentoxide and potassium hydroxide; yield 22.1 g, a light brown powder. Phosphorus: 14.3 %. Inhibition of hyaluronidase 95 % (5 μg per ml).

II. *Polyphloroglucinol phosphate.* 500 g of phloroglucinol was dissolved in a mixture of 2 l of dry acetone and 2 l of dry chloroform. 400 ml of phosphorus oxychloride was added at a temperature of − 10°. 400 ml of dry pyridine was then added with stirring and cooling at a rate such that the temperature remained below 0° (about 20 minutes). A further 1 000 ml of dry pyridine was added as rapidly as possible, causing a rise of temperature to about 25°. After a further 10 minutes the mixture became viscous and the

stirring was continued for about 1 hour. 15—20 hours later the precipitate was filtered by suction, washed with acetone and suspended in water. After 24 hours the precipitate was filtered again, washed with acetone and dried at 60—70°; yield 1 060 g. Analysis:

Moisture	6.4 %	
Pyridine	25.0 %	(on dried sample)
Chlorine	3.9 %	» » »
Phosphorus	12.8 %	» » »

This product was insoluble and may be regarded as a very high molecular weight, presumably cross-linked polymer. According to this assumption the product could be partially depolymerized thus yielding water soluble products with a very high inhibitory effect on hyaluronidase. 35 g of the insoluble polymer was boiled with 800 ml of 0.1 *N* hydrochloric acid. After 3 hours a clear, light yellow solution resulted containing polyphloroglucinol phosphates of different degrees of condensation. Inhibition of hyaluronidase 80 % (0.13 μg per ml). By heating the insoluble polymer to 160° for 4 hours a product was formed which gradually dissolved in water. Inhibition of hyaluronidase 70 % (5 μg per ml).

III. *Polyphloroglucinol thiophosphate.* A solution of 2.2 ml of thiophosphoryl chloride was added to 2.6 g of phloroglucinol in 20 ml of dry pyridine at — 10° (2 minutes). After a further 1.5 hour in the ice-salt bath a precipitate was formed and after another 15 hours at room temperature the mixture became semi-solid. After hydrolyzing with crushed ice the mixture was allowed to stand until the precipitate had dissolved (24 hours). On evaporating *in vacuo* a red viscous oil was obtained. Inhibition of hyaluronidase 100 % (5 μg per ml).

IV. *Polyhydroquinone phosphate.* At — 5° a solution of 12.5 ml of phosphorus oxychloride was added to 22 g of hydroquinone in 50 ml of dry pyridine (11 minutes). The mixture was then treated as in III. On evaporating *in vacuo* a yellow oil resulted, which by washing with dilute hydrochloric acid and water became semi-solid; yield after drying in a desiccator 29 g. Analysis:

Moisture	21.7 %	(60° *in vacuo*)
Phosphorus	12.1 %	(dried sample)
Chlorine	0.9 %	» »
Pyridine	14.1 %	» »

The substance was soluble in sodium bicarbonate solution. Inhibition of hyaluronidase 95 % (5 μg per ml). An aqueous solution is, however, not stable. A solution, kept at room temperature for a week showed an inhibition of 40 % and after warming in an autoclave to 120° for 20 minutes the inhibition had decreased to 15 %. No inorganic phosphorus was liberated.

If the synthesis was performed with equimolecular amounts of phosphorus oxychloride and hydroquinone the resulting product had a similar inhibitory effect but the stability was somewhat better. A water solution of this product was dialyzed against running water for 5 days. After this time 90 % of the original phosphorus contents had not dialyzed and the solution had an inhibitory effect of 70 %. A control solution held at the same temperature had after the same time a similar inhibitory effect. Initially all phosphorus was organically bound and no inorganic phosphorus was liberated.

If the synthesis was performed with 1.5 mole of phosphorus oxychloride the inhibition decreased to 20 % (5 μg per ml) while with 2 moles no inhibition could be observed with 50 μg per ml.

Polyhydroquinone phosphate could also be prepared at a higher temperature. If the addition of phosphorus oxychloride was made at 25 to 50° and the mixture hydrolyzed after a further 30 minutes, a product was obtained with similar properties.

V. *Polyresorcinol phosphate.* To a solution of 1.25 ml of phosphorus oxychloride in 10 ml of pyridine there was added 2.2 g of resorcinol in 20 ml of dry pyridine (½ minute, — 10°). The mixture was treated as in III. The resulting oil had an inhibitory effect of 100 % (5 μg per ml). A solution kept at room temperature for a week showed an inhibition of only 20 %.

In another run portions of the mixture were withdrawn 3 and 120 minutes after the addition of phosphorus oxychloride. The samples were worked up as usual and showed

Table 1. The inhibitory effect on hyaluronidase of various inhibitors.

Polymer prepared from	Tertiary amine, pyridine (Py) or quinoline (Qu)	Concentration of inhibitor μg per ml	Inhibitory effect on hyaluronidase, %
Resorcinol	Py	5	100
» + $PSCl_3$	Py	5	80
Orcinol	Py	5	75
4-Hexylresorcinol	Py	8	90
2-Nitroresorcinol	Py	5	95
4,6-Dibromo-2-nitroresorcinol	Py	1.3	100
4-Nitroresorcinol	Py	50	100
Resorcinol-4-sulphonic acid	Py	50	50
4-Nitrobenzeneazoresorcinol	Py	5	45
Hydroquinone	Py	5	95
Gentisic acid	Py	5	80
Phloroglucinol	Py	0.13	80
»	Qu	5	95
» + $PSCl_3$	Py	5	100
Phloracetophenone	Py	5	60
2,4,6-Trihydroxybenzaldehyde	Py	50	80
Hydroquinone + phloroglucinol	Py	5	100
Pyrogallol	Py	50	100
Gallic acid	Py	8	75
m-Aminophenol	Py	5	40
p-Aminophenol	Py	5	75
p-Methylaminophenol	Py	50	45
p-Aminothiophenol	Py	5	90
5-Aminoresorcinol	Py	0.5	75
p-Phenylenediamine	Qu	5	55
p,p'-Dihydroxybibenzyl	Py	5	95
4,4'-Dihydroxydiphenyldimethyl-methane	Py	0.05	80
4,4'-Dihydroxy-γ,δ-diphenyl-β,δ-hexadiene	Py	5	95
2,2',4,4'-Tetrahydroxydiphenyl-methane	Py	5	85
2,4,4',6-Tetrahydroxybenzophenone	Py	0.5	40
Benzidine	Qu	25	50
4,4'-Diaminodiphenylmethane	Qu	40	90
p,p'-Diaminobibenzyl	Py	5	75
p-Aminodiphenylamine	Qu	50	50
4,4'-Diaminodiphenylsulphone	Py	5	80
1,3-Naphtalenediol	Py	5	80
1,5- »	Py	5	100
2-Methyl-1,4-naphtalenediol	Py	5	100
1,2,7-Trihydroxyanthraquinone	Py	5	95
1,2,5,8-Tetrahydroxyanthraquinone	Py	10	80

As has already been pointed out even small variations in the experimental conditions may give rise to products with varying activities. A study of the effect on other enzymes is in progress.

an inhibition of 0 % (50 μg per ml) and 40 % (5 μg per ml) respectively. After a further 15 hours at room temperature the inhibition was the same as above.

VI. *Polyhexylresorcinol phosphate.* A solution of 0.65 ml of phosphorus oxychloride in 10 ml of dry pyridine was added to 1.95 g of hexylresorcinol in 20 ml of dry pyridine

at −10° (2 minutes). The mixture was treated as in III. The resulting oil was dissolved in a mixture of 25 ml of acetone and 40 ml of 2 *N* sodium hydroxide solution. Upon acidifying with 60 ml of 2 *N* hydrochloric acid the solution became turbid. It was extracted several times with ether, the combined ether extracts were washed once with saturated brine and then evaporated *in vacuo*, yielding a viscous oil; yield after drying 2 g, inhibition 90 % (8 μg per ml). The inhibition did not decrease if an aqueous solution was kept for 7 days at room temperature. After dialyzing (cellophane membrane) for 5 days against running water all the activity remained in the inner solution.

VII. *Poly-4,4'-dihydroxydiphenyldimethylmethane phosphate.* A solution of 0.62 ml of phosphorus oxychloride in 5 ml of dry pyridine was added to 2.28 g of 4,4'-dihydroxydiphenyldimethylmethane in 20 ml of dry pyridine at −15° (5 minutes). After a further 15 hours in the ice-salt bath the viscous mixture was hydrolyzed with crushed ice. Upon addition of 75 ml of 5 *N* hydrochloric acid a powder was obtained, which was filtered by suction and dissolved in sodium hydroxide solution. The solution was washed several times with ether and then acidified with hydrochloric acid, thus yielding a fine white precipitate, which was filtered by suction, washed with water and dried, yield 3.2 g; inhibition 80 % (0.05 μg per ml).

Enzyme experiments

As mentioned above the polymers, with one exception, have been investigated only as to their inhibitory effect on hyaluronidase. The same method was employed as in the previous paper [1]. The results are summarized in Table 1.

SUMMARY

High molecular weight polyesters and polyamides of phosphoric acid and aromatic hydroxy and amino compounds, containing at least two groups, capable of reacting with phosphorus oxychloride, have been prepared. They have been shown to exert a very high inhibitory action on hyaluronidase.

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The Anomalous Electric Dipole Moment of *p*-Quinones

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The electric dipole moment of 2,5-di-tert-butyl-1,4-benzoquinone has been measured in benzene solution at 25° C. It is 0.81 ± 0.03 D. The origin of the unexpectedly large apparent moments of *p*-benzoquinone and various symmetrical derivatives is discussed in the light of the new result.

The conventional formulation of *p*-benzoquinone is symmetrical. X-ray work [1] has revealed that in the crystalline state the ring is an irregular hexagon, but the molecule including the oxygen atoms is coplanar and centrosymmetrical. It is reasonable to assume that the same structure prevails in dilute solution, because of *inter alia* the resonance in the system. Consequently *p*-benzoquinone should have a zero dipole moment. Hassel and Naeshagen [2], however, found the value 0.67 D in benzene solution. They suggested that the observed orientation polarisation is an apparent one, and that an abnormally high atom polarisation is responsible for it.

Hammick, Hampson and Jenkins [3] put forward the hypothesis that the molecule including the ring is distorted out of plane by thermal impacts to polar forms, which should be retained long enough to give rise to an orientation polarisation. A calculation of the period of vibration, however, showed this to be much smaller than the time of relaxation, and the explanation was further weakened by observed dipole moments of substituted quinones. By extending the measurements to solvents other than benzene still the same polarisation was found, so that also an explanation on grounds of solvent effect was rendered unlikely. It was finally ruled out by Coop and Sutton [4], who found practically the same value in the vapour phase and moreover observed temperature invariancy of the polarisation within a considerable range, which confirms that the thermal bending postulate was an invalid explanation. Atom polarisation, as suggested by Hassel and Naeshagen [2], was therefore deemed to be responsible for the anomaly, as the only possibility left. The type of vibration giving rise to the abnormally high atom polarisation is probably not the one suggested by Hammick *et al.*[3] as a part of the thermal bending hypo-

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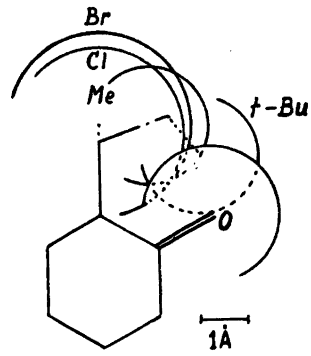

Fig. 1. Steric interference between the oxygen atom and ortho-substituents in benzoquinone. For the methyl and tert-butyl groups only one hydrogen contour is indicated in the position of maximum overlap.

thesis, since the vibration period disagrees with that calculated from absorption frequencies in the infra red. The mode of vibration suggested by Coop and Sutton [4] was the following. Each C = O bond vibrates relative to the rest of the molecule in the plane of the ring *i.e.* perpendicularly to the plane of the π-bond orbitals. If this view is correct, a decrease of atom polarisation and hence of the apparent dipole moment should be expected, when the freedom of vibration is sterically decreased by the presence of bulky substituents in the *ortho* positions. Hammick *et al.*[3], however, working along other lines, observed much the same moment, within the experimental accuracy, of a number of substituted quinones (*vide* Table 1).

In 1,4-di-tert-butyl-2,5-benzoquinone the steric interference between adjacent groups is much larger than in any other compound listed in Table 1 (*cf.* the scale diagram, Fig. 1). This substance was at hand as an intermediate in some synthetic research. It has been measured in the hope of throwing further light on the problem of the quinone moment.

The results are recorded in the bottom line of Table 1 together with the earlier measurements by the authors quoted.

Table 1. Polarisations of substituted benzoquinones in benzene solution at 25° C.

	$_TP_{vap.}$*	$_TP$	R_D	$_TP-R_D$	$\mu_{app.}$
1,4-Benzoquinone	36.6	37.1	28.3	8.8	0.65
2,5-Dichloro-1,4-benzoquinone	46.3	47.2	38.4	8.8	0.65
2,5-Dimethyl-1,4-benzoquinone	47.4	47.8	38.4	9.4	0.68
2,5-Dibromo-1,4-benzoquinone		54	44.0	10	0.7
2,5-Di-tert-butyl-1,4-benzoquinone		79.7	66.3	13.4	0.81 ± 0.03

* Coop and Sutton [4]; bottom line by the present author; all other values by Hammick *et al.*[3]

The substances in Table 1 are arranged in the order of increasing steric interference, applying the usual van der Waals radii and atomic distances [5], *cf.* Fig. 1. $_TP_{vap.}$ is the total polarisation in the vapour phase, $_TP$ is the corresponding term in benzene solution. The refractivities R_D are experimental

values except for the bromo compound, for which it was calculated from atom refractivities. ${}_TP-R_D$ thus represents the apparent orientation polarisation with the conventional minor correction for atom polarisation implied in not extrapolating to infinite wave length.

It is evident from the table that the introduction into the quinone of the two tert-butyl groups does not diminish its electric moment, on the contrary it is slightly increased. The increase is too small relative to the experimental accuracy to justify quantitative treatment, but the following conclusions may probably be drawn from the results.

If the apparent moment of *p*-benzoquinone is due to abnormally high atom polarisation, then this is unaffected by even very voluminous groups as tert-butyl. Since the envelope of the freely rotating groups must have a considerable overlap with the oxygen atoms, *vide* Fig. 1, the hypothesis must involve that the rotation of the butyl group is stopped, and it is fixed in a position of minimum interference. Such an arrangement can be realized without appreciable strain.

An alternative hypothesis is that atom polarisation involving the oxo-groups is responsible only for the moment of benzoquinone itself, whereas this effect is sterically suppressed in at least some of the substituted compounds. The apparent moment of those should then be due to other reasons. The rise in ${}_TP-R_D$ of the butyl-compound over the value of the unsubstituted quinone is 4.6 cm^3. It cannot be explained solely as an atom polarisation of the tert-butyl groups since ${}_TP-R_D$ for 1,4-di-tert-butyl-benzene was found to be as small as 1.6 ± 0.5 cm^3 (Kofod *et al.*[6]). Subtracting the atom polarisation of the benzene ring, 0.4 cm^3, from the upper limit we get the maximum value 1.7 cm^3 as the contribution of the butyl groups in that compound. 1.7 is likely to be valid as a maximum value also in di-tert-butyl-benzoquinone on the assumption that the steric interference reduces the freedom of vibration of interfering groups. One therefore has to explain an apparent orientation polarisation of at least 2.9, which is well beyond the experimental error. Induced moments are no doubt operative in the molecule, but they will almost certainly cancel out because of the symmetry of the molecule. Neither does an explanation on grounds of solvent-solute interaction seem to be plausible, because it should be most marked in connection with the polar-oxo groups, much less with the butyl groups, and benzoquinone itself does not show any solvent effect. Finally the exaltation could be explained as a true orientation polarisation if the interfering groups or some of them were permanently deflected out of the ring plane, or if the entire ring was distorted, giving rise to polar forms. The latter distortion is unlikely because of the resonance in the molecule and since it is perfectly planar in the crystalline state. The former type of distortion, again because of the pronounced resonance in the quinone system, probably would mean deflection of chiefly the butyl groups out of plane. Their group moment is hardly big enough to account for the experimental facts, and secondly, if they were deflected out of plane it would most likely happen in a symmetrical way with no effect at all on the dipole moment. Although the compounds are not strictly analogous, it may be mentioned that in 2,5-di-bromo-1,4-di-tert-butylbenzene it was concluded [6] that either no deflection out of plane occurred or, if so, centrosymmetrical

forms predominated, since the dipole moment of the compound was indistinguishable from zero.

The author is thus inclined to favour the former hypothesis, according to which abnormally high atom polarisation is responsible for the apparent dipole moment of both benzoquinone and the substituted compounds. The atom polarisation is due to vibrations of the oxo groups possibly including distortion of the ring and it is practically unaffected by the presence of the substituents Cl, Br, CH_3 and $(CH_3)_3C$. The free rotation of the tert-butyl group is either stopped or we arrive at the same conclusion as in a previous paper [6] that the conventional van der Waals radii are too large in this context. It is intended to measure duroquinone for comparison.

EXPERIMENTAL

Benzene. Analytical grade benzene was frozen out three times, about one-fifth being poured off and rejected each time, dried over phosphorus oxide, distilled just before use, and stored under dry air; it had f.p. 5.2° C.

2,5-Di-tert-butyl-1,4-benzoquinone. The corresponding di-tert-butyl-hydroquinone was prepared by alkylation of hydroquinone with isobutyl alcohol using oleum as a dehydrating agent, according to Verley [7] (*cf.* Smyth *et al.*[8]). The substituted hydroquinone was oxidized by suspending 50 grammes of the finely ground product in 300 ml dilute nitric acid, 1 part concentrated and 3 parts water. The suspension was heated to the boiling point with frequent stirring for three hours after the colour of the solid had changed into bright yellow, and the evolution of nitrogen oxides ceased. The quinone was collected on a filter, washed with water and recrystallized from benzene. M.p. 150 – 151°, identical with that observed by E. Boedtker [9], who obtained this compound in a different way. The identity was further established by reduction in alkaline medium with hydroxylamine, which led back to the di-tert-butylhydroquinone.

Physical measurements. All measurements were made at 25° C. The electric dipole moment was obtained from the equation $\mu = 0.2212\,(_TP - R_D)^{\frac{1}{2}}$. No correction was made for atom polarisation apart from that implied in using R_D to give the distortion polarisation. The total polarisation $_TP$ was computed from observed values of dielectric constant and the specific volume v for benzene solutions by Halverstadt and Kumler's procedure [10]. Symbols have the same significance as in their paper. The dielectric constants ε were measured with a heterodyne beat capacity meter designed by Hill and Sutton. Specific volumes were measured with a modified Sprengel-Ostwald pyknometer. The molar refractions R_D were computed from the refractive indices, n_D and specific volumes. The refractive index n_D of a solution relative to that of the benzene used for preparing it was measured with a Pulfrich refractometer fitted with a centrally divided cell. Even the most concentrated solution had n_D identical with that of the pure solvent. The standard dielectric constant of pure dry benzene was taken as 2.2727 [11]. The results are given in Table 2.

Table 2. 2,5-di-tert-butyl-1,4-benzoquinone.

$10^5\omega$	ε	V	$10^5\Delta n_D$
1020	2.2755	1.14365	0
920	2.2755	1.14379	0
870		1.14384	0
573	2.2746	1.14418	
221	2.2731	1.14470	

$\varepsilon = 2.2725 + 0.314\,\omega$; $v = 1.14494 - 0.127\,\omega$; $_TP = 79.7$; $R_D = 66.3$; $\mu = 0.81 \pm 0.03$ D.

The investigation was carried out during a two year stay at Oxford University. The author is greatly indebted to Dr. L. E. Sutton, F. R. S. for helpful advice and discussion and to *The British Council* for a scholarship.

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Brønsted's Congruence Principle and Mixtures of *n*-Alkanes of Widely Different Chain Lengths

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Previously reported results of an investigation of the swelling of linear polymethylenes in low-molecular *n*-alkanes have been applied in an attempt to test the congruence principle under extreme conditions. The mixed state of aggregation of the polymer and the difficulties in determining independently the effective index did, however, prevent a quantitative test in this region. It is suggested that the congruence principle may apply to *n*-alkanes only up to a certain critical index, and possible relationships above that index are discussed.

In a previous paper[1] the swelling of linear polymethylenes (polythenes) in low molecular weight alkane vapours was investigated. Since polymethylene swelled in *e.g.* *n*-hexane vapours is essentially a binary mixture of normal alkanes, and the phenomenon of swelling is virtually governed by the same fundamental laws of thermodynamics as the process of dissolution, it should be justified to compare the findings with earlier results reported from this laboratory on other binary mixtures of *n*-alkanes. In addition the polymethylene data might afford a convenient test of Brønsted's theory of congruence [2,3] under extreme conditions.

According to this theory a mixture of isochemical compounds may be characterized by its index $\nu = \Sigma x_i \cdot \nu_i$, x_i being the mole fraction and ν_i the number of carbon atoms (chain length) of a component i. Any index or "average chain length" may be reproduced in an infinite number of ways using different individual components in the required mole fractions. The principle of congruence postulates that certain thermodynamic properties are determined alone by the index of the mixture, in the sense that mixtures having the same index, *congruent mixtures*, have identical properties, although their composition may be entirely different.

Experimental evidence in support of the principle has been obtained in this laboratory by measurements comprising two properties, solubility and

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vapour pressure, and two homologous series, methyl esters of normal fatty acids ($4 \leq \nu \leq 15$) and normal alkanes ($6 \leq \nu \leq 36$). Data by J. N. Brønsted, recently reported by J. Koefoed [3] proved that the solubility of *sym*-tetraphenylbutane was identical in an ester of a certain index ν and in binary mixtures of homologues, if only the mixtures had the same index ν. Vapour pressure measurements by Brønsted and Koefoed [2] on binary *n*-alkane mixtures ($6 \leq \nu \leq 16$) disclosed that the activity coefficient f_1 of a component 1 could be represented by the simple relation

$$\log f_1 = B\ (\nu_{12}-\nu_1)^2. \qquad (1)$$

It thus depended only on the index ν_1 and on the index ν_{12} of the mixture, whereas it was independent of the index of the second compound in accordance with the congruence principle. B is a constant depending upon temperature and on the nature of the component 1. For *n*-hexane at 20° B is -0.00048.

In their paper [2] Brønsted and Koefoed pointed out that although the relation (1) satisfied the experimental data in the region $6 \leq \nu \leq 16$, it could not be expected to hold by unlimited increase in chain length. This appears to be confirmed by our measurements on mixtures of polymethylenes of index up to 2 860 and low molecular *n*-alkanes. As shown below these extreme mixtures do not satisfy equation (1).

Apparent activity coefficients and indices have been calculated from some of the hexane-polymethylene data. They are given in Table 1.

Table 1. Activity coefficients in mixtures of n-*hexane and polymethylene at 22° C.*

Series *	Film * No.	ν_P	x_6	-log f'_6	ν_{LP}	log ν_{LP}
4	10d	2 860	0.9725	0.05	84	1.92
			.9567	.17	129	2.11
			.9256	.35	218	2.34
			.8228	.70	512	2.71
			.9053	.43	276	2.44
			.8820	.51	341	2.53
			.6811	.99	916	2.96
6	1	1 430	.6736	.75	470	2.67
			.8340	.48	242	2.38
			.9202	.23	120	2.08
			.9583	.08	65	1.81
			.8760	.39	182	2.26

* Ref. 1, Table 4

The mole fraction x_6 was calculated from $x_6 = \left[1 + \frac{86}{M_P} \cdot \frac{g_P}{g_6}\right]^{-1}$ as usual.

It will be noted that, owing to the extreme ratio of the molecular weights of the components, even dilute solutions of hexane in polymethylene have large mole fractions. Thus in a 0.5 % solution of *n*-hexane in polymethylene of

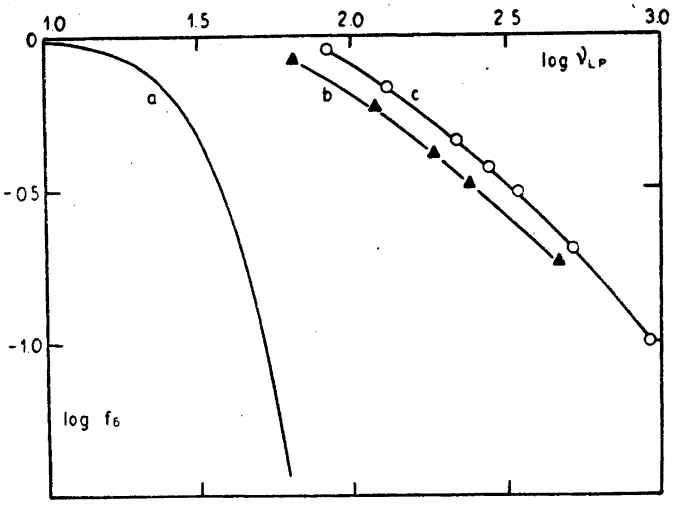

Fig. 1. Activity coefficient f_6 in n-hexane/polymethylene mixtures against the index of the mixtures. a. Extrapolated from the region $6 \leqq \nu \leqq 16$ (Brønsted and Koefoed); b, c. Experimental curves.

a molecular weight 40 000 ($\nu_P = 2\,860$) the mole fraction is $x_6 = 0.7$. Log f'_6 was obtained from $\log f'_6 = \frac{p_6}{p_{6(6)}} - \log x_6$, the necessary data being taken from Ref. 1 Table 4. For the present purpose, the deviation of the hexane vapours from ideality may be neglected, since the correction would only affect the third decimal place. The sixth column contains the index ν_{LP} of the binary mixtures calculated from $\nu_{LP} = 6\ x_6 + (1-x_6)\ \nu_P$.

The experimental data from Table 1 are plotted in Fig. 1 (b, c), together with the curve a of Brønsted and Koefoed (equation 1, $B = -0.00048$ $\nu_1 = 6$) extrapolated to higher indices. The discrepancy between prediction and experiment is evident.

It must, however, be pointed out that this does not necessarily imply any invalidation of the theory of congruence in general, it only emphasizes that this theory cannot be subjected to strict experimental test in the high polymeric region, the chief reason being the mixed state of aggregation of solid polymethylenes, which are not even in thermodynamic equilibrium *cf.* ref. 1 p. 243. The dissolved low-molecular component is probably only contained in the amorphous parts of the polymer. Consequently the index of the mixture ν_{LP}, calculated as above, does not represent the effective value, since the mole fraction of the polymer should not enter by its full value. Adequate correction for the presence of inactive crystalline material is not possible. It must also be borne in mind that the index and average molecular weight of polymethylene samples cannot be determined independently with any great accuracy, and finally that the presence of branched-chain material cannot be quite excluded. The difficulties arising from the mixed order-disorder state may possibly be

eliminated by working at temperatures above the melting point of polymethylene. In this region, however, the experimental technique is complicated.

So far the theory of congruence has been neither proved nor disproved in the region of high polymer *n*-alkanes. A priori the mixtures investigated by us [1] might be assumed to comply with even simpler laws than that implied in the expression (1), and not to follow the principle of congruence.

Imagine an isotropic mixture of (non-volatile) polymethylene (P) and a volatile low-index *n*-alkane (L). The long chains of P, consisting of say 2 000 carbon atoms each, are randomly curled up, the small molecules of L filling in the cavities *cf.* ref. 1 page 244. No appreciable change in the properties of the mixture should be expected if the polymethylene chains were each divided into two parts containing 1 000 atoms, since the relative effect of the terminal methyl groups would still be very small. The equilibrium vapour pressure over the mixture, for instance, may be assumed to remain unaltered. In other words the activity of a given low-index *n*-alkane in mixtures with polymethylene should be independent of the index ν_P (chain length, molecular weight) of the polymethylene, and alone depend on weight fraction and temperature.

This is contradictory to the principle of congruence, as may be realized in the following way. The process of cleavage mentioned does not affect the weight fraction and diminishes the mole fraction x_L only to a slight degree, which is easily seen from the expression $x_L = \left[1 + \frac{M_P}{M_L} \cdot \frac{g_L}{g_P}\right]^{-1}$. Since the index ν_P is reduced to half its value by the cleavage, it follows that the index of the mixture $\nu_{LP} = x_L \cdot \nu_L + x_P \cdot \nu_P$ is different before and after the cleavage. As the vapour pressure is assumed to be unaltered, we thus have two mixtures with equal equilibrium vapour pressure but differing as regard to the index, which obviously is not in accordance with the congruence principle.

When trying to verify the alternative hypothesis experimentally we run into the same difficulties as before. Nevertheless there are indications in the material which seem to favour the hypothesis. In Fig. 15 (Ref. 1, p. 266) the vapour pressure (as fractional parts of the saturation pressure) $p_6/p_{6(6)}$ is plotted against composition (weight by weight) of the mixture. The different curves represent mixtures of *n*-hexane and polymethylenes of the indices indicated to the right. It is striking that the vapour pressure of the mixtures *n*-hexane + "2 500" and *n*-hexane + "1 430" are nearly identical for any given weight fraction, in accordance with the above views. The curves "2 860" and "360", however depart strongly from the other ones. As pointed out previously (Ref. 1, p. 266) it is reasonable to assume, that this is a result of differences in crystallinity, and that all the curves would in fact coincide if the same degree of crystallinity could be secured in the various polymethylenes. This again suggests that measurements should be carried out at temperatures above the melting region of the polymer, where the latter should be isotropic or nearly so.

It is obvious that if the chain length (index) of the polymer component is repeatedly reduced by a factor two as outlined above, ultimately a critical index ν^* is reached, below which the above hypothesis does not hold any more, because the relative weight of the terminal groups will have become significant.

Unpublished measurements in this laboratory show that liquid mixtures of n-alkanes of index up to 36 satisfy equation (1) with reasonable accuracy, so that the critical index ν^* must be larger than 36. Preliminary calculations by Brønsted suggested a value $\nu^* = 54$ (unpublished).

For further elucidation of the problems outlined in this paper it would be desirable to examine liquid n-alkane mixtures containing components of indices from about 50 to about 100. Such work has been in progress in this laboratory, but it was discontinued after the death of professor Brønsted.

The basic ideas in this paper were developed in discussions between the late professor J. N. Brønsted and the author.

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On the Isomerism of Hydroxyurea

II. Preparation of the Isomers

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An improved method of preparation of the alleged isomers has been devised. They are obtained in almost equal amounts and the combined yield is 30—50 %.

Reported methods of preparation of the two known isomers of hydroxyurea * are based on the ionic reaction

$$^{-}OCN + HONH_3^{+} \rightarrow CH_4O_2N_2 \qquad (1)$$

Dresler and Stein [1], the discoverers of the higher melting isomer, on mixing concentrated aqueous solutions of potassium cyanate and hydroxylammonium sulphate observed an immediate rise of temperature and evolution of ammonia. The reaction products were urea and various other substances but no hydroxyurea. When, however, the process was conducted at low temperature, the decomposition was repressed, and the reaction (1) appeared to be favoured. Dresler and Stein, by repeated fractional precipitations, succeeded in isolating minute quantities of a substance analyzing as hydroxyurea and melting at 128—130°. Their compound, as we now know, was an impure specimen of the higher melting isomer.

During their process of isolation the reaction mixture was exposed to room temperature for a considerable time, and finally heat was applied during the evaporation of the solvent. For these reasons Dresler and Stein failed to notice the thermolabile lower melting isomer. This was first prepared by Francesconi and Parrozzani [2] by letting *solid* potassium cyanate and hydroxylammonium chloride react in test tubes at low temperature. The reaction mixture was treated with dry acetic ester, in which both isomers are tolerably soluble. Upon evaporation of the solvent the lower melting isomer was isolated from

* In a paper, which has appeared after the present one had been delivered for publication, C. Runti and R. Deghenghi report that hydroxyurea, m.p. 140°, can be obtained in 63 % yield, when ethyl urethan is treated with excess of hydroxylamine. (*Annali Triestini* **23** (1953) 5).

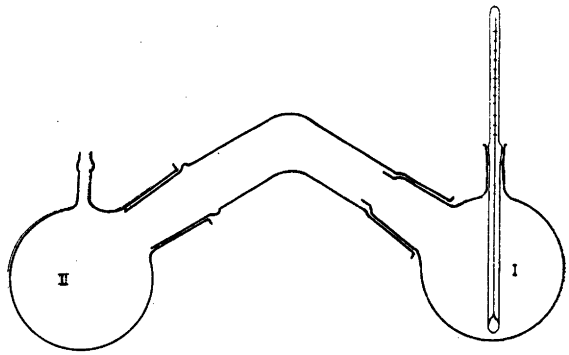

Fig. 1. Freeze-drying apparatus for removal of the solvent.

the extract by means of dry ether. The reaction in solid phase seems rather irrational. Attempts by the present author to reproduce this way of preparation gave extremely small yields and proved that a certain amount of moisture was necessary to initiate the reaction. Most of the material was decomposed.

Conduché [3] obtained a solution of the lower melting isomer, probably containing some of the higher melting isomer as well, by slowly mixing aqueous solutions of the reactants at low temperature. He did not isolate the crystalline material as such but employed the solution for preparation of derivatives.

The above material and a number of preliminary experiments by the present author seem to indicate that the isomers are formed simultaneously by the reaction (1) in aqueous solution, when the heat of reaction is removed. Kinetic measurements [4] showed that the reaction is sufficiently rapid at 0° C for preparative purposes, so that the small yields are not due to incomplete reaction. It was further established that no decomposition involving formation of ions took place within 20 h in dilute aqueous solution at 0° C. It is therefore reasonable to assume that the unsatisfactory yields are due to a pronounced lability of the isomers or of some intermediates in aqueous solution at higher temperatures. For this reason it was decided not only to conduct the reaction but also to remove the solvent, water, at low temperature. A kind of primitive freeze-drying technique was used as described below in the experimental section. Upon complete removal of the solvent the lower melting isomer was extracted by means of dry ether. From the remaining mixture of potassium chloride and the higher melting isomer the latter was isolated by means of absolute ethanol.

The crystalline isomers were obtained in almost equal proportions and in overall yields varying from 30—50 % of the theoretical. They corresponded to the descriptions given in the literature.

EXPERIMENTAL

Materials. The hydroxylammonium chloride and potassium cyanate were identical with those described in the preceding paper [4].

Procedure. The 100 ml flask I was removed from the rest of the apparatus shown in Fig. 1 and used as reaction vessel. It was equipped with a mechanical stirrer and placed

in a freezing mixture of salt and ice. A solution of 13.9 g (0.2 mole) hydroxylammonium chloride in 30 ml water was placed in the flask. When the temperature had reached −5° C, a freshly prepared solution of 16.2 g (0.2 mole) potassium cyanate in 30 ml water was slowly run into the flask with stirring, the temperature being kept below −5° C throughout the reaction (about 1 h).

The time t_a required for the bimolecular reaction to proceed to a degree a is $t_a = \frac{1}{k \cdot c_0} \cdot \frac{a}{1-a}$. In the present preparation the initial concentration of the reactants is $c_0 = 3.33$ moles/l, the rate constant for this unit of concentration and using the minute as time unit has previously [4] been determined as $k = 8$ at 0° C. Inserting these values we get $t_{0.999} = 37$ min. as the time required for 99.9 % of the starting materials to be transformed into hydroxyurea, which is satisfactory for the preparative purpose.

The reaction mixture was therefore left for another hour at 0° C, and should now contain much less than 0.1 % of the starting materials. The flask was then reconnected with flask II as in Fig. 1, the ground glass joints being carefully lubricated with high-vacuum grease. Flask II was cooled in a mixture of solid carbon dioxide and acetone; the system was evacuated with an oil rotary pump, yielding a residual pressure less than 0.01 mm Hg. Gentle heat was applied to flask I (an electrically heated mercury-bath was found useful) so as to provide for a rapid sublimation of the ice from the reaction mixture into flask II. By this primitive freeze drying technique the solvent was removed in less than half an hour, without exposing the hydroxyureas to temperatures higher than 0° C. In the later stages, when the evaporation became insufficient to keep the temperature down, flask II was surrounded by a water bath at 0°. The final drying was effectuated by replacing flask II by another flask containing phosphorus oxide, and the temperature of the reaction mixture was only allowed to rise to 20° after removal of the last traces of water.

The reaction mixture was immediately extracted with 200 ml boiling anhydrous ether, in which the lower melting isomer is slightly soluble. The ether was decanted and the lower melting isomer obtained as colorless plates on cooling to −10°. Two batches of 200 ml ether were used alternately for extraction. After five to eight extractions no more crystalline material could be obtained. The remanence was extracted with 100 ml absolute ethanol at 40° C for 1 min. Colorless rosettes of the higher melting isomer were obtained on cooling. The mother liquor was used for two more extractions. The yields are given below.

It is of utmost importance that all equipment is dry, and that the solvents are absolutely anhydrous. Extractions must be carried out rapidly and heating of the compounds in contact with solvents must be reduced to the shortest possible time. Even with these precautions loss of material in form of gaseous destruction products cannot be quite avoided, as indicated by a smell of ammonia, which has been noticed in nearly all stages of the isolation and particularly in less successful experiments. The lability of the substances is also clearly shown in Table 1, representing the one experiment, which gave the highest yields.

Table 1. Preparation of hydroxyurea.

Reactants	Products			
		calculated	obtained	
			freeze-dried mixture	after fractionation
KOCN 0.2 mole = 16.2 g	hydroxyurea 72°	15.2 g	28.7 g	3.3 g
$HONH_3Cl$ 0.2 mole = 13.9 g	hydroxyurea 140°			4.2 g
	KCl	14.9 g		14.5 g
30.1 g		30.1 g	28.7 g	22.0 g

Loss of material during reaction and freeze-drying 1.4 g

Loss of material during isolation 6.7 g

Nothing is known about the proportions in which the two isomers are formed, but taking into account the greater lability of the lower melting isomer, it appears to be 1 : 1.

Characterization. The identity of the above substances with the hydroxyureas described in the literature was proved by their melting points, or rather destruction points. In an electrically heated melting-point apparatus, essentially as described by F. Halstrøm [5], 71° C was observed for the lower melting isomer (lit. 70—72° C[3]) and 140° C for the higher melting one (lit. 130° C[1]; 139° C[2]; about 141° C[3].) Details of the behaviour of the substancees when heated under the microscope will be presented in a subsequent paper together with other physical properties. Determination of nitrogen by the "micro-Kjeldahl" method [6] gave the following equivalent weights. Lower melting isomer 49.0, 45.5, 55.4, 50.5, 49.7, and 49.8. Higher melting isomer 49.5. Calculated for $CH_4O_2N_2$ 38.0. The reason for the irreproducible and much too high results probably is that some of the nitrogen content is lost in elementary form during the destruction. The average value, about 50, does not, however, correspond to any stoichiometric fraction of the nitrogen content.

The author is greatly indebted to the head of this laboratory, professor H. Baggesgaard Rasmussen for the kind interest he has shown my work. My thanks are due to Dr. Tso Yueh Huang for valuable assistance.

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Studies on the Biosynthesis of *p*-Aminobenzoic Acid by Symbiosis Experiments

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In a previous paper [1] it has been reported that two different strains of lactic acid bacteria can be grown together in symbiosis in a synthetic medium, each producing growth factors needed by the other. These investigations have been continued in this laboratory with the special aim of obtaining information concerning the relationships between these growth factors, especially vitamins and amino acids.

It was first shown by Woods [2] in 1940 that *p*-aminobenzoic acid was an essential metabolite in living organisms, and that it inhibited the bacteriostatic action of sulfonamide drugs. It has since been shown to be a growth factor for certain micro-organisms (reviewed by Knight [3] and Peterson and Peterson[4]). However, almost nothing is known of the mechanism by which it is synthesized in nature. The data recently presented by Davis [5–7] on aromatic biosynthesis indicate that shikimic acid serves as a precursor of *p*-aminobenzoic acid in the mutant strains of *E. coli*.

In the present work using the technique of symbiosis it was found that L-phenylalanine can replace *p*-aminobenzoic acid as growth factor for the phenylalanine-requiring strain *Lactobacillus arabinosus* 17—5. This finding suggests that L-phenylalanine functions as a precursor for the synthesis of *p*-aminobenzoic acid in this organism. In an attempt to obtain some information on possible intermediates in this biosynthesis, tests were made on whether certain compounds related to *p*-aminobenzoic acid and phenylalanine can replace *p*-aminobenzoic acid. In these experiments some observations have been made indicating a possible mechanism that may be involved in the conversion of L-phenylalanine to *p*-aminobenzoic acid.

EXPERIMENTAL

Cultures and method. The organisms used were *Lactobacillus arabinosus* 17—5 and *Streptococcus faecalis* R (obtained from Professor E. E. Snell, University of Wisconsin, U.S.A.). The stock cultures were maintained by stab inoculation in the glucose citrate tryptone yeast extract agar as described previously [1]. The inocula were prepared by transferring the organism from stab culture to 7 ml of this medium, without agar.

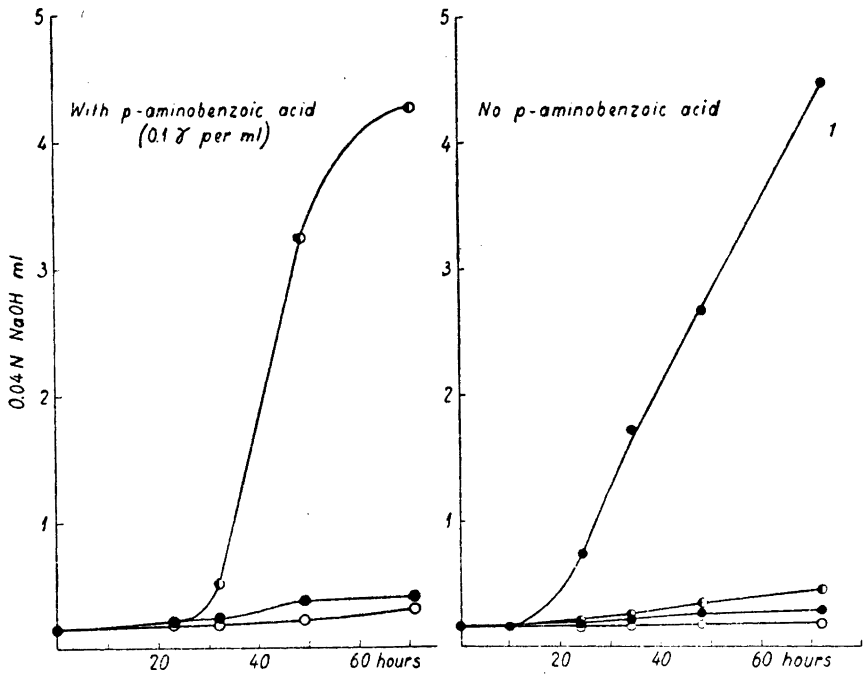


Fig. 1. The replacement of p-*aminobenzoic acid by* L-*phenylalanine. Basal medium without folic acid, phenylalanine, adenine, guanine, xanthine, uracil, and* p-*aminobenzoic acid (Medium B). Curve 1. Medium B supplemented with 10 γ of* L-*phenylalanine per ml.*
● *L. arabinosus 17—5 (phenylalanine-requiring strain)*
○ *Str. faecalis R (folic acid-requiring strain)*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

After incubating for 16—18 hours at 37° C the cells were centrifuged out and washed with 10 ml of 0.9 % sterile saline. This process was repeated, the cells being washed 2—3 times. The cells were finally suspended in saline and the suspension diluted to contain approximately half a million organisms per ml. One drop of this suspension, containing about 2×10^4 bacteria, was used as inoculum for 2 ml of the final medium.

The experimental procedure was essentially the same as that described in an earlier paper [1]. The basal medium of Henderson and Snell [8] in slightly modified form and with appropriate omission was also used in this study. The basal medium was added to test tubes in 1 ml portions, diluted with water or supplements to 2 ml, and autoclaved at 112° C for 5 minutes. In addition, filter-sterilized solutions of the test compounds were prepared (excluding sulfanilamide, benzoic acid, and *p*-aminohippuric acid) and added, with sterile technique, to the autoclaved basal medium in order to avoid possible decomposition of the compounds due to autoclaving. The incubation temperature was 37° C. The growth response was followed titrimetrically. The acid produced was titrated electrometrically directly in the test tubes with 0.04 *N* sodium hydroxyde, using a Cannon automatic titrator.

RESULTS

In an earlier study [1] it was demonstrated that *Str. faecalis* R and *L. arabinosus* 17—5 are able to grow together, but not alone, in a medium from which folic acid and phenylalanine have been omitted, although folic acid is required

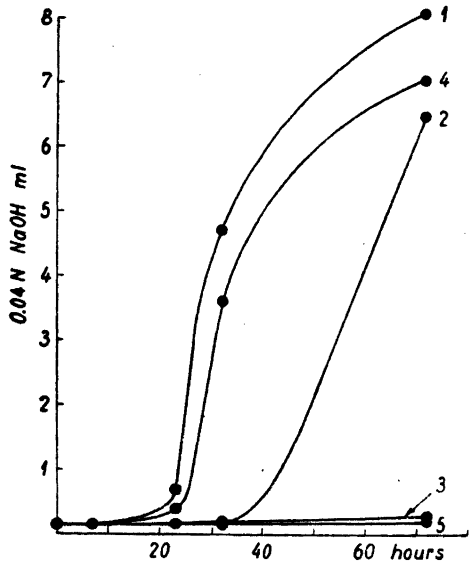


Fig. 2. The replacement of p*-aminobenzoic acid by* L*-phenylalanine is inhibited by sulfanilamide. Test organism L. arabinosus 17—5. Medium B added with sulfanilamide 0.1 γ per ml.*

Curve 1. 1 000 γ of L*-phenylalanine per ml.*
Curve 2. 100 γ » » » »
Curve 3. 10 γ » » » »
Curve 4. 10 γ » » » », but without sulfanilamide.
Curve 5. No L*-phenylalanine and sulfanilamide.*

by *Str. faecalis* R and phenylalanine by *L. arabinosus* 17—5. It was suggested that good growth results because each organism produces a growth factor needed by the other, phenylalanine and folic acid respectively. If, besides these compounds, all purine and pyrimidine bases (adenine, guanine, xanthine and uracil) were also omitted, both bacteria could still grow together, as can be seen from Fig. 1. However, if *p*-aminobenzoic acid, in addition to folic acid, phenylalanine, purines, and uracil, was omitted from the basal medium, the two organisms could not be grown together at all (Fig. 1). It appears, therefore, that the omission of *p*-aminobenzoic acid was the limiting factor for the growth of the two strains in association.

Since *Str. faecalis* R does not require *p*-aminobenzoic acid and since, moreover, this compound cannot replace the folic acid requirement of this organism, it was obvious that the inhibition of the symbiotic growth was due to the inability of *L. arabinosus* 17—5 to synthesize *p*-aminobenzoic acid and folic acid in the absence of phenylalanine. The fact that L-phenylalanine can substitute for *p*-aminobenzoic acid as a growth factor for *L. arabinosus* 17—5 is illustrated in Fig. 1.

As can be seen from Fig. 2 and Fig. 3 the replacement of *p*-aminobenzoic acid by phenylalanine was inhibited by sulfanilamide and also by benzoic acid.

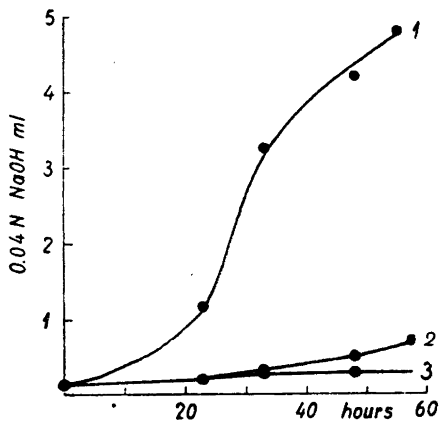


Fig. 3. The replacement of p-*aminobenzoic acid by* L-*phenylalanine is inhibited by benzoic acid. Test organism L. arabinosus 17—5. Medium B. Curve 1. 10 γ of* L-*phenylalanine per ml. Curve 2. 10 γ of* L-*phenylalanine and 10 γ benzoic acid per ml. Curve 3. No* L-*phenylalanine and benzoic acid.*

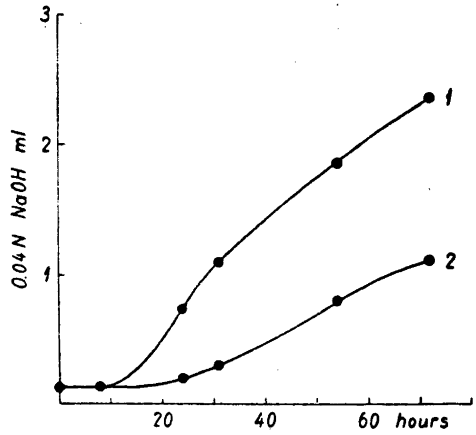


Fig. 4. The promoting effect of p-*nitrobenzoic acid on the utilization of* L-*phenylalanine with L. arabinosus 17—5. Medium B. Curve 1. 1 γ of* L-*phenylalanine and 1 γ of* p-*nitrobenzoic acid per ml. Curve 2. 1 γ of* L-*phenylalanine per ml.*

However, benzoic acid was a distinctly weaker inhibitor than sulfanilamide. The inhibitory effect of both compounds disappeared as soon as minute amounts (0.1 γ per 2 ml) of *p*-aminobenzoic acid were added to the medium.

Since it was found that *p*-nitrobenzoic acid had a promoting effect on the utilization of L-phenylalanine by *L. arabinosus* 17—5, but only in the absence of *p*-aminobenzoic acid (Fig. 4), certain compounds were tested for their ability to replace *p*-aminobenzoic acid in associations of these bacteria. The results of these experiments are summarized in Table 1 and in Figs. 5 and 6. In addition, the ability of these same compounds to replace phenylalanine was tested on *L. arabinosus* 17—5 alone. The results of these experiments are given in Table 1 and in Fig. 7.

As shown in Figs. 5 and 6 and in Table 1, *p*-nitrobenzoic acid, *p*-iodobenzoic acid, *p*-aminophenylacetic acid, *p*-nitrophenylacetic acid and *p*-aminohippuric acid could replace *p*-aminobenzoic acid as growth factor in symbiosis experiments. In the presence of *p*-nitrophenylacetic acid or *p*-aminophenylacetic acid it was shown microscopically that the *L. arabinosus* 17—5 symbiont grew relatively more rapidly than *Str. faecalis* R as compared with the experiments in which these substances were replaced with *p*-aminobenzoic acid [1]. Only one compound, shikimic acid, could replace phenylalanine for *L. arabinosus* 17—5 in the complete basal medium, as can be seen from Table 1 and Fig. 7.

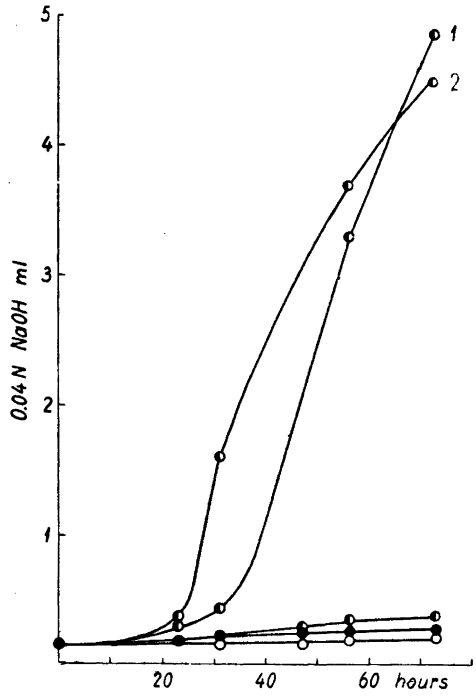


Fig. 5. The replacement of p-*aminobenzoic acid by* p-*iodobenzoic acid and* p-*nitrobenzoic acid. Medium B. Curve 1. 1 γ of* p-*iodobenzoic acid per ml. Curve 2. 10 γ of* p-*nitrobenzoic acid per ml.*
● *L. arabinosus 17—5*
○ *Str. faecalis R*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

DISCUSSION

The data obtained from the present symbiosis experiments can readily be explained by assuming the participation of phenylalanine as a precursor in *p*-aminobenzoic acid formation in *L. arabinosus* 17—5. In the medium used, this organism requires *p*-aminobenzoic acid only in the absence of L-phenylalanine, which is an essential amino acid for it (for the synthesis of the cell proteins). When L-phenylalanine is available it can rapidly synthesize *p*-aminobenzoic acid, which will then be a non-essential vitamin for this organism. On the basis of this fact it is also very understandable why these two bacteria cannot be grown in symbiosis in the absence of *p*-aminobenzoic acid, folic acid, and phenylalanine.

L. arabinosus 17—5 cannot synthesize folic acid for *Str. faecalis* R in a medium lacking these three growth factors, and the latter strain is therefore unable to produce phenylalanine, with the result that the growth of both bacteria is prevented. In addition, it is of interest to note that folic acid contains *p*-aminobenzoic acid as a structural unit and therefore it must be assumed that *p*-aminobenzoic acid is one essential component for the biosynthesis of folic acid in *L. arabinosus* 17—5. It also seems possible that the function of *p*-aminobenzoic acid is inhibited by sulfanilamide and benzoic acid, these compounds preventing the participation of *p*-aminobenzoic acid in the synthesis of folic acid *(Cf. e. g.* [9–11]*)*.

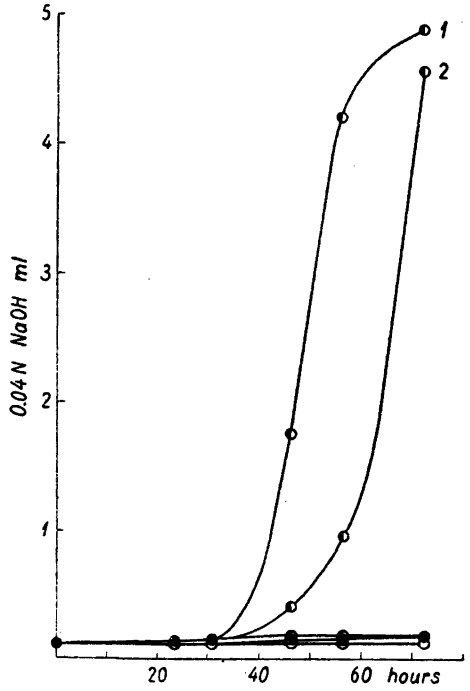


Fig. 6. The replacement of p-*aminobenzoic acid by* p-*aminophenylacetic acid and* p-*nitrophenylacetic acid. Medium B. Curve 1.* p-*Aminophenylacetic acid 10 γ per ml. Curve 2.* p-*Nitrophenylacetic acid 10 γ per ml.*
● *L. arabinosus 17—5*
○ *Str. faecalis R*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

Table 1. Activity of certain compounds to substitute for p-*aminobenzoic acid and phenylalanine.*

Compound	Activity to substitute for *p*-aminobenzoic acid *	Activity to substitute for phenylalanine *
p-Nitrobenzoic acid	active	inactive
m- » »	inactive	
o- » »	»	
p-Iodobenzoic acid	active	i nactive
m- » »	inactive	
o- » »	»	
p-Aminophenylacetic acid	active	inactive
p-Nitrophenylacetic acid	»	»
Phenylacetic acid	inactive	»
Phenylpropionic acid	»	»
Phenylpyruvic acid	»	»
p-Hydroxybenzoic acid	»	»
p-Aminohippuric acid	active	»
Shikimic acid	inactive	active
Quinic acid	»	inactive
Benzoic acid Sulfanilamide		inhibitory effect on the utilization of phenylalanine in the absence of *p*-aminobenzoic acid

* By the technique of symbiosis (with *L. arabinosus* 17—5 and *Str. faecalis* R).
** With *L. arabinosus* 17—5.

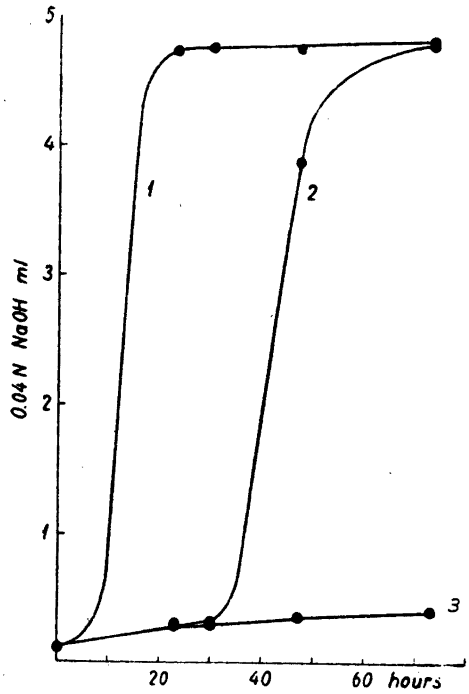
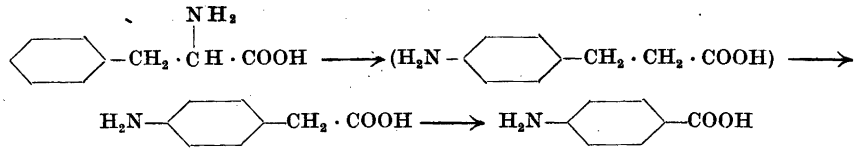


Fig. 7. The replacement of phenylalanine by shikimic acid in L. arabinosus 17—5. From the original basal medium is lacking phenylalanine. Curve 1. L-*phenylalanine 10 γ per ml. Curve 2. Shikimic acid 20 γ per ml. Curve 3. Medium without phenylalanine and shikimic acid.*

Evidence indicates that one of the cellular functions of *p*-aminobenzoic acid is to catalyze the synthesis of purine bases in certain micro-organisms[9,12,13]. In view of this fact it seems possible that the omission of purines has increased the requirement of *L. arabinosus* 17—5 for *p*-aminobenzoic acid in these symbiosis experiments. Neither of the strains required purines and uracil in the original basal medium.

The ability of certain compounds related to *p*-aminobenzoic acid to replace this growth factor in the symbiosis experiments indicates a possible mechanism allowing the conversion of phenylalanine to *p*-aminobenzoic acid. Because *p*-aminophenylacetic acid and *p*-nitrophenylacetic acid could replace *p*-aminobenzoic acid, it seems probable that the carbon chain of phenylalanine undergoes oxidation (presumably after deamination) and gives rise to a carboxyl group. However, since phenylpropionic acid and phenylacetic acid were inactive, before oxidation the hydrogen atom in the *para*-position of the benzene ring is presumably replaced by the amino group (or, by some other reactive group, which again would be replaced by an amino group). The activity of *p*-nitrobenzoic acid and *p*-iodobenzoic acid indicates that the organism concerned is able to replace the nitro group and iodine by the amino group. It should be noticed that only *para*-compounds of nitrobenzoic acid and iodobenzoic acid were active. It seems, therefore, reasonable to postulate the following mechanism:



The activity of *p*-aminohippuric acid must be due to the hydrolysis of this compound, presumably by *L. arabinosus* 17—5, by which *p*-aminobenzoic acid will be liberated.

The inactivity of *p*-hydroxybenzoic acid indicates that this compound cannot be converted into *p*-aminobenzoic acid by the lactic acid bacteria used. Similar observations have also been made with *E. coli* mutants, in which, however, the conversion of *p*-aminobenzoic acid to *p*-hydroxybenzoic acid seems to be possible [6].

According to Davis [5-7] shikimic acid (and certain related compounds) function as a common precursor in the synthesis of aromatic amino acids, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid in *E. coli* mutants. As shown in Table 1 and in Fig. 7, shikimic acid could not replace *p*-aminobenzoic acid, but instead phenylalanine in *L. arabinosus* 17—5. It must therefore be concluded that shikimic acid can serve as a precursor of phenylalanine in this organism, but not directly as a precursor of *p*-aminobenzoic acid, as is evidently the case in *E. coli* mutants.

SUMMARY

Using the technique of symbiosis it was found that L-phenylalanine may be substituted for *p*-aminobenzoic acid as a growth factor for *L. arabinosus* 17—5, which cannot synthesize phenylalanine at all in the basal medium used. The replacement of *p*-aminobenzoic acid by L-phenylalanine is very susceptile to sulfanilamide inhibition. It is concluded that L-phenylalanine functions as a precursor for the synthesis of *p*-aminobenzoic acid in this organism and tests have therefore been made on the ability of various compounds to replace *p*-aminobenzoic acid. *p*-Aminophenylacetic acid, *p*-nitrophenylacetic acid, *p*-nitrobenzoic acid, *p*-iodobenzoic acid, and *p*-aminohippuric acid were active. In addition shikimic acid could substitute for L-phenylalanine. The mechanism that may be involved in the conversion of phenylalanine to *p*-aminobenzoic acid has been discussed.

The author wishes to acknowledge his indebtness to Professor A. I. Virtanen for his kind interest in this study.

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Consecutive Formation of Aquo Metallic Ions in Alcoholic Solution

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In some cases the absorption spectra of metallic ions are distinctly different, according to the solvation. Thus, as early as 1909, Jones *et al.*[1] discovered that many rare earth salts in solutions of alcohol containing relatively small amounts of water showed features of the spectra in pure water as well as in pure alcohol. The phenomenon is largely independent of the anion concentration and, therefore, can be caused only by the changes in the solvation. The most remarkable case of this kind is neodymium chloride in methanol and water. Neodymium chloride in aqueous solution among many other bands has one at 427.5 mμ corresponding to a band at 429.5 mμ in alcoholic solution. We have examined this case thoroughly with a Beckman DU spectrophotometer and found, in contradistinction to the conclusion which Jones *et al.* draw from their measurements, that the spectra cannot be described by two juxtaposed bands with gradually changing intensity alone. This agrees well with the modern views of consecutive complex formation [2], and in this paper attention is drawn to the manner in which measurements of this kind can be used for determining formation curves of aquo ions in alcoholic solution by use of J. Bjerrum's [3] principle of corresponding solutions.

In the experiments, a preparation of anhydrous didymium chloride [4] (about 58 per cent of the total rare earth being neodymium) was dissolved in anhydrous methanol [5], and the optical density was measured at 25° C. (Curve 1 in Fig. 1). The solution was subsequently diluted with small quantities of water and measured after each addition. One part of the anhydrous solution was diluted with four parts of CH_3OH and treated analogously (Curves 2 and 4). The observed optical densities were reduced to a quantity D_{corr} by referring to a 2 cm thick layer of 0.600 molar $DiCl_3$ (or in case of the diluted solutions 10 cm of 0.120). Fig. 1 shows some of the spectra. The influence of the Di-concentration on solutions with nearly the same water concentration may be seen from curves 3 and 4.

By the principle of corresponding solutions two solutions with the same absorption spectrum have the same percentage distributions of the solvated ions and the same ligand number $\bar{n}$, *i.e.*, average number of water molecules bound

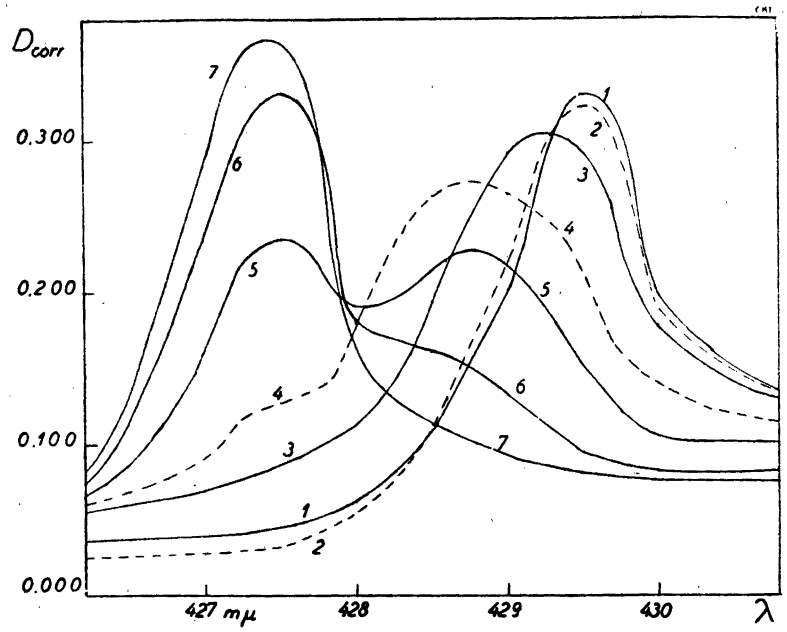

Fig. 1. The corrected optical density as function of wavelength in the interesting interval.

Curve	C_{Di}	C_{H_2O}	$\bar{n}$ (calc. from $N = 6$, $K = 0.333$)	$[H_2O]$
1	0.600	0	0	0
2	0.120	0	0	0
3	0.580	1.81	1.5	0.96
4	0.116	1.92	2.2	1.68
5	0.533	6.20	3.5	4.25
6	0.477	11.20	4.5	9.05
7	0.82	55	6	—

per metal ion, and, consequently, according to the mass action law, also the same concentration of free water, $[H_2O]$. If the total concentrations, C'_{H_2O}, C'_{Di} and C''_{H_2O}, C''_{Di}, respectively, in two corresponding solutions are known, the ligand number and the concentration of free water can be determined by the formulae:

$$\bar{n} = \frac{C'_{H_2O} - C''_{H_2O}}{C'_{Di} - C''_{Di}}, \qquad [H_2O] = C_{H_2O} - \bar{n} \cdot C_{Di}$$

Total concentrations of corresponding solutions were obtained by interpolation as previously described [3], and the data obtained by this principle plotted in Fig. 2. From the experimental points at the lowest water concentrations the first complexity constant

$$K_1 = \frac{a_{Di(H_2O)(alc.)_{N-1}} \cdot a_{alc.}}{a_{Di(alc.)_N} \cdot f_{H_2O}[H_2O]} \sim \frac{[Di(H_2O)^{+++}]}{[Di^{+++}] \cdot [H_2O]}$$

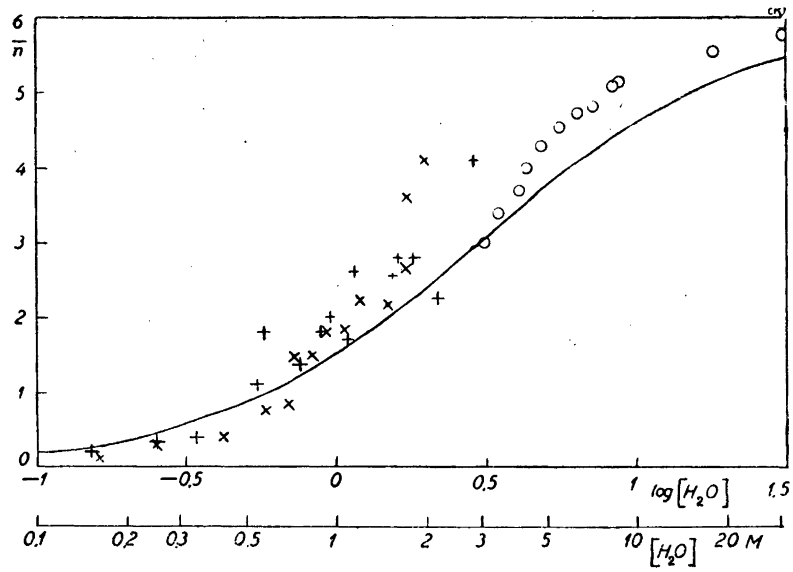

Fig. 2. Formation curve of aquo-didymium ions in methanol at 25° C. Points determined by the principle of corresponding solutions: × from the wavelength 427.5 mμ alone, + average of values for several wavelengths. Full-drawn the statistical case for N = 6 *and* K = 0.333. O*-points from the hypothesis of relative band intensity.*

is estimated to be of the order of magnitude of 1.7. For $[H_2O] \gtrsim 1$, $a_{alc.}$, the activity of alcohol, and f_{H_2O}, the water activity coefficient in alcohol, deviate measurably from 1, but vapour pressure measurements [6] show that the ratio $f_{H_2O}/a_{alc.}$ in salt-free solutions of methanol and also fairly well in ethanol is close to 1, until $[H_2O] \sim 30$ molar. For related ligands, as here, the formation curve for $\bar{n}$ as function of $\log \frac{f_{H_2O}[H_2O]}{a_{alc.}} \sim \log [H_2O]$, often follows the statistical formula

$$\bar{n} = \frac{NK[H_2O]}{1 + K[H_2O]},$$

where N is the coordination number, and K is the average consecutive constant. The full-drawn curve in Fig. 2 shows that $N = 6$ and $K = \frac{1}{3}$ agrees reasonably with the data. The coordination number is possibly higher, *e.g.* 9 as in $Nd(BrO_3)_3$, 9 H_2O,[7] but the estimated value for the 1st complexity constant ($K_1 \cong 1.7$), which in the statistical case is equal to NK, makes $N = 6$ plausible. The o-points on the upper part of the formation curve are roughly estimated under the assumption that the integrated intensities of the water and alcohol bands in the mixed solutions are in the ratio $\bar{n}$ to $6-\bar{n}$. As a whole the experimental points correspond to a formation curve slightly steeper than the statistical curve. This may be explained by assuming that the metal ion to

some extent prefers to solvate with alcohol alone or water alone in preference to forming mixed solvates, but the deviations may also be caused by the great experimental uncertainty and systematic errors. Among the experimental difficulties with the very narrow bands (which necessitate a constant slit-width for all measurements, here 0.0125 mm) is the bad reproduction of the wavelengths in the Beckman DU spectrophotometer. A piece of didymium glass was placed in the third filter-place of the instrument, and the extinction of this used as internal wavelength standard. The background in the spectra is partly due to a trace of ferric ion, which in these strong chloride solutions has a rather strong absorption, and also caused Jones' description of his chloride as yellow-brown.

Cobaltous nitrate gives another example of greatly varied absorption according to the solvation. The extinction coefficient of this salt in various organic solvents is often many times (in ethanol 5 times) higher than in aqueous solution, with only a slight shift of the broad band near 512 mμ towards longer wavelengths. This was discovered by Katzin and Gebert [8], who explain the great colour change by addition of small amounts of water as an exchange of complex-bound nitrate ions with solvent molecules. In our opinion the main effect consists in an exchange of solvated alcohol molecules with water, and this view is supported by the fact that the extinction coefficients of 0.005 molar $Co(NO_3)_2$ in pure ethanol as well as in alcohol containing a few per cent of water increase only about 10 per cent upon addition of 0.5 mole ammonium nitrate per liter. This is not more than corresponds to a normal salt effect [9] and excludes the possibility of nitrato complex formation. On the other hand, some preliminary conductivity measurements show that $Co(NO_3)_2$ in ethanol behaves as a medium dissociated electrolyte, and that the conductivity increases twice in the interesting range from 100 to 92 per cent alcohol, and totally 8 times until pure water is reached. This is a quite normal behaviour for many strong electrolytes in ethanol, and it is to be explained mainly by ion-pair formation. In media with lower dielectric constant this effect is more pronounced. *E.g.* in 75 volume per cent carbon tetrachloride the conductivity is about 50 times smaller than in pure alcohol. Addition of one per cent water increases the conductivity only 2—3 times, but the colour is nearly totally changed to the pink of the aquo ions *.

The independent behaviour of the conductivity and the absorption spectra seems to show that the association with nitrate ions occurs outside the solvation shell, and for this reason does not interfere much with the exchange of alcohol with water, which determines the change in colour. It is also of interest in this connection that Katzin and Ferraro [10] have prepared the following mixed solvates as solid salts: $[Co(alc.)_n(H_2O)_{6-n}](NO_3)_2$ with $n = 2$, 3, 4, and 6 (alc. = *t*-butanol).

The formation curve of the aquo-cobaltous ions themselves is not easily determined due to the great optical density of the more concentrated cobaltous solutions, but the cobaltous ion, *e.g.* in ethanol, is a good indicator [3] for the determination of the hydration of colourless ions. Using the cobaltous system

* The solutions are becoming blue by standing, but this is due to chloro-complex formation caused by HCl from the hydrolysis of CCl_4, which proceeds quite fast in this mixture.

as an indicator, we have found that $Th(NO_3)_4$ hydrates with $K_1 \sim NK \sim 1.5$, and perhaps $N = 8$. For $LiNO_3$ in ethanol was found $K_1 \sim 0.5$. The didymium chloride system in methanol has in a similar way been used as indicator for LiCl, which was found to bind 3 or 4 molecules of water with $K_1 \sim 1.5$. As also indicated by other methods [11], the metal ions seem to bind water molecules about 10 times ($= K \cdot C_{alc.}$) more strongly than methanol and ethanol, and only the exceptional hydrogen ion binds one water molecule much more strongly [12] ($K_1 \cdot C_{alc.} \sim 200$).

The experiments on this subject are being continued.

SUMMARY

It is known that addition of small amounts of water to alcoholic solutions of metal salts cause characteristic changes in the absorption spectra. In some cases these changes seem independent of the anion concentration. In this paper is shown that these changes are caused by consecutive formation of aquo ions. It is also shown how spectrophotometrical measurements can be used for estimation of the relative affinity of water and alcohol molecules to the metal ions.

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Polarographic Determination of Traces of Metals in Organic Material. Determination of Pb, Cu, Cd, Ni, Zn, and Fe

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The most prominent features of polarography are rapidity and selectivity. Furthermore it is often possible to determine, simultaneously, several elements in the same sample. It will be shown that only two samples are necessary for the determination of the six elements mentioned in the heading, whereas when using colorimetric methods, the determination of each element consumes one sample. Unfortunately most previous work on polarographic determination of traces has been carried out without making full use of this favourable feature of polarography.

The determination of traces of metals in organic material is a routine problem in to-days' analytical laboratory. In this work cellulose, carboxymethyl cellulose (CMC) and dry yeast have been investigated. In cellulose it is important to know for example the copper and cadmium content because of the influence of copper on the bleaching of cellulose and of cadmium on the manufacture of viscose. CMC is nowadays used for pharmaceutical purposes and accordingly the lead content must be controlled, and for the same reason it is necessary to estimate the lead content of dry yeast. The other metals mentioned in the heading are usually ascertained in order to establish the degree of cleanliness of manufacture.

In order to prevent uncontrollable losses of the minute amounts of elements being determined, a leading principle in the present investigation has been to avoid achieving separations by the method of precipitation of interfering elements.

Another important aim in this work has been to device and combine the separate unit operations so that the working time is cut down to a minimum. As a result of this effort, the determination of the six elements described in this paper, including disintegration of the sample takes less than 60 minutes in routine work.

A. PROCEDURE

A method for wet combustion of cellulose, CMC, and dry yeast and subsequent polarographic determination of the amount of lead, copper, cadmium, nickel, zinc and iron in the organic material is described below. Two samples

are used. In one of them lead is determined in acid solution after separation by precipitation with ammonia, using ferric hydroxide as a collector. In the other sample, iron is determined in perchloric acid solution and copper, cadmium, nickel, and zinc after adding hydroxylammonium sulphate and pyridine.

Reagents of the highest grade of purity often contain too much lead and copper and need to be purified. Especially important is the purification of those reagents which are used in large amounts, *e.g.* the nitric and perchloric acids employed for wet combustion. The following reagents are used in this procedure.

Nitric acid is distilled in pyrex glass apparatus without stopcock grease, and stored in pyrex containers.

Perchloric acid is distilled in pyrex apparatus under reduced pressure, and stored in a pyrex container. Often "Baker Analyzed" perchloric acid can be used without purifying.

Hydrochloric acid is diluted with an equal volume of water and distilled in pyrex apparatus.

Pyridine: 100 ml pyridine are mixed with 100 ml conc. hydrochloric acid and distilled in a fractionating flask with a wide air-cooled condenser. The fraction boiling between 218 —222° C consists of pure pyridinium chloride, melting point 82° C. The distillate is mixed with 50 ml 50 % sodium hydroxide and pyridine is separated by decantation.

Hydroxyl ammonium chloride: Reagent grade.

Hydroxyl ammonium sulphate: Reagent grade.

Sodium chloride: Reagent grade.

Sodium carbonate: Reagent grade.

Iron (III) nitrate: Reagent grade.

Nitrogen gas.

Distilled water is redistilled in pyrex apparatus or purified by passing through a cation exchange resin, saturated with H^+ by thorough treatment with 4 *N* hydrochloric acid and subsequent washing with distilled water.

Wet combustion of cellulose and CMC. A 5.00 g sample is weighed out in a 250 ml pyrex beaker, and a mixture of 18 ml conc. nitric acid and 15 ml conc. perchloric acid is added. The beaker is covered with a watch glass with ribs and placed on a hot plate with a temperature of 250—300° C. The easily oxidized parts of the organic material first react with the nitric acid. When all this acid is gone and the temperature has risen to about 160° C, the perchloric acid begins to foam and the rest of the sample is oxidized. If charring occurs when the cellulose samples are combusted, the reaction can be accelerated by adding a few drops of conc. nitric acid. The perchloric acid is evaporated and, when only a salt cake remains, 5 ml of water are added. The solution is boiled for 2 mins. to drive off volatile oxidizing matter and hydrochloric acid formed during the reaction. Usually, enough perchloric acid remains on the walls of the beaker to dissolve the residue. If not, 1 ml 0.1 *N* perchloric acid is added for this purpose.

Time required for the above procedure: 40—60 mins. Note: If the sample contains only minute amounts of salts, a salt cake is formed in the beaker by the addition of 0.5 g sodium chloride before wet combustion.

Wet combustion of dry yeast. A 250 ml beaker is placed on a hot plate at about 150° C, and a mixture of 15 ml conc. perchloric acid and 35 ml conc. nitric acid is added. A 5.00 g sample of dry yeast is added in small portions to the acid. If too much of the sample is allowed to react, the foam may rise over the edges of the beaker. Even if sufficiently small amounts are added, it is necessary to stir with a glass rod to prevent the foam rising. When the

foaming has ceased, the beaker is covered with a watch glass with ribs and the procedure described above is followed. If the sample shows a tendency to char, nitric acid is added drop-wise till the charring disappears.

Determination of lead. The wet combusted sample is diluted with 50 ml water in the beaker and 3 ml conc. ammonia is added to precipitate ferric iron and lead. The solution is filtered through pyrex glass wool in a small funnel and washed with 10 ml water. The filtrate is discarded. (If the sample does not contain enough iron to give an easily filterable precipitate, 0.5 ml 0.1 *M* iron (III) nitrate should be added.) The precipitate is moistened with 2 *N* hydrochloric acid and iron and lead is washed out with 3 ml water. The filtrate is collected in a 10 ml measuring flask. The flask is heated on a steam bath and saturated sodium carbonate solution is added until ferric hydroxide begins to precipitate. The solution is acidified drop by drop with 1 *N* hydrochloric acid until the solution becomes clear. At the pH thus obtained iron (III) can easily be reduced by adding hydroxyl ammonium chloride (about 50 mg) and heating on steam bath for a few minutes. After cooling and diluting to the mark with water, the solution is transferred to the polarographic vessel. A blank is run all through the procedure. Note that in the blank, iron (III) nitrate should always be added in order to provide a collector for lead. The polarogram is run according to section B.

Determination of iron. Another sample, previously submitted to wet combustion and dissolved, is neutralized by adding saturated sodium carbonate solution to the hot sample until ferric hydroxide begins to precipitate. The precipitate is dissolved by acidifying with 1 ml 0.1 *N* perchloric acid and the solution is cooled and diluted to the mark in a 10 ml measuring flask.

The sample is transferred quantitatively by pouring it into a polarographic vessel without using any wash water. This operation is possible if the flask is previously made water-repellent with silicone [1].*

The solution is deaerated for 10 mins. by bubbling with nitrogen gas without submerging the dropping mercury electrode in the vessel. A polarogram must be run within a few minutes, as otherwise the ferric iron is reduced by mercury gathering on the bottom of the vessel. The sample is kept for the subsequent determination. The polarogram is run according to section B.

Determination of copper, cadmium, nickel and zinc. Hydroxyl ammonium sulphate, 0.1 g, is added to the polarographic vessel from the iron determination and ferric iron is reduced to ferrous iron by heating on a steam bath. After cooling, 1.00 ml pyridine is added and the solution is carefully deaerated with nitrogen. The polarogram is run according to section B.

B. RUNNING AND EVALUATING THE POLAROGRAMS

The polarograms of lead, copper, cadmium and nickel have the usual shape. The zinc wave, however, is not so good. The upper part is too steep and short to be described as approximating to a straight line (Fig. 3). The steepness results from the proximity of the Fe^{2+}-wave which follows. If the rate of increment of applied voltage is decreased, the

* The flask is impregnated with Desicote according to the description provided by Beckman Instruments, South Pasadena 3, California, USA.

wave becomes easier to evaluate. Evaluation of the polarograms is effected by measuring the heights of the waves at the half-wave potentials.

The different elements are polarographed between the following potentials referred to the saturated calomel electrode at 20° C.

Lead:	−0.15 volts	−0.65 volts
Copper:	−0.20 »	−0.70 »
Cadmium:	−0.50 »	−0.80 »
Nickel:	−0.50 »	−1.05 »
Zinc:	−0.75 »	−1.35 »

Note that if lead and copper are present in comparable quantities, a correction must be made when computing the copper content.

The polarogram of Fe^{3+} is run in quite a different way. As can be seen in Fig. 2 no part of the curve is horizontal before the rise of the Fe^{3+}-wave, and therefore the polarogram is run by recording the diffusion current at a constant voltage of 0.0 v. The zero line of the instrument is then recorded. The distance along the current axis between these two lines is proportional to the ferric ion concentration.

C. STANDARD CURVES AND BLANKS

The standard samples are made in the following way: Filter paper is thoroughly washed with 4 *N* hydrochloric acid to displace cations present in the paper. Since filter paper absorbs metal ions, it should not be washed with distilled water. A 5 g sample is weighed out and known amounts of the elements concerned are added. The analysis is then carried out in the manner described for the samples. After correction for the blanks, standard curves (diffusion current against concentration) are plotted. All standard curves are approximately straight lines. If a blank amounts to more than 10 % of the element concerned, the reagents should be purified further.

It is also possible to use the "pilot ion" and standard addition technique, but in the present investigation the method with standard curves for each element was found more reliable. The computation of the cadmium content, however, was made by comparison with the height of the nickel wave, as the cadmium content was so low, that precision was not very important. Cadmium ions were shown to give approximately the same wave-height as nickel ions at the same molarity. In Fig. 1 are given examples of the standard curves obtained.

D. APPARATUS AND EXPERIMENTAL CONDITIONS

The *polarograph* used in the present investigation was an electronic pen-recording instrument (Radiometer, Copenhagen, Type: PO 3e). Maximum sensitivity: $2.7 \cdot 10^{-4}$ μA/mm. It was provided with a device for prewave compensation and another device for compensation of condenser current. The former is necessary when polarographing several ions in the same solution and the latter is indispensable when employing the high sensitivity necessary in trace analysis.

The capillary. Drop time at 1.0 V in the sample solutions 4.1 sec./drop. The mercury flow was 2.2 mg/sec. It is advantageous to have a capillary with a longer drop time than 4 sec. because otherwise maxima of the second type may develop [2,3]. Moreover, when using short drop time the capillary often drops irregularly at elevated potentials.

The *polarographic vessel* was a short test-tube provided with a stopper in which were bored holes for capillary, agar bridge and deaeration tube. The agar bridge connected the polarographic vessel with a saturated calomel electrode. The electrolyte in the agar bridge was 3 *M* sodium nitrate. Potassium chloride or ammonium nitrate cannot be used

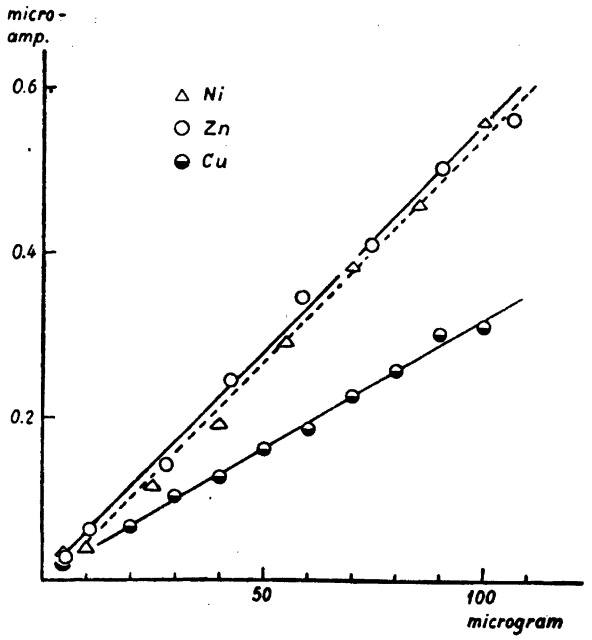

Fig. 1. Standard curves for copper, nickel, and zinc.

in the bridge because potassium and ammonium ions precipitate with perchlorate. Moreover, chloride interferes in the iron and copper determination.

The *temperature* was measured in the sample after the determination, and if not within 20° ± 0.5, the theoretical factor 1.6 % change of diffusion current per degree was used to correct the results.

E. DISCUSSION

1. Decomposition of sample.

Dry ashing is a lengthy process and during the ashing random losses of the elements to be determined can hardly be avoided. Moreover it is often difficult to dissolve the ash without previously melting it with, for example potassium pyrosulphate. Intractable residues are often produced when iron and aluminium are present in the samples. Another defect that is particularly important when traces of metals are involved is the danger of loss by combination with the material of the crucible.

Wet ashing with a mixture of perchloric and nitric acids is a rapid procedure that has been found suitable for trace analysis. The main objection to this method has been that the large volumes of reagents employed might introduce significant amounts of impurities, but in the present investigation purified reagents have been used and difficulties from this source have been easily overcome. The methods of purifying the reagents have been very simple.

The oxidation of organic material by means of this mixture consists of two separate processes. First the nitric acid reacts with the easily oxidized parts

of the organic material present, and not until most of the nitric acid is volatilized does the perchloric acid react (at about 160°) with the more resistant parts. The final oxidation is then carried through by the constant boiling acid (72 %) at about 200° C. This process proceeds smoothly and without bumping. At the temperatures employed the volatility of the perchlorates of the metals with which this work is concerned is insignificant.

A mixture of perchloric and nitric acids has been used for oxidation of the organic parts of many very different materials, *e.g.* rubber [4,5], carbon [6], skin and leather [7], blood [8], plants *etc.* [9,10], textiles [11], sulfite waste liquor and lignosulphonates [12], slate and kolm [13], copper pyrite [14].

According to Kahane [15] there is risk of explosion if the oxidizable substance does not mix well with the acid, *e.g.* mineral oils and fats. Almost all accidents reported with perchloric acid have had the same cause, *viz.*, the evaporation of the alcoholic filtrate from potassium determinations [16]. The general use of perchloric acid has been discouraged by the view that all kinds of perchloric acid are dangerous. This opinion, however, stems from confusion of constantboiling perchloric acid with waterfree acid, which is unstable and explosive. Constant-boiling perchloric acid (72 %) is commercially available, and may be distilled without risk and is stable indefinitely.

In the laboratory, nitric and perchloric acids for wet combustion should be stored ready mixed to avoid the risk of using perchloric acid alone, which might be dangerous. Wet combustion with perchloric acid should not be applied to previously uninvestigated materials without thorough testing. A careful study of the literature is recommended.

Whilst working out this analysis about 1 500 wet combustions have been carried out without any sample having reacted with unreasonable violence. According to Hamlin [11] the tendency to char is diminished by prolonged oxidation with nitric acid. However, the CMC samples in the present investigation did not show any tendency to char when submitted to a very brisk oxidation with nitric acid, whereas when oxidation was done at lower temperatures charring was often observed. This can be explained by the fact that there is, during the heating of the perchloric acid, a period of reduction just after the nitric acid has been evaporated and before the reaction with the perchloric acid begins and this period should be as short as possible. The charring disappears slowly when the perchloric acid reacts, but the reaction is a lengthier process than usual.

The losses are sometimes measurable when using the present method of wet combustion, mainly because of mechanical loss during reaction in the beaker, but usually the agreement is good. (See Table 1.)

Table 1. Determinations of lead and cadmium before and after wet combustion.

	Before		After	
Lead	28.5	27.0 p.p.m.	27.0	28.5 p.p.m.
	15.0	16.5	15.0	17.0
Cadmium	54.3	54.8	55.5	54.0
	26.3	26.4	26.3	26.0

If instead of a beaker a flask provided with an air-cooled reflux condenser is used, losses are less frequent but evaporation of the acids after removing the condenser takes about twice as long. However, as the method is sufficiently precise for determination of traces, the more rapid procedure was chosen. It was found advantageous to add sodium chloride to the samples before wet combustion, because the salt cake left at the bottom of the beaker then became easier to dissolve and losses due to undissolved residues were avoided.

2. Polagraphy.

Table 2. Half-wave potentials (volts) against saturated calomel electrode.

	Aqueous solution of perchloric acid pH 0—2	Aqueous solution of pyridine and hydroxylammonium sulphate pH 5—6
Fe (III)	+ 0.47	—
Fe (II)	— 1.32	— 1.4
Cu (II)	— 0.3	—
Cu (I)	—	— 0.43
Pb (II)	— 0.43	— 0.45
Cd (II)	— 0.62	— 0.73
Ni (II)	— 1.1	— 0.82
Zn (II)	— 1.02	— 1.06

Lead. If a perchloric acid solution containing iron, copper, lead, cadmium, nickel and zinc in the proportions usual in the samples is polarographed, the resulting curve has the appearance shown in Fig. 2. As can be seen in Fig. 2 only Fe^{3+} can be determined with accuracy.

Large amounts of iron (III) and copper interfere in the determination of lead, since their waves precede that of lead. Generally, in the samples employed in the present investigation, the quantities of iron and copper are much larger than those of lead, and these interfering substances must be rendered harmless.

With this purpose in view it was decided that lead should be separated before determination. For various reasons methods involving extraction of lead from the ash were not attempted [17,18]. The main objection to this procedure is that it is always difficult to ensure complete extraction. It was considered more convenient to get all the sample into solution and then make the separation.

Lead may also be separated by electrolysis [19] which is a rapid procedure. The method finally chosen, however, is equally rapid and the equipment required is somewhat simpler.

Kolthoff and Matsuyama [20] recommend the precipitation of copper by potassium thiocyanate. When testing their method in this laboratory, using very low concentrations of lead (10^{-6} *M*), a small wave coinciding with the lead wave was always found, even when the pH was closely adjusted to the value recommended by these authors. Another procedure — especially common when colorimetric methods are being used — is the extraction of the lead dithizonate with chloroform [21]. This procedure was found to take too long and was therefore rejected.

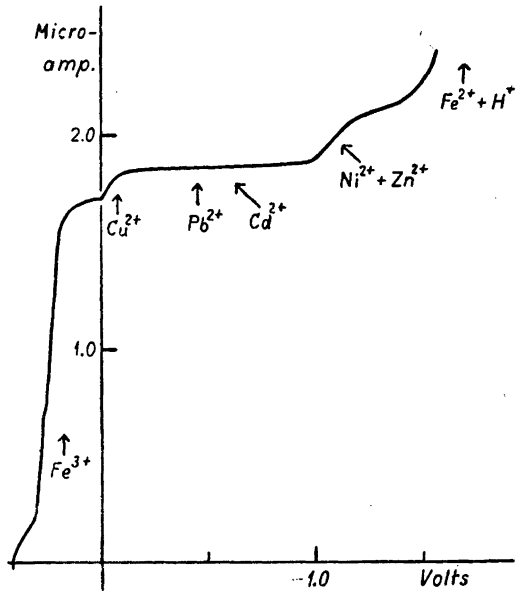

Fig. 2. Characteristic polarogram of metallic impurities in carboxymethyl cellulose after wet combustion with perchloric acid. pH 0—2.

Precipitation of lead with ammonia in the presence of ferric iron [22] and subsequent dissolution in acid makes quantitative recovery of traces of lead possible, as can be seen below.

Lead added (microgram)		20.0			50.0	
Lead found (microgram)	21.1	20.1	19.2	46.2	46.2	50.1

Taking up ferric hydroxide on glass wool instead of filter paper speeds up the procedure, and loss of lead by absorption in the paper is avoided. However, when large amounts of aluminium are present, as was the case with the dry yeast samples, the filtration of the combined ferric and aluminium hydroxides becomes rather tedious. Furthermore it is difficult to dissolve the precipitate in the limited amount of acid that may be used in order to keep the volume of the sample well within 10 ml.

When large amounts of copper are present, some of it is coprecipitated in the ferric hydroxide. The waves of copper and lead, however, are well separated in the acid medium used and thus copper does not interfere when present in the small quantities possible after separation.

The only metal giving a wave partly coinciding with the lead wave is thallium. No attempt was made to detect this element because of the improbability of thallium occurring in the samples.

No maximum was observed on the lead wave in the samples, which is an advantage, because the presence of maximum supressors like gelatin and methylcellulose may decrease the size of the lead wave [23].

Iron. Iron is usually determined in complexing solutions like tartrate [24], citrate [25], oxalate [26] and triethanol amine [27] where the ferric iron wave has the conventional shape of a polarographic wave. In the present investigation, however, it was necessary to consider the fact that copper, cadmium, nickel, and zinc should preferably be determined in the same sample, and the number of possible methods was accordingly restricted. If all methods involving precipitation of the excess iron were excluded, only one procedure was satisfactory *i.e.*, that of determining the iron in a non-complexing solution, where copper cannot interfere [20]. After reducing the ferric iron to ferrous iron, it is possible to determine the other metals simultaneously in a suitable complexing solution where the half wave potentials are well separated.

The iron determination in perchloric acid solution can be made in the presence of large amounts of copper if the solution is free from chloride. Chloride interferes because an anodic wave from the reaction $2Hg + 2Cl^- = Hg_2Cl_2 + 2e^-$ is obtained at + 0.2 volts.

Molybdenum and vanadium, even at low concentrations, give very high steps, coinciding with the ferric wave. If these metals are present in the sample the iron determination should be made in another background solution.

The ferrous iron wave cannot be used because the upper part of the wave coincides with the hydrogen wave.

Copper, Cadmium, Nickel and Zinc. Pyridine buffers have been used in polarographic analysis, mainly because of their ability to precipitate ferric iron without any considerable coprecipitation [28]. Such a solution, however, has many other favourable features. As can be seen from Table 2 and Fig. 3, the half-waves are well separated. Nickel in noncomplexing solution gives an irreversible wave coinciding with the zinc wave. This wave moves to a more positive potential when pyridine is added and a halfwave potential is obtained that is well separated from those of zinc and cadmium [29].

Moreover, it was found that hydroxylamine easily reduced copper to a stable copper (I)-pyridine complex giving a wave consisting of one step instead of the double-wave usually obtained in complexing solutions. Here the height of the copper wave is, for the same molar concentration, about half that of the waves of the other ions concerned. This is an advantage, as copper gives the first wave (See Fig. 3) and may be determined with an adequate degree of accuracy, whereas the following ions cannot be determined with sufficient accuracy if too much copper is present. Furthermore the single copper wave is very easy to evaluate.

Another advantage of the pyridine-hydroxylamine solution is that no iron is precipitated and, in addition it was found that Fe (II) and Cu (I) are not oxidized by air at the pH obtained (pH = 5—6). The waves obtained are well developed and there are no maxima. The only irregularity observed was that if the molarity of copper exceeds 10^{-3} *M* the copper wave shows a peculiar minimum at potentials more negative than −0.5 v.

Many other background solutions have been suggested for the polarography of copper, cadmium, nickel and zinc, but the pyridine-hydroxylamine solution

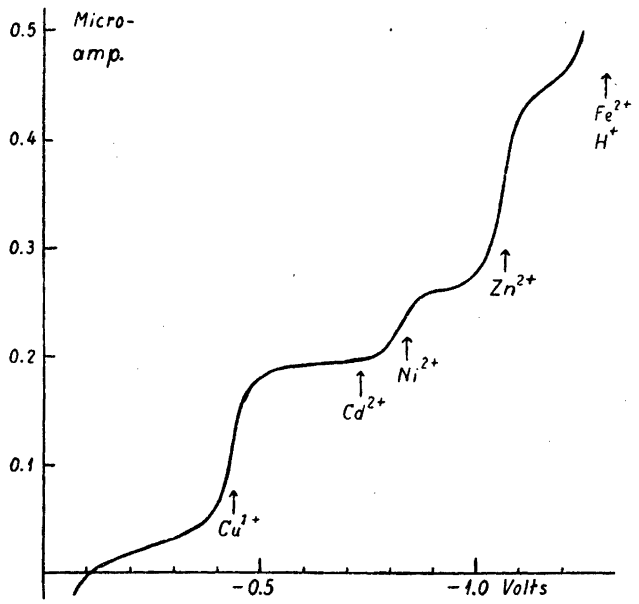

Fig. 3. Polarogram in aqueous solution containing pyridine and hydroxylammonium sulphate.

proved to be the most versatile. For example SCN^- is a complexing agent frequently used [30], but unfortunately copper precipitates and cannot be determined. Tartrate and citrate buffers are frequently employed for the same purpose, but if much iron is present it interferes with all the elements to be determined in the solution except copper, thus making more separations necessary [31,32].

The copper and lead waves coincide in pyridine buffered solution but, since lead is determined separately, a correction can be made for the lead content, which is usually small compared with copper. The amount of pyridine added to the solution is not very critical and as the samples may contain different amounts of perchloric acid after the wet combustion, it was decided to use an excess of pyridine to get the same pH from time to time. If too much perchloric acid is left after evaporation, pyridinium perchlorate precipitates. Care should be taken to avoid the presence of chloride, because copper is mono-valent in this solution and copper (I) chloride is precipitated.

No wave coincides with the cadmium wave and thus the cadmium determination is very selective.

If the pyridine is not purified by distillation as pyridinium chloride as described above, there is interference through some impurities in the pyridine having their waves at the same potentials as nickel and zinc. Distillation of analytically pure pyridine in a column does not yield satisfactory results. After a few days the pyridine then gives the same characteristic interfering waves.

Cobalt gives a wave partly coinciding with the zinc wave. If the former is present, determination of these two metals should be made, for example according to Gagliardo [33].

The sample for determination of iron, copper, cadmium, nickel, and zinc is transferred quantitatively from the measuring flask to the polarographic vessel, the reason being that when pyridine is added after the determination of iron, the volume of the sample must be known exactly. Transferring of liquid by using pipettes in this case is not so rapid and gives less precision than the former procedure. Thus it has been found very useful to render the vessel water-repellent when transferring small volumes of liquid quantitatively from one vessel to another without diluting with washwater [1].

F. ANALYSES

About 200 determinations of each element have been made, and below a few of them are given.

Carboxymethyl Cellulose. Three subsequent analyses of a typical sample

Pb	Fe	Cu	Cd	Ni	Zn
4.6	180	22.4	0	4.8	7.6 p.p.m.
4.7	180	24.3	0	5.0	7.5
4.4	211	23.8	0	5.0	7.4

An homogenous lot of CMC was divided in two parts. One of them was analysed in its original condition and the other after addition of known amounts of metals (Table 3). In this table, each value represents a mean of three determinations.

Table 3.

	Original p.p.m.	Added p.p.m.	Found p.p.m.	Theoretical p.p.m.
Pb	0.8	10.0	12.2	10.8
	1.3	10.0	12.4	11.3
Fe	132	99.5	230	232
	136	99.5	224	236
Cu	7.0	24.2	27.5	31.2
	8.0	24.2	29.2	32.2
Cd	0.0	20.1	17.0	20.1
	0.0	20.1	17.6	20.1
Ni	3.6	17.8	21.6	21.4
	4.0	17.8	22.4	21.8
Zn	11.4	20.2	36.0	31.6
	15.6	20.2	33.4	35.8

Cellulose. Three subsequent analyses of a typical sample

Pb	Fe	Cu	Cd	Ni	Zn
4.7	180	24.3	0.0	5.0	7.5 p.p.m.
4.4	180	24.8	0.0	5.0	7.4
5.0	174	24.4	0.0	5.0	6.9

The lead and copper contents of this sample were determined by dithizone methods at the Institute of Wood research, Stockholm, and were found to be 25, 23, and 25 p.p.m. for copper and 7, 7, and 6 p.p.m. for lead in good, respectively fair correspondence with the values given above.

Dry Yeast. For reasons mentioned in section E 2 only lead was determined in these samples. Results for determination of lead in three different samples:

1:st sample	2:nd sample	3:rd sample
1.7	0.8	3.0 p.p.m.
1.6	0.8	3.4
1.5	0.5	3.2

G. PRECISION

The precision of the method as derived from 180 determinations is about $\sigma = \pm 2$ at 50—100 p.p.m., and $\sigma = \pm 0.4$ at 2 p.p.m. The main sources of error are to be found in the wet combustion and above all in the subsequent chemical processes preceding the polarographic determination. Care should be taken, that impurities are not introduced from the vessels.

The polarographic measurements are made rapidly, and even when the concentrations are as low as 10^{-5} M the precision is 4—5 %. However, it should be observed that the precision is considerably influenced by the presence of preceding waves [34,35].

SUMMARY

A rapid method is described for wet combustion of cellulose, carboxymethyl cellulose and dry yeast and subsequent polarographic determination of the amount of lead, copper, cadmium, nickel, zinc and iron in the organic material. Two samples are used. In one of them lead is determined in acid solution after separation by precipitation with ammonia, using ferric hydroxide as a collector. In the other sample, iron is determined in perchloric acid solution and copper, cadmium, nickel, and zinc after adding hydroxyl ammonium sulphate and pyridine. The time required for the complete analysis in routine work is about 60 minutes. Precision: $\sigma = \pm 2$ at 50—100 p.p.m. and $\sigma = \pm 0.4$ at 2 p.p.m.

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Hydrolytic Degradation of Diheterolevulosan I and II Hexamethyl Ethers

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Our interest in the chemistry of fructose anhydrides originated from a study of crystallisation inhibitors in the beet sugar refining process. Apparently, however, they do not act as inhibitors in this process.

Three difructose anhydrides have been isolated from inulin hydrolysates, and a further two, diheterolevulosan I and II[1,2] have been obtained by the action of strong hydrochloric acid on fructose. (The field of difructose dianhydrides has been reviewed in more detail elsewhere[3–5].) In the present paper improved methods are described for the methylation of the diheterolevulosan acetates, for the hydrolytic degradation of the hexamethyl ethers and the identification of the fructose trimethyl ethers formed.

By methylation of the diheterolevulosan hexaacetates with methyl sulphate and aqueous sodium hydroxide in acetone, rather unsatisfactory yields were obtained, probably due to the fact that incompletely methylated fractions are difficult to extract from the aqueous phase. Green, Myers and Grant[6] recommended the use of solid sodium hydroxide in dry acetone, and by using dioxane, which gives no condensation products by the action of alkali instead of acetone, we have further improved the method, which, of course, is of general application.

The structure of diheterolevulosan I as a 1,2′ : 2,1′-di-D-fructopyranose dianhydride was elucidated by Schlubach and Behre[1] by hydrolysis of the hexamethyl ether and isolation of D-fructose 3,4,5-trimethyl ether, which, however, was of doubtful purity. We have repeated these experiments as well as the preparation of D-fructose 3,4,5-trimethyl ether by the method of Irvine and Patterson[7] and in both cases products of improved purity, $[\alpha]_D^{20}$ −128° (present investigation) compared with −116° (Irvine and Patterson) and −70.5° (Schlubach and Behre) were obtained. That the compounds were identical was established by means of paper chromatography and by conversion to the *p*-nitrophenyl osazones. The melting point reported in the literature[8] for the osazone (206—210°) is considerably lower than that now obtained

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(235—236°), probably due to insufficient purification. However, the validity of Schlubach and Behre's conclusions is confirmed.

During the course of our studies Wolfrom *et al.*[9] showed by oxidative hydrolysis of the hexamethyl ether, that diheterolevulosan II is a D-fructopyranose D-fructofuranose 1.2′ : 2.1′-dianhydride. We arrived at the same structure by an independent course of reactions. On hydrolysis diheterolevulosan II hexamethyl ether yielded fructose 3,4,5-trimethyl ether and fructose 3,4,6-trimethyl ether, which could be easily separated and identified by partition chromatography. As the hexamethyl ether is resistant to hydrolysis and the fructose trimethyl ethers are rather sensitive to acids, it proved convenient to carry out an incomplete hydrolysis and separate the trimethyl ethers from unchanged starting material by partition between water and chloroform. The trimethyl ethers were oxidised with lead tetraacetate, the resultant arabonolactones were purified by distillation and converted to the corresponding arabonamides by treatment with liquid ammonia. The yields of pure arabonamides were satisfactory, 75—80 % and 38 % calculated on the basis of fructose trimethyl ether and of diheterolevulosan II, respectively.

EXPERIMENTAL

Preparation of diheterolevulosan I and II. D-Fructose (50 g) was dissolved in cold (0°) concentrated hydrochloric acid (d = 1.19, 200 ml) and left in the refrigerator (0°) for three days. The dark coloured solution was then filtered through asbestos wool; part of the hydrogen chloride was removed *in vacuo* and the solution was neutralized with lead carbonate, filtered and treated with hydrogen sulphide. The solution was then passed through a column of Amberlite IR 4B. The volume was made up to 400 ml with water, and baker's yeast (25 g) in 200 ml 0.9 % NaH_2PO_4 added. After standing overnight at 35° the solution was filtered, treated with lead acetate and hydrogen sulphide and, after filtration, passed through columns of the Amberlite resins IR 120 and IR 4 B. Evaporation and drying with ethanol-benzene *in vacuo* afforded a thick syrup which was dissolved in a small volume of hot methanol. Crystalline material (6 g) immediately began to separate and was collected when crystallisation was complete. The methanolic mother liquours were poured into a large volume of acetone, whereby an amorphous material (13 g) was precipitated.

On paper chromatograms (solvent: n-butanol-ethanol-water 5 : 1 : 4, upper layer) the crystalline fraction gave two spots, the R_F values of which were less than that of fructose. They were detected with the following three reagents: Resorcinol-hydrochloric acid, silver nitrate followed by sodium hydroxide in ethanol and sodium periodate followed by Schiff's reagent.

The crystalline fraction was recrystallised by evaporating an aqueous solution to a strongly supersaturated syrup, which was diluted with methanol and left to crystallise. By following the separation chromatographically one of the compounds was obtained almost pure after a few recrystallisations. The substance (3.4 g) was dissolved in a warm mixture of pyridine (70 ml) and acetic anhydride (35 ml) and left overnight. The bulk of the solvent was removed by distillation under reduced pressure, the residue was taken up in chloroform and washed with dilute sulphuric acid, then with sodium bicarbonate solution and finally run through a short column of alumina. The acetate crystallised from methanol in needles, m.p. 168—9°*. $[\alpha]_D^{20}$ −58° (chloroform, c = 2). Deacetylation of the pure acetate with catalytic amounts of sodium ethoxide in absolute ethanol yielded pure diheterolevulosan I. M.p. 270—3°. $[\alpha]_D^{20}$ −44° (water, c = 2). Melting point determinations were difficult to reproduce owing to decomposition which appeared to proceed autocatalytically. The R_F-value on paper chromatograms was about half of that of fructose.

* All melting points uncorrected.

The second component which was enriched in the mother liquours could not be obtained in a pure state by recrystallisation. However, when the amorphous anhydride mixture mentioned above (13 g) was dissolved in methanol it yielded crystalline material (4.9 g) which upon recrystallisation from pyridine-methanol gave chromatographically pure diheterolevulosan II (2.2 g). M.p. 255—6°, $[\alpha]_D^{20}$ —40° (water, $c = 2$). The process could not be reproduced. On paper chromatograms diheterolevulosan II travels with a speed intermediate that of diheterolevulosan I and fructose.

Acetylation of diheterolevulosan II in pyridine-acetic anhydride at room temperature gave an amorphous acetate which crystallised in needles from absolute ethanol on long standing at 0°. M.p. 121—2°, $[\alpha]_D^{20}$ —41° (chloroform, $c = 2$) in good agreement with the values reported by Wolfrom *et al.* Later a sample of the crystalline acetate after standing in its ethanolic mother liquour was transformed to stout prisms which softened below 70° on rapid heating and probably contained alcohol of crystallisation. When an airdry and obviously not quite homogenous sample was dried *in vacuo* at 95° the loss of weight corresponded to 0.8 mole of ethanol pro mole acetate. When a sample of the prisms was allowed to stand slightly above room temperature in a small amount of ethanol overnight it was completely converted to the needle form.

Diheterolevulosan I hexamethyl ether. Dioxane (35 ml) and methyl sulphate (1 ml) were added to diheterolevulosan I hexa-acetate (1.7 g) and powdered sodium hydroxide (3.6 g). The mixture was then heated to 70° with vigorous stirring and methyl sulphate (3.6 ml) added in 0.5 ml portions over a period of 45 minutes. The mixture was heated for two hours and left at room temperature overnight. It was then filtered under suction care being taken to prevent cracking of the gelatinous filter cake which was then thoroughly washed with dry benzene. The combined filtrates were evaporated under reduced pressure and the resulting pale syrup dissolved in liquid ammonia (4 ml). Potassium was added to produce a stable blue colour, and, after the ammonia had evaporated methyl iodide (2 ml) was added. After standing overnight the mixture was heated to boiling for a short time, the unreacted methyl iodide was removed by distillation and the residue extracted with chloroform. The solution was filtered and the filtrate was run through a short column of alumina and then evaporated to dryness. The residue, on addition of light petroleum, crystallised in needles, m.p. 152—9°. The product was distilled (0.7 mm, bath temperature 210°) and the distillate recrystallised from chloroform-light petroleum. Yield 0.98 g or 80 % of theory.

Twice recrystallised from acetone-*iso*propyl ether it formed fine needles, m.p. 160—1°, $[\alpha]_D^{20}$ —49° (chloroform, $c = 2$). A further crystallisation resulted in the formation of small prisms together with the needles. When the mixture was left overnight at room temperature in acetone-*iso*propyl ether only prisms remained. On heating to 146—7° they melted to a liquid which then solidified and finally melted over a wide range (150—160°). The lowmelting form is probably identical with the α or β modification reported by Sattler *et al.*, m.p. 143—5° and 145—7°. Efforts to regain the new highmelting form with a sharp melting point failed, the products obtained melting in the range 155—160°.

Hydrolysis of diheterolevulosan I hexamethyl ether. The hexamethyl ether (0.5 g) was dissolved in 1.5 *N* sulphuric acid (10 ml) and the solution heated to 80° for 18.5 hours. After neutralisation with barium carbonate and filtration the hydrolysate was extracted with chloroform (4 × 5 ml). The aqueous phase was evaporated to dryness under reduced pressure and the resulting syrup was dissolved in chloroform and run through a short column of alumina. The column was thoroughly washed and the chloroform solution evaporated to a syrup (0.3 g) which was distilled. A distillate (215 mg), $[\alpha]_D^{20}$ —125° (after 15 minutes) and —132° (final value) (water, $c = 2$), was obtained at 142—3°/0.42 mm bath temperature.

An authentic sample of fructose 3,4,5-trimethyl ether prepared according to Irvine and Patterson distilled under the same conditions and mutarotated slightly to the final value $[\alpha]_D^{20}$ —128° (water, $c = 2$).

p-Nitrophenyl osazone of fructose 3,4,5-trimethyl ether. Fructose 3,4,5-trimethyl ether (100 mg), *p*-nitrophenylhydrazin (250 mg) and acetic acid (1.5 ml) were mixed with water (5 ml) and heated on a water bath for 45 minutes. The reaction mixture was diluted with water (5 ml) and cooled. The crystalline precipitate was collected, washed with hot dilute acetic acid and dissolved in a small volume of chloroform. The solution was added to the

top of an alumina column (1.8 × 20 cm) and the chromatogram developed with the same solvent. The chromatogram was very complicated, but from an orange coloured zone two fractions (50 mg, m.p. 235—6° and 23 mg, m.p. 229—30°) were obtained which probably consisted of the desired osazone. This corresponds to 23 % and 9 % of theory. Analysis (main fraction): Found CH_3O, 18.1. Calc. for $C_{18}H_{17}O_5N_6(OCH_3)_3$: CH_3O, 19.0 %.

By this procedure fructose trimethyl ether from the hydrolysis of diheterolevulosan I hexamethyl ether gave the same osazone in about the same yield. M.p. 234—5°, alone or in admixture with an authentic sample.

Diheterolevulosan II hexamethyl ether. Methylation of diheterolevulosan II hexaacetate (1.7 g) as described for diheterolevulosan I hexamethyl ether (omitting the distillation) afforded prisms from acetone-*iso*propyl ether, m.p. 100—100.5°, $[\alpha]_D^{20}$ −21° (chloroform, c = 2). Yield 1.06 g or 86 % of theory.

Hydrolysis of diheterolevulosan II hexamethyl ether. A preliminary experiment showed that hydrolysis of diheterolevulosan II hexamethyl ether in *N* sulphuric acid reached half completion in about 20 hours at 80°. Paper chromatograms (solvent: benzene-ethanol 52 : 9, saturated with water) indicated the presence of fructose 3,4,5-trimethyl ether and fructose 3,4,6-trimethyl ether. R_F values and colour reactions were identical with those of authentic materials. Spraying reagents were: Resorcinol-hydrochloric acid, aniline hydrogen phtalate and *p*-anisidin phosphate. There also were two fast-moving components, probably furan derivatives, which gave a yellow colour with the *p*-anisidin reagent even in the cold.

The hydrolysis was repeated using larger quantities of material (hexamethyl ether (500 mg) in *N* sulphuric acid (10 ml)). The filtrate, after neutralisation of the hydrolysate with barium carbonate, was evaporated to 7 ml volume and extracted with chloroform (4 × 1 ml). The chloroform extract was washed with 1 ml water which was finally extracted with chloroform (2 × 0.3 ml). On the addition of *iso*propyl ether to the combined and concentrated chloroform extracts unchanged hexamethyl ether (246 mg, m.p. 99.5—100.5°) and an oil (22 mg) were obtained. The oil was combined with the aqueous solutions.

After evaporation of the aqueous solution, the mixture of the fructose trimethyl ethers was separated on a hydrocellulose column (2 × 20 cm) using benzene-ethanol 50 : 4, saturated with water, as solvent. The eluate was collected in 3 ml fractions which were examined by paper chromatography.

Fractions 9—15 were evaporated giving 27 mg of an oil from which crystalline diheterolevulosan II hexamethyl ether (8 mg) was recovered.

Fractions 16—21 yielded an oil (108 mg) which contained the bulk of the fructose 3,4,6-trimethyl ether.

Fractions 22 and 23 contained small amounts of both methyl ethers and were discarded.

Fractions 24—32 yielded an oil (77 mg) containing the fructose 3,4,5-trimethyl ether.

D-*Arabonamide 2,3,5-trimethyl ether from fructose 3,4,6-trimethyl ether.* Authentic fructose 3,4,6-trimethyl ether, chromatographically pure, $[\alpha]_D^{20}$ +21° (chloroform, c = 2.1) was prepared by hydrolysis of fully methylated inulin and subsequent chromatography on a cellulose column, followed by distillation.

The trimethyl ether (120 mg) was dissolved in acetic acid (2 ml) and water (1 ml). The stoichiometric amount (240 mg) of lead tetraacetate was added. After two hours at room temperature the solution gave a faint blue spot on potassium iodide-starch paper. Water (1 ml) was added and the solution was then left overnight. After small amounts of lead dioxide had been removed by filtration the solution was saturated with hydrogen sulphide, and centrifuged. The clear solution was evaporated under reduced pressure and the residue distilled under water pump pressure (bath temperature 130—5°) for complete conversion of the arabonic acid derivative to the corresponding lactone. The distillate was dissolved in liquid ammonia which was then allowed to evaporate. The crystalline residue was dissolved in boiling acetone from which on cooling the amide crystallised in needles, m.p. 136—7°. More material was obtained from the mother liquors by addition of *iso*propyl ether, the total yield being 85 mg or 75 % of theory. After recrystallisation from acetone, the melting point was unchanged. $[\alpha]_D^{20}$ −13° (water, c = 1.8). The values reported for the corresponding L-derivative is 138° and $[\alpha]_D^{20}$ +18° (water, c = 1.3) [10].

On application of this procedure to the fructose 3,4,6-trimethyl ether from the hydrolysate there was obtained 47 mg (overall yield 38 % of theory) of the amide. M.p. 135.5—6.5°. Recrystallised once from acetone-*iso*propyl ether it melted at 136—7° alone or admixed with authentic substance.

D-*Arabonamide 2,3,4-trimethyl ether from fructose 3,4,5-trimethyl ether.* Prepared by the method described above for D-arabonamide 2,3,5-trimethyl ether, the lactone distilled at 150—60° bath temperature under water pump pressure. The amide obtained from authentic fructose 3,4,5-trimethyl ether melted at 102.5—3°, $[\alpha]_D^{20}$ —28° (water, c = 1.5) compared with 96° and $[\alpha]_D^{20}$ +26° (water) reported for the corresponding L-derivative [11]. The yield was 80 % of theory.

From the hydrolysate there was obtained 47 mg (38 % of theory) of the amide, m.p. 101—2°. Recrystallised once from acetone-*iso*propyl ether it melted at 102.5—3°, alone or admixed with authentic sample.

SUMMARY

Diheterolevulosan I and II hexamethyl ethers have been hydrolysed and the D-fructose 3,4,5- and 3,4,6-trimethyl ethers obtained characterised by oxidation with lead tetraacetate and subsequent conversion of the resultant arabonolactones by means of liquid ammonia, to the corresponding arabonamide trimethyl ethers.

The methylation procedure has been modified by using methyl sulphate and solid sodium hydroxide in dry dioxane.

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Studies of Growth Factors for *Streptococcus faecalis* Occurring in Marine Algae

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It has been reported from this laboratory that water extracts of marine algae contain factors that stimulate the growth of *Streptococcus faecalis* ATCC 8 043 [1]. The nature of these factors was not further investigated and no attempt was made to release possible bound forms of the active substances. Different methods of extracting the growth factors and of separating and identifying the active substances bioautographically have now been tried.

EXPERIMENTAL

The algae investigated are *Sphacelaria fastigiata, Laminaria saccharina* and *Fucus vesiculosus* of the division *Phaeophycophyta* and *Furcellaria fastigiata, Rhodomela subfusca* and *Polysiphonia nigrescens* of the division *Rhodophycophyta* [1]. The algae were dried at room temperature and ground.

The Difco folic acid assay medium with addition of 1.6 % Bacto-agar was used in an agar cup plate method with *Streptococcus faecalis* ATCC 8 043 as test organism [2]. The same medium and the same organism were employed for the bioautographic studies. For the chromatographic separation of the factors different solvent systems were tried, *i. e.* (I) water-saturated sec. butanol containing 3 % acetic acid and 25 mg KCN/l, (II) *n*-butanol-water-acetic acid 125 : 125 : 30 v/v, (III) 70 % ethanol, and (IV) 5 % water solution of Na_2HPO_4 in *iso*-amyl alcohol (aqueous phase). Whatman No. 1 paper was used and the chromatograms were run at 21° C for 7—18 hours, depending on the solvent system used.

Six different methods of extracting the growth factors for *S. faecalis* were tried, *i. e.* water extraction for 24 hrs at 20° C and at 37° C, boiling the algal material under reflux at 100° C for 30 min., treatment with chicken pancreas homogenate, with hog kidney homogenate and with papayotin. The samples were heated at 100° C for 5 min. before extraction in order to destroy the algal enzymes.

The water extracts were prepared by adding 25 ml of water to 2 g of dried and ground algal material. The chicken pancreas and the hog kidney were removed and frozen immediately after the animals had been killed. The organs were homogenized in a Waring blendor to give suspensions containing about 400 mg fresh tissue material per ml. The homogenates were then centrifuged to remove solid particles. To 2 g of alga 1 ml of these enzyme preparations was added and the volume was taken to 25 ml with 0.2 *M* phosphate or acetate buffers. For the chicken pancreas enzyme a pH of 7.5 (phosphate buffer) was

used, for the hog kidney the pH was 4.5 (acetate buffer). The buffer for the latter enzyme was 0.01 *M* in respect of cysteine hydrochloride. — In the experiment with papayotin 10 ml of a 0.4 % papayotin solution in 0.2 *M* phosphate buffer having a pH of 6.5 was employed per 2 g of alga, and the solution was taken to 25 ml with the same buffer.

An example of the effects obtained with these different methods of extraction is given in Table 1, which shows the results with one brown *(Laminaria saccharina)* and one red alga *(Furcellaria fastigiata)*. It can be seen that much higher values are obtained when enzymatic digestion with chicken pancreas or hog kidney homogenate is employed. The activity of the enzyme homogenates was insignificant. The enzyme complex of the chicken pancreas homogenate appears to be the most effective in releasing growth factors for *S. faecalis*. Treatment with papayotin did not significantly increase the total folic acid activity of the algae.

Table 1. Effect of different methods of extraction on the total activity for S. faecalis.

Algae	Activity expressed as μg folic acid/g dry weight					
	20° C 24 h	37° C 24 h	100 °C 30 min.	Chick.pancr. 37° C pH 7.5 24 h	Hog kidney 37° C pH 4.5 24 h	Papayotin 37° C pH 6.5 24 h
Laminaria saccharina	0.13	a)	0.20	0.40	0.40	0.16
Furcellaria fastigiata	0.10	0.16	0.08	0.87	0.33	0.11

a) Due to liberation of inhibitory substances, no estimation of the folic acid content could be made.

RESULTS

Table 2 summarizes the results of a quantitative estimation of the activity for *S. faecalis* in the algae investigated. The maximum figures obtained after enzymatic treatment of the samples with chicken pancreas homogenate as described above are given. Folvite, Lederle, was used as a standard. The activity found corresponds to from 0.25 to 1.10 μg folic acid per gram dry weight of alga.

Table 2. Quantitative estimation of S. faecalis activity in algae.

Algae	Activity expressed as μg folic acid per g dry weight
Sphacelaria arctica	0.90
Laminaria saccharina	0.40
Fucus vesiculosus	0.25
Furcellaria fastigiata	0.85
Rhodomela subfusca	0.90
Polysiphonia nigrescens	1.10

In order to study the type of factors released by various treatments of the algal material and to investigate the naturally occurring forms of the active substances, the growth factors in four of the six different extracts of *Laminaria*

saccharina and *Furcellaria fastigiata* were separated chromatographically. A standard solution containing pteroic acid (7—9 113 Lederle; 2 μg/ml), folic acid (pteroylglutamic acid, Folvite, Lederle; 0.1 μg/ml), pteroyltriglutamic acid (Teropterin 7—9 111, Lederle; 0.2 μg/ml), N_{10}-formylfolic acid (Ro 1—5 681, Hoffman-La Roche; 0.1 μg/ml), N_{10}-formylpteroic acid (Rhizopterin 7—9 112, Lederle; 0.1 μg/ml), N_5-formyl, 5,6,7,8,-tetrahydroformylfolic acid (Leucovorin, Lederle; 0.2 μg/ml) thymine-desoxyriboside (thymidine; 100 μg/ml) and thymine (100 μg/ml) was employed for comparison. The position of the growth zones of the factors in the algal extracts and in the standard solution is shown in Figure 1. Solvent system I was employed.

All extracts, whether treated with enzymes or not, contained factors with the same R_F-values as N_{10}-formylfolic acid, folinic acid, N_{10}-formylpteroic acid and thymidine respectively. A growth factor for *S. faecalis* having a R_F of 0.36 was also found (Factor V) in all samples except those treated with hog kidney homogenate. Pteroyltriglutamic acid but only traces of folic acid were observed in the aqueous extracts. A factor with R_F 0.07 (Factor I) could be detected both in the aqueous extracts of *Furcellaria fastigiata* and in a sample of *Furcellaria* treated with hog kidney enzyme.

Both the brown alga *Laminaria saccharina* and the red alga *Furcellaria fastigiata* contain a factor having a R_F-value of 0.12 (Factor II) after boiling or treatment with chicken pancreas homogenate. Chicken pancreas also releases one factor with R_F 0.17 (Factor III) and another with R_F 0.24 (Factor IV), not found in the boiled samples or in samples that had been incubated with hog kidney homogenate. The hog kidney enzyme appears to release folic acid, whereas little or no folic acid seems to result from the treatment with chicken pancreas. An intensified growth due to pteroyltriglutamic acid was observed in the algal samples that had been incubated with the hog kidney enzyme. The enzyme complex of the hog kidney homogenate also gave rise to a spot having a R_F of about 0.40 (Factor VI).

Factors with the same R_F-values (solvent system I) as pteroyltriglutamic acid, N_{10}-formylfolic acid, folinic acid, N_{10}-formylpteroic acid and thymidine were found in the aqueous extracts of all the seaweeds investigated. Factor I was observed in the aqueous extracts of *Sphacelaria arctica*, *Fucus vesiculosus* and in *Furcellaria* as mentioned above, Factor II in all seaweeds except *Fucus vesiculosus*. Factor V appeared in the aqueous extracts of *Sphacelaria* and *Fucus* as well as in *Laminaria* and *Furcellaria* as just described.

An increased activity due to the formation of Factor III and Factor IV was observed in all seaweeds after treatment with chicken pancreas homogenate, which also released a factor with the same R_F-value as Factor V.

All algal extracts were also chromatographed in solvent systems II and III. Factors with the same R_F as pteroyltriglutamic acid, N_{10}-formylfolic acid, folinic acid, N_{10}-formylpteroic acid and thymidine were found. This agrees with the results obtained with solvent system I. Thymidine has the same R_F-value as thymine in solvent system I, but separates from thymine in system III. No thymine was found in the algal extracts.

When the algal samples were treated with chicken pancreas enzyme and chromatographed in solvent system III, factors with low R_F-values appeared which is in agreement with the observations made with solvent system I.

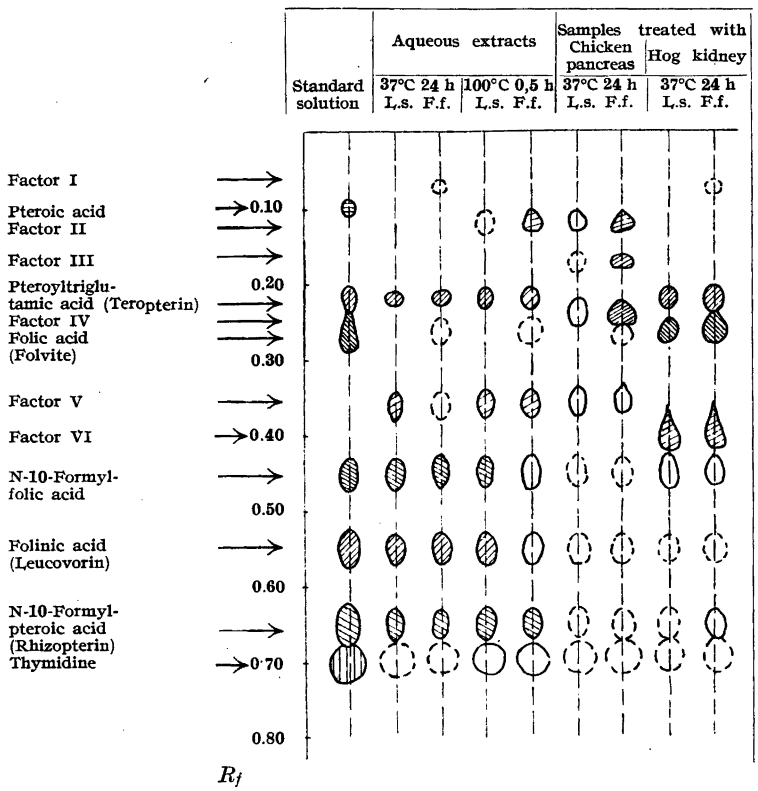

Fig. 1. Chromatographic separation of growth factors for Streptococcus faecalis (ATCC 8 043) in different extracts of Laminaria saccharina (L.s.) and Furcellaria fastigiata (F.f.). Solvent system I was employed. Thymine has the same Rf-value as thymidine in this solvent.

Cutting chromatograms lengthwise through the original spots and placing one half on plates seeded with *S. faecalis* and the other on plates seeded with *Leuconostoc citrovorum* revealed that the same areas on the chromatograms were active both for *S. faecalis* and *L. citrovorum*. This was observed both when solvent I and solvent III were employed. The existence of growth factors — other than folinic acid and thymidine — active both towards *S. faecalis* and *L. citrovorum* is thus probable.

A preliminary study of the action of chicken pancreas homogenate on several pure substances has been carried out. For that purpose solutions of crystalline pteroic acid, folic acid, pteroyltriglutamic acid, folinic acid, N_{10}-formylpteroic acid and N_{10}-formylfolic acid were incubated with chicken pancreas homogenate at 37° C for 24 hours at pH 7.5, and the solutions thus obtained assayed bioautographically with *S. faecalis* and *L. citrovorum*. The treated compounds were converted into several new growth factors for these two microorganisms.

No significant release of folic acid from pteroyltriglutamic acid could be observed. Instead a factor with the same R_F as Factor IV had been formed. Formylation of pteroic acid and folic acid appeared to have taken place as well as a conversion of some factors for only *S. faecalis* to growth factors for both *S. faecalis* and *L. citrovorum*. Treatment of plant extracts with chicken pancreas homogenate can thus not be used when the naturally occurring forms of growth factors for *S. faecalis* and *L. citrovorum* are to be studied.

DISCUSSION

The results of the present investigation establish the occurrence in marine algae of a number of compounds that can stimulate the growth of *S. faecalis* in a medium used for the estimation of folic acid [2]. Pteroyltriglutamic acid, N_{10}-formylpteroic acid, N_{10}-formylfolic acid, folinic acid, thymidine and small amounts of folic acid as well as three other factors — Factors I, II and V — appear to be present in the aqueous extracts. Factor II was observed first when boiling was used in the extraction process.

Treatment of the algae with chicken pancreas or hog kidney homogenates gives rise to three new factors, provisionally called Factors III, IV and VI. Factors III and IV appeared only after incubation with chicken pancreas. Factor VI only after treatment with hog kidney homogenate. This demonstrates a different action of the enzyme complexes of chicken pancreas and of hog kidney homogenates, a phenomenon that was also observed by Doctor and Couch, in their recent study of a conjugated form of the citrovorum factor [3]. To what extent this depends on the differences in the pH at which the two enzyme mixtures were employed has not been further investigated.

At least twelve different growth factors for *S. faecalis* in addition to thymine have thus been observed. Chromatography of the extracts in different solvent systems and comparison of the activity of the factors for *S. faecalis* and for *L. citrovorum*, indicates that the number of naturally occurring substances that can support the growth of *S. faecalis* may be still higher. This can be expected considering the fact that formylated (position N_5 or N_{10}) and/or reduced (di- or tetrahydro-) forms of pteroic acid, pteroylglutamic acid, and the pteroylpolyglutamic acids can act as growth factors for this microorganism. It seems likely that at least some of the unidentified growth factors in marine algae are such formylated and/or reduced forms of known factors.

Wieland *et al.* have studied the natural occurrence of folic acid and the citrovorum factor [4]. They mention only two substances other than folic acid and thymidine (found in mouse liver homogenate and in the charcoal eluate of a liver preparation) that can stimulate the growth of *S. faecalis*. They also demonstrate the multiple nature of the *L. citrovorum* activity, but do not describe any factor other than folinic acid and thymidine that are active for both microorganisms. The existence of such substances seems likely, considering the results of the present study.

The action of chicken pancreas or hog kidney homogenate (or other enzyme preparations) on different substrates is generally referred to as only a release of folic acid or folinic acid from bounds forms [5–10]. Such enzyme mixtures

appear, however, to carry out several different reactions that result in a number of active compounds.

Dabrowska *et al.* have shown that digestion of pteroyltriglutamic acid with the chicken pancreas enzyme leads to pteroyldiglutamic acid [11]. This compound is thus likely to be one of the unidentified growth factors (Factors I to VI) that are present in the algae investigated.

SUMMARY

Aqueous extracts or marine algae have been shown to contain at least nine factors stimulating the growth of *S. faecalis*, among them pteroyltriglutamic acid, N_{10}-formylpteroic acid, N_{10}-formylfolic acid, folinic acid, thymidine and small amounts of folic acid as well as three other as yet unidentified factors. Three further factors appeared when the algal samples were treated with chicken pancreas or hog kidney homogenates. It was also observed that some crystalline substances stimulating the growth of *S. faecalis* were converted to new growth factors for this microorganism and also to growth factors for *L. citrovorum* on incubation with chicken pancreas homogenate. As the chicken pancreas homogenate causes not only a release of growth factors but also a conversion of some factors to others, it cannot be employed in studying the naturally occurring factors for *S. faecalis* or *L. citrovorum*. Evidence has been gathered for the existence of factors, other than folinic acid and thymidine, stimulating the growth of both *S. faecalis* and *L. citrovorum*.

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Electrophoretic Studies of Cobalamins. I

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The present paper deals with electrophoretic experiments on vitamin B_{12} (cyanocobalamin) and on the vitamin B_{12}-cyanide complex (di- or tricyanocobalamin).

EXPERIMENTAL

The apparatus employed was of Tiselius' type with the improvements designed by Svensson [1]. The experiments were run in preparative cells at 0° C. Buffer solutions having an ionic strength of 0.1 and covering a pH range from 1.1 to 10.55 were used. Weighed amounts of crystalline vitamin B_{12} (Cobemin, Merck & Co. Inc.) were dissolved in the buffer solutions to give a concentration of about 100 μg/ml. The electrophoretic mobility was calculated from the content of vitamin in the different cells, which was determined spectrophotometrically, using the maxima at 550 and 361 mμ. For every estimation of vitamin B_{12} the spectrum in the neighbourhood of these peaks was measured to make certain that no change of the molecule had taken place during the experiment, *e. g.* conversion to hydroxo- or aquocobalamin. For more extreme pH-values, *i. e.* pH 1.1, 1.8 and 10.55, the vitamin B_{12} content of the cells was also determined microbiologically, using *E. coli* 113—3[2] as test organism in the agar cup plate method. The results of these microbiological estimations always agreed with those obtained spectrophotometrically.

It was observed that in some experiments, especially at high pH-values, both upper chambers contained vitamin B_{12}, as if the vitamin dissociated into two oppositely charged components. This confusing phenomenon — which may be electrokinetic in nature — could be avoided if the electrophoresis was carried out in a sugar gradient, which was accomplished by dissolving the vitamin in a buffer solution containing 2 % glucose, pushing the cells into position for electrophoresis and allowing the sugar to diffuse into the upper chambers. The diffusion of glucose, which is much faster than that of vitamin B_{12} could be observed by the ordinary optical method. The current was turned on after 5 hours and the movement of vitamin B_{12} could then also be observed optically — despite the low concentration — as the light refractive index curve of the not migrating glucose was overlapped by the red colour of the vitamin and the movement of the coloured front could be observed. When the vitamin B_{12} boundary had to move in a glucose gradient a definite electrophoretic movement was established *.

* We are grateful to Dr. H. Svensson, LKB Produkter, Fabriksaktiebolag, Stockholm, for the suggestion to try this method.

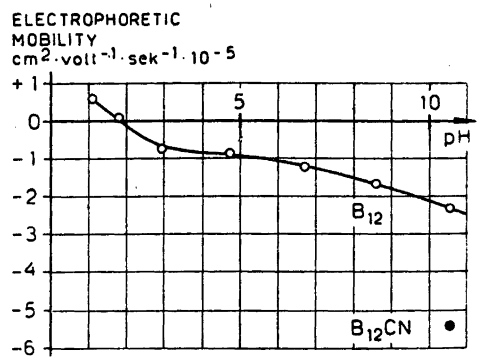

Fig. 1. Electrophoretic mobility of vitamin B_{12} (cyanocobalamin) at different pH-values and of the vitamin B_{12}-cyanide complex (di-or-tri-cyanocobalamin) at pH 10.55.

RESULTS

Fig. 1 shows the curve obtained by plotting the mobility values for vitamin B_{12} against pH. The vitamin B_{12} molecule moved towards the anode in experiments where the pH was 2.95 or higher, but towards the cathode at the experiments run at pH 1.1 and 1.8. The isoelectric point appears to be near pH 1.9. Vitamin B_{12} recrystallized once (run at pH 2.95 and 10.55), three (pH 1.1 and 6.75) and five times (pH 4.75) from aqueous acetone gave results which were in agreement with those observed with the original B_{12}-preparation (Cobemin, Merck).

The mobility of the cyanocobalamin at pH 7 ($\mu = 0.1$) is $1.3 \cdot 10^{-5}$ cm^2 volt^{-1} sek^{-1}. Due to diffusion, accurate mobility figures are difficult to obtain with a compound having a comparatively low molecular weight and a low mobility.

The vitamin B_{12}-cyanide complex was run in a buffer solution of potassium cyanide alone which had a pH of 10.55 and an ionic strength of 0.01. The solution of vitamin B_{12} in this buffer showed the characteristic maxima at 578 and 368 mμ, both before and after the electrophoresis. The mobility of the B_{12}-cyanide compound was found to be $5.4 \cdot 10^{-5}$ cm^2 volt^{-1} sek^{-1}.

DISCUSSION

Brink *et al.*[3] have shown that vitamin B_{12} is a polyacidic base, as revealed by potentiometric titration in glacial acetic acid solution. They also stated that the basic groups were too weak to be detected when the compound was titrated in aqueous solution. Alicino [4] observed that vitamin B_{12} could form with perchloric acid a salt, having the composition $B_{12} \cdot 6\ HClO_4$, indicating the presence of six basic groups in the vitamin. Recently H. Schmid, A. Ebnöther and P. Karrer [5] reported that 5—6 molecules of ammonia per molecule of vitamin B_{12} could be obtained on catalytic reduction in hydrochloric acid of vitamin B_{12}. The results of the present investigation in which vitamin B_{12}

was observed to move towards the cathode at low pH values provides further evidence for the existence of weak basic group(s) in the molecule.

Vitamin B_{12} is generally referred to as a neutral compound [6-8]. As the vitamin appears to have an isoelectric point at pH 1.9, this is strictly true only at this pH value. The acidic group or groups that were observed when the electrophoresis was carried out at pH values higher than 1.9 are probably comparatively weak as judged from the mobility values. Lester Smith *et al.*[7] report no change with pH (varying from 2 to 9.5) in the partition coefficients of vitamin B_{12} between benzyl alcohol and water, which could mean that the acidic groups are too weak to influence measurably the partition of the compound between these two solvents. The failure of one of us and other authors [9,10] to demonstrate any definite electrophoretic mobility of vitamin B_{12} by paper electrophoresis may possibly be explained by the same reason.

I II

Fig. 2. Formula I represents vitamin B_{12} (cyanocobalamin). Formula II the vitamin B-cyainde complex (di-cyanocobalamin).

Bush *et al.*[8] have proposed the formula I (Fig. 2) for the cyanocobalamin. In this formula the „zeros denote a neutral group or groups coordinated to cobalt by dative bonds". It is evident that these O-groups, whether occurring together with the minus-groups in a single molecular aggregate or not, represent groups that can ionize, being basic or acidic depending on the pH. Cooley *et al.*[11] suggest that introduction of a second cyanide group into the cyanocobalamin leads to displacement of the N_3 of the 5,6-dimethylbenzimidazole from the coordination with cobalt. The vitamin B_{12}-cyanide complex formed can thus be written as shown in formula II (Fig. 2), where a second CN^--group replaces one of the O-groups rendering the molecule as a whole negative. Consequently the vitamin B_{12}-cyanide complex behaves as an acid [7]. This is in accord with the observed mobility values at pH 10.55 for the vitamin B_{12}-cyanide complex ($5.4 \cdot 10^{-5}$) as compared with that of the vitamin B_{12} itself ($2.4 \cdot 10^{-5}$) at the same pH. It should be mentioned, however, that Conn *et al.*[12] have provided polarographic evidence for the uptake of two cyanide molecules per molecule of vitamin B_{12} in a buffer consisting of 0.1 *M* sodium cyanide and 0.1 *M* lithium borate and having a pH of 10.99.

SUMMARY

Electrophoretic studies of vitamin B_{12} (cyanocobalamin) at pH values varying from 1.1 to 10.55 revealed both acidic and basic groups and showed that the vitamin has an isoelectric point near pH 1.9. The mobility of vitamin B_{12} was observed to be $1.3 \cdot 10^{-5}$ cm^2 $volt^{-1}$ sek^{-1} at pH 7.0. The vitamin

B_{12}-cyanide complex showed a mobility value of $5.4 \cdot 10^{-5}$ cm^2 $volt^{-1}$ sek^{-1} at pH 10.55 whereas the mobility value for vitamin B_{12} at this pH was $2.4 \cdot 10^{-5}$ cm^2 $volt^{-1}$ sek^{-1}.

The authors wish to express their thanks to Dr. S. Brohult, LKB Research Laboratories, and to Professor H. Lundin, Division of Food Chemistry, Royal Institute of Technology, for their encouraging interest.

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Investigations on the Phosphorus Metabolism in *Rhodotorula gracilis*. I. The Influence of the Phosphate Content of the Nutrient Solution on the Formation of Fat

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A number of microorganisms can be brought to form large quantities of fat under special cultural conditions. This fat formation takes place for example if there is a low nitrogen content in the nutrient solution, providing that plentiful quantities of sugar are present. In this way, for instance, *Rhodotorula gracilis* can be brought to contain up to 63 % fat, whereas the normal proportion of fat in this yeast is only about 8 % (Enebo, Anderson and Lundin [1]).

Fat formation can also be brought about, however, if the amounts of nutrient substances other than nitrogen are decreased below a certain level. In the case of *Rhodotorula gracilis*, Nielsen and Rojowski [2] have shown that a low sulphur or iron content can also bring about fat formation. As the fat content of the yeast increases, the protein content decreases, as is the case when the nutrient solution is nitrogen deficient. It can perhaps be assumed that the cause of fat formation is the same with low quantities of nitrogen, sulphur or iron, namely, a reduced protein formation with a correspondingly increased fat formation as a result thereof.

The investigation presented here has been made to show how fat formation in *Rhodotorula gracilis* is affected if the phosphate content of the nutrient solution is reduced. As phosphates are necessary for protein synthesis, a low amount of phosphate in the nutrient solution should cause a decrease in the protein content of the yeast, which in turn should result in an increased fat formation. At the same time, however, phosphates stimulate fat formation and therefore two different, opposing tendencies must be expected after reducing the phosphate content of the nutrient solution.

Our experiment was performed in the following way.

Rhodotorula gracilis was cultivated in a nutrient solution of varying phosphate content. The composition of the nutrient solution was as follows: 15 g asparagine — 3 g $MgSO_4$, $7H_2O$ — 1.5 g NaCl — 1.5 g $CaCl_2$, $6H_2O$ — 0.015 g $FeCl_3$ — 60 g glucose to 1 litre. Varying quantities of KH_2PO_4 were added to the solution, so that it contained per litre: 4.7 — 2.0 — 1.0 — 0.5 — 0.2 and 0.1 g KH_2PO_4. The pH was adjusted to 4.8 in all solutions.

The phosphate amount usually used for experiments with *Rh. gracilis* is 4.7 g KH_2PO_4 per litre, which is a rather excessive quantity.

The cultivation took place in 750 ml Erlenmeyer flasks each containing 300 ml of nutrient solution. The flasks were shaken at 25° C. After 5 days the protein and fat contents of the yeast were determined [3].

Table 1. Nutrient solution with 15 g asparagine per litre. Time of experiment: 5 days.

KH_2PO_4 g per litre	Fat %	Protein %	Fat + Protein %
4.7	10.9	50.6	61.5
2.0	12.2	47.5	59.7
1.0	11.8	51.3	63.1
0.5	24.2	35.6	59.8
0.2	37.8	21.3	59.1
0.1	39.2	18.8	58.0

From Table 1 it is clear that if the phosphate content of the nutrient solution falls below a certain level, the protein content of the yeast begins to decrease. This decrease in the protein content begins if the quantity of KH_2PO_4 sinks to 0.5 g per litre. At the same time as the protein content decreases, the proportion of fat in the yeast begins to rise and reaches 39 % as compared with 10 % in yeast which has been cultivated with an excess of phosphate.

A phosphate deficiency, therefore, brings about a decrease in the protein content of the yeast parallel with an increase in its fat content, as is also the case when the yeast is cultivated in a nutrient solution low in nitrogen, sulphur or iron. The fat formation is not so great, however, as when yeast is cultivated in a nitrogen-deficient nutrient solution. This question will be further discussed later. The above experiment was repeated and gave the same results.

In order to determine the effects of a low content of both phosphate and nitrogen occurring simultaneously, we have carried out an experiment in which the quantities of nitrogen and phosphate in the nutrient solution were varied at the same time.

As Table 2 indicates, if the nitrogen content of the nutrient solution (with optimal phosphate content) is decreased to 1 g of asparagine per litre the fat content of the yeast increases to 52.5 %. If the phosphate content is decreased to 0.2 g of KH_2PO_4 per litre (with optimal nitrogen content) the fat content of the yeast increases to 39.4 %. The fat content is consequently appreciably less in the latter case than with a low nitrogen content. If the

Table 2. Nutrient solution with varying quantities of asparagine and KH_2PO_4. Time of experiment: 5 days.

Aspargine g per litre	KH_2PO_4 g per litre	Fat %	Protein %	Fat + Protein %
15	4.7	11.2	48.8	60.0
15	0.2	39.4	18.8	58.2
1	4.7	52.5	14.4	66.9
1	0.2	43.2	15.6	58.8

nutrient solution is low in both nitrogen and phosphate at the same time (solution with 1 g asparagine and 0.2 g KH_2PO_4) the fat content of the yeast is lower than in a nutrient solution low in nitrogen only, namely 43.2 % fat as compared with 52.5 %. Two corresponding experiments gave the same results.

The reason why a lack of phosphate appears to decrease fat formation which has already resulted from a lack of nitrogen seems to be the fact that phosphate is essential to the formation of fat. When the maximum fat production has been reached as a result of the lack of nitrogen the low phosphate content, on the other hand, slows down the further formation of fat, and the fat content will therefore be lower than is the case with the normal quantity of phosphate. This conclusion also accounts for the fact that the fat formation brought about by a lack of phosphate is lower compared with that produced by a lack of nitrogen. The above experiments confirm the earlier results of Smedley MacLean and Hoffert [4,5] and Kleinzeller [6] concerning the significance of phosphates for the formation of fat.

SUMMARY

Fat production in *Rhodotorula gracilis* can be brought about by a low phosphate content in the nutrient solution, in the same way as by a low content of nitrogen, sulphur or iron. On account of the unfavourable effects of a lack of phosphate on the formation of fat, however, the production of fat is comparatively small.

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Studies on Ionic Solutions in Diethyl Ether

IV. Properties of $LiClO_4$-Ether Solutions

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In parts I—III of this series [1-3] it has been shown how lithium perchlorate may be used for obtaining solutions in diethyl ether of a constant ionic strength. By the addition of a large and constant amount of $LiClO_4$ to the solutions, the activity factors of various ions such as Ag^+ and halogenide ions may be kept constant so that their concentrations may be measured with emf methods.

The solutions of $LiClO_4$ in diethyl ether are remarkable in many ways. At 25° C, ether dissolves as much as 53.21 per cent by weight of $LiClO_4$, corresponding to a molar fraction of 0.44 (Willard and Smith[4]). The solutions are very viscous especially at the highest concentrations.

On the addition of a small quantity of water to such a solution, one gets immediately a precipitate of $LiClO_4(H_2O)_3$, whose solubility in ether is only 0.196 %[4]. This large difference in solubility would perhaps not have been expected.

A number of other salts, such as lithium chloride and lithium acetate, which are only slightly soluble in pure ether, are considerably soluble in $LiClO_4$-ether solutions. This may be compared with the "neutral salt" effect on the solubility of electrolytes in water. However, the effect seems to be much larger in $LiClO_4$-ether.

To understand better the properties of $LiClO_4$-ether as solvent, it seemed desirable to study the properties of solutions of lithium perchlorate in ether at different concentrations.

From the fall of 1948 to the summer of 1949 we undertook measurements of the vapor pressure and electric conductivity of such solutions. When our experimental work was completed, at the end of September, 1949, one of us had the pleasure of listening to a lecture by Dr. Oliver Johnson at the meeting of the American Chemical Society at Atlantic City [5]. He then learned that Dr. Johnson [6] and another of Professor Fajans's co-workers, Dr. Chu[7,8], had also made a number of measurements on $LiClO_4$-ether solutions, though their approach to the problem was quite different from ours. We exchanged our data and found that they were in many ways complementary. In the

Table 1. Density ϱ of ether (1)-$LiClO_4$ (2) mixtures of varying mole fraction x_2, according to Johnson (⁶p. 39); partial molar volumes v_1 and v_2 calculated by three methods (see text); molar concentration c_2.

		Method 1 (from d)		Method 2 (from φ_2)		Method 3 (from v)		
x_2	ϱ	v_1	v_2	v_1	v_2	v_1	v_2	c_2
0	0.70776	104.7	15.2	104.7	14.2	104.7	13.0	0
0.0608	0.76595	104.6	17.5	104.4	19.0	104.3	19.0	0.612
0.0872	0.79265	104.3	19.5	104.3	19.9	104.2	19.9	0.8983
0.1256	0.83301	104.0	22.6	104.1	21.6	104.0	21.7	1.3381
0.1552	0.86661	103.9	23.5	103.8	23.1	103.2	26.0	1.6970
0.2363	0.95999	102.7	28.2	102.3	29.2	101.4	31.6	2.7750
0.2898	1.02559	101.3	31.8	100.7	33.7	99.8	35.2	3.5608
0.3264	1.07330	99.4	36.2	98.9	37.9	98.0	39.2	4.1383
0.4401	1.20807	89.7	52.4	92.0	48.9	94.7	45.6	6.0196

following, with the kind permission of Drs Fajans, Johnson and Chu, we shall compare their measurements with ours and also use some of their data in the discussions.

EXPERIMENTAL

Reagents. Diethyl ether and lithium perchlorate were obtained as described in Part I[1]. The amount of $LiClO_4$ in the ether solutions was determined in principle as described in part II[2] by tipping the ethereal solution into water and evaporating the ether (occasionally by evaporating the ether and dissolving in water), diluting to a defined volume, taking out a sample with a pipet, passing it through a H^+ saturated ion exchanger, and titrating for the liberated hydrogen ions with NaOH.

Temperature. All measurements were carried out in a thermostat room, the temperature of which was kept at 23.5 ± 0.2° C. The choice of this temperature, instead of 25° C or 18° C which would have been preferable, may be ascribed to the law of mental inertia.

Concentration units, densities, molar volume. In our measurements, the concentration of the solutions was generally obtained as the molar fraction x_2 of lithium perchlorate (1 = ether, 2 = lithium perchlorate). When it was desirable to convert x_2 to the molarity, c_2, we used Johnson's values [6], which are given in Table 1. The density change between 23.5° C and 25° C was neglected.

From Johnson's figures [6] the partial molar volumes of ether and $LiClO_4$, v_1 and v_2, were calculated by three methods (see *e. g.* Lewis and Randall [11], Ölander [12], Sillén, Lange and Gabrielson [13]): 1) by plotting the "shrinkage" d versus x_2, 2) by plotting the apparent molar volume φ_2 versus log n_2 ([11]method III), 3) by plotting the volume of 1 mole solution, v, versus x_2 ([11]method IV). The results are given in Fig. 3a.

Vapor pressure measurements. Fig. 1 shows the apparatus used. The $LiClO_4$-ether solution to be studied is kept in the bulb A. C is a flask of nearly 1 l volume, intended to act as a buffer volume and thus to eliminate the influence of small unavoidable leaks in the apparatus. The vessel B contains pure diethyl ether, some of which may be let in to build up the ether pressure in C and avoid large evaporation losses in A.

In the beginning of an experiment, the apparatus was mounted and the vessel A, which was closed by means of a stop-cock, dipped into liquid air (about −190° C). The freezing point of diethyl ether is −116.3° C, and at this temperature the vapor pressure is about 0.008 torr [14]. It must therefore be still lower at −190° C.

The whole system was then evacuated by an oil pump, first with closed stopcocks at A and B, then opening the stop-cock at A. The pumping was continued until on closing

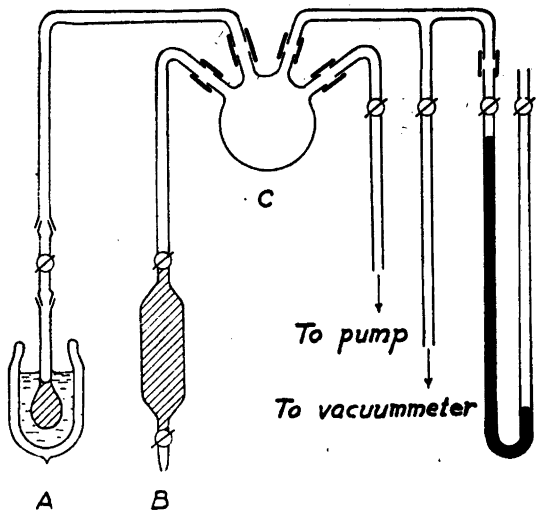

Fig. 1. Apparatus for measuring vapor pressure p_1. A *contains the* $LiClO_4$*-ether solution,* B *pure ether.* C = *buffer volume.*

the stop-cock to the oilpump, the pressure given by the vacuummeter was 0.2 torr or generally less.

Now the stop-cock at *A* was closed, the liquid air bath taken away, and sufficient ether let in from *B* so that the pressure at *C*, as measured by the manometer, was not far from the expected equilibrium pressure of the solution at *A*. Then the stop-cock at *A* was opened, and the system allowed to attain equilibrium at the temperature of the thermostat room, which generally took 4—5 hours.

At the end of the experiment, vessel *A* was weighed together with its content of solution, which had been in equilibrium with ether vapor of the measured pressure. Then the whole solution was tipped into water, and the amount of $LiClO_4$ determined using a known fraction of the solution, and an ion exchanger as described above.

Table 2. Activity factors f_1 *of ether in* $LiClO_4$*-ether solutions, calculated from the ether pressure* p_1 *at 23.5° C (present work), and from the boiling point elevation as measured by Chu and Fajans* [8] *1949.*

x_2	p_1	f_1	x_2	p_1	f_1
0	503	1.000	0.241	453	1.187
0.047	489	1.020	0.257	433	1.159
0.059	484	1.023	0.280	426	1.176
0.101	482	1.066	0.280	432	1.193
0.141	470	1.088	0.284	418	1.161
0.177	470	1.135	0.287	398	1.110
0.199	466	1.157	0.317	370	1.077
0.221	451	1.151	0.319	341	1.019
0.237	454	1.183	0.321	361	1.057

x_2	ΔT_e	f_1
0.00903	0.142°	1.0043
0.02935	0.427°	1.0154
0.04542	0.625°	1.0255
0.06881	0.880°	1.0422
0.08149	0.955°	1.0539
0.12859	1.368°	1.0954

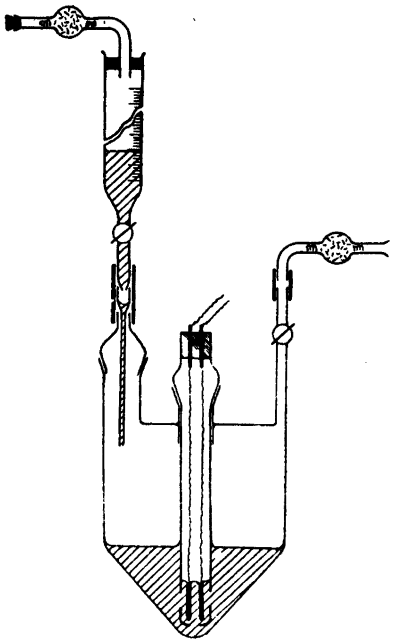

Fig. 2. Cell for measuring conductivity.

The results are given in Table 2 and Fig. 4 (circles). The vapor pressure $p_1^0 = 503$ torr for pure ether, measured with this apparatus, compares very well with the value 503.5 torr at 23.5° C, calculated using the formula of Taylor and Smith [15]. From the p_1 values found, the activity factors $f_1 = p_1/x_1 p_1^0$ were calculated from Chu's and Fajans' boiling point data[8], using the equation $-\log(f_1 x_1) = \Delta T_e \cdot 0.1477_5$ corresponding to a molar heat of evaporation 6.38 kcal mole^{-1}. The results are given as dots in Fig. 5; the range is smaller but the agreement good.

Conductivity measurements. Fig. 2 shows the conductivity cell, its cell constant being 4.77 cm^{-1}. After each experiment it was cleaned with nitric acid and water, and the constant checked using 0.0200 C KCl. A fresh KCl solution was prepared every week. Between measurements, the conductivity cell was stored in ethanol.

As seen from Fig. 2 the vessel was conical, to allow measurements of conductivity over a large range of volumes. The dip cell was inserted into the vessel through a standard ground-glass joint. A burette, containing pure ether, and a calcium chloride tube (for letting out excess air) were connected to the vessel by ground-glass joints with rubber joints as shown in the figure.

At the beginning of an experiment, the vessel was weighed with the dip cell but without burette and calcium chloride tube. A certain amount of concentrated $LiClO_4$-ether was introduced and the vessel weighed again. After waiting for equilibrium, the resistance of the dip cell was measured. Then pure ether was added in portions of 1—5 ml from the burette. After each addition of ether, the vessel was shaken, equilibrium waited for and the resistance measured. Finally when about 50 ml had been added, the vessel was weighed again, the apparatus disconnected, and the total amount of $LiClO_4$ present determined as described above. Two different stock solutions of $LiClO_4$-ether were used.

The resistance was measured using a Philoscope. The accuracy was only about 1 %, which was sufficient in our case since the conductivity varied by a factor of more than 10^4 in the concentration range studied and since larger errors came from the concentration calculations.

The primary data $\varkappa$ and x_2 are given in Table 3 (every third one of our points).

Table 3. Conductivity $\varkappa$ and molar conductance Λ of $LiClO_4$ in ether at various concentrations and 23.5° C (present work) or 25° C (Chu [7]).
23.5° C (present work). Every third point is given; (all points are given in Fig. 8.)

x_2	c_2	$\varkappa$	Λ	x_2	c_2	$\varkappa$	Λ
0.00664	0.060	$2.33 \cdot 10^{-8}$	$3.88 \cdot 10^{-4}$	0.113	1.19	$1.87 \cdot 10^{-5}$	$1.57 \cdot 10^{-2}$
0.00896	0.084	3.67	4.37	0.125	1.33	3.10	2.33
0.0141	0.132	6.35	4.81	0.143	1.55	4.99	3.22
0.0174	0.165	8.74	5.30	0.157	1.72	7.59	4.41
0.0284	0.27	$2.05 \cdot 10^{-7}$	7.59	0.174	1.94	$1.13 \cdot 10^{-4}$	5.83
0.0347	0.34	3.27	9.62	0.205	2.35	2.10	8.94
0.0491	0.49	8.93	$1.82 \cdot 10^{-3}$	0.226	2.64	2.56	9.70
0.0583	0.58	$1.55 \cdot 10^{-6}$	2.67	0.249	2.96	3.23	$1.09 \cdot 10^{-1}$
0.0695	0.71	2.62	3.69	0.275	3.34	3.75	1.12
0.0836	0.86	6.16	7.16	0.286	3.50	3.96	1.13
0.0975	1.02	$1.08 \cdot 10^{-5}$	$1.06 \cdot 10^{-2}$	0.307	3.84	4.11	1.07

25° C (Chu)

x_2	c_2	$\varkappa$	Λ
0.0608	0.6120	$9.86 \cdot 10^{-7}$	$1.611 \cdot 10^{-3}$
0.0872	0.8983	$4.659 \cdot 10^{-6}$	$5.186 \cdot 10^{-3}$
0.1256	1.3381	$2.144 \cdot 10^{-5}$	$1.602 \cdot 10^{-2}$
0.1552	1.7101	$5.913 \cdot 10^{-5}$	$3.458 \cdot 10^{-2}$
0.2363	2.7750	$2.552 \cdot 10^{-4}$	$9.196 \cdot 10^{-2}$
0.2898	3.5608	$3.969 \cdot 10^{-4}$	$1.115 \cdot 10^{-1}$
0.3264	4.1383	$4.339 \cdot 10^{-4}$	$1.049 \cdot 10^{-1}$
0.4401	6.0196	$3.111 \cdot 10^{-4}$	$5.169 \cdot 10^{-2}$

In the way our experiments were performed, we immediately obtained the weight percentage, and thus the mole fraction x_2 of $LiClO_4$. Using Johnson's densities [6] (Table 1), x_2 can be converted to give the concentration c_2 in C (moles l^{-1}), neglecting the difference in density between 23.5° C and 25° C. By means of c_2 we can calculate the molar conductance Λ of $LiClO_4$. In Fig. 8 Λ is given as a function of x_2; our data (circles 23.5° C) are seen to agree well with those of Chu [7] (dots 25° C).

Viscosity measurements. For comparison with the conductance data, we wished to know how the viscosity η varies with the concentration c_2. Since very accurate data were not needed, a Höppler viscosimeter was used. The measurements were made by Miss Christina Mannerskantz, M.Sc. Professor Paul Nylén was kind enough to provide us with a Höppler viscosimeter with calibration tables, the accuracy of which was stated to be 1–3 %.

What is measured in a Höppler viscosimeter is the time needed by a large steel ball to roll along a tilted tube, filled with the liquid, of only slightly larger diameter than the ball.

No elaborate equipment was used to avoid the evaporation of ether, but the viscosimeter was closed quickly after introducing the solution, and after the measurement of η the solution was quickly transferred to another vessel, where its density was determined by an aerometer. The concentration c_2 was read from a curve (Fig. 3 b) giving c_2 as a function of the density ϱ, as obtained from Johnson's data [6].

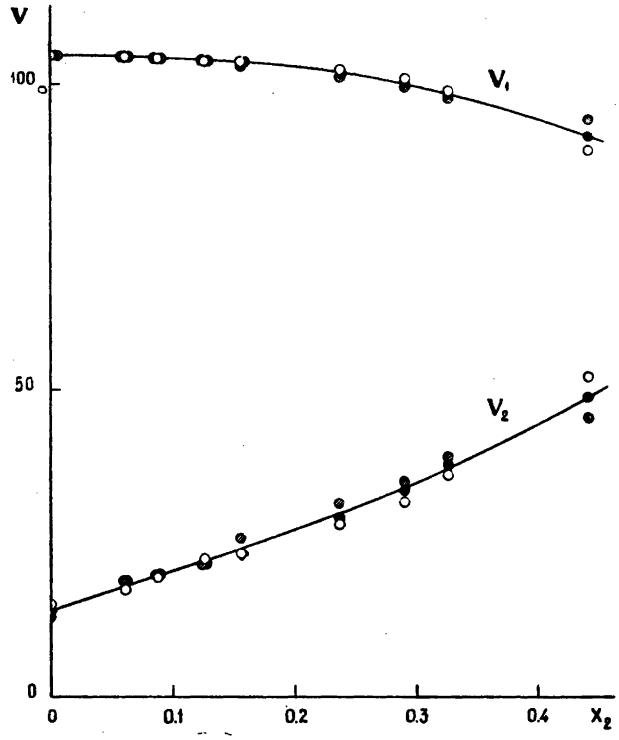

Fig. 3 a) Partial molar volumes v_1 *and* v_2 *(in ml) calculated from Johnson's density values* [6]*: 1)* ○ *Open circles from the shrinkage* d, *2)* ● *black dots from the apparent molar volume* φ_2, *3)* ◍ *shaded dots from the molar volume* v *(see text).*

This curve was checked using solutions which had been mixed from accurately known amounts of ether and concentrated $LiClO_4$-ether solution. The lithium content of the latter had been determined by the ion exchange method [2]. The agreement was good (see Fig. 3b).

In Fig. 7 η (in cP) is given on a logarithmic scale as a function of x_2 at 20° C and 25° C (see also Table 4). The diagram shows the enormous increase of viscosity with concentration; it was not thought worth while to attempt to increase the accuracy.

DISCUSSION

Association $LiClO_4$-$LiClO_4$ or complexes $LiClO_4$-ether? Chu and Fajans [8] measured the boiling point of $LiClO_4$-ether solutions, and from their data calculated the "apparent molecular weight" of $LiClO_4$ in the solution. They found values between 2 and 3 times the formula weight of $LiClO_4$, but did not consider these figures as exact because the solutions are really non-ideal. The process was described as an "association of $LiClO_4$", and it was pointed out that the conductivity data of Chu [7] indicated the formation of $(LiClO_4)_m$ Li^+ and $(LiClO_4)_nClO_4^-$ with m and n perhaps = 4 and 3 ([7] p. 129 and 145).

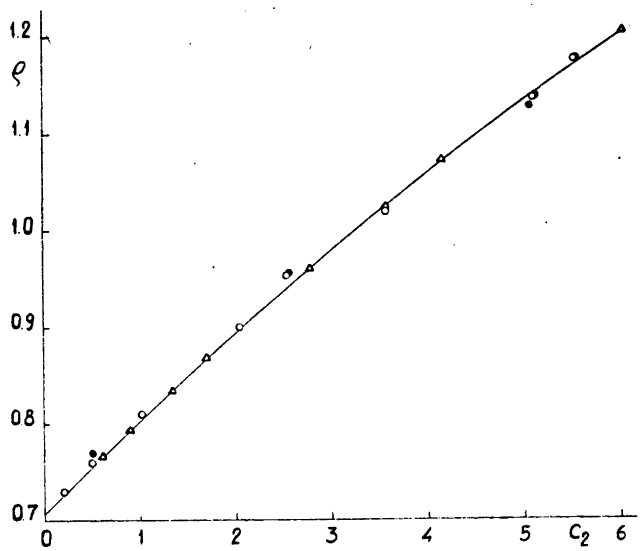

Fig. 3 b) Density ϱ (mg ml^{-1}) as a function of the concentration c_2 (in C). Triangles △ and curve from Johnson's density data[6] at 25° C. Dots ● (20° C) and circles ○ (25° C) from direct measurements with an aerometer. (Present work).

This is one of the many examples in literature (see also *e.g.* [16]), where measurements of the activity of one component in a mixture have been used for drawing conclusions on the molecular state of the other component. In order to find out how farreaching conclusions can be drawn in this way, Högfeldt [9,10] has recently made a theoretical study of the activity and activity factor curves of binary mixtures A—B under various simple assumptions: formation of compounds A_uB_v, of polymer complexes $(A_uB_v)_n$, association of only A to A_n or of only B to B_n etc.

Högfeldt has assumed that the solutions are ideal as did Fajans and Chu in their rough calculations of "apparent molecular weight". Then all deviations in the activity factors from 1 are caused by the formation of associated groups of like molecules such as A_n or B_n, or of complexes of unlike molecules such as A_uB_v.

Table 4. Viscosity η (cP) of $LiClO_4$-ether solutions at 20° C and 25° C.

x_2	η_{20}	x_2	η_{20}	x_2	η_{25}	x_2	η_{25}
0.013	0.230	0.232	1.49	0.026	0.220	0.328	11.7
0.015	0.234	0.244	1.95	0.056	0.257	0.345	18.2
0.026	0.242	0.275	3.54	0.104	0.345	0.373	50.1
0.036	0.272	0.285	4.74	0.185	0.815	0.382	63.5
0.066	0.288	0.301	7.02	0.232	1.33	0.417	180
0.075	0.330	0.324	16.3	0.248	2.16		
0.104	0.394	0.349	24.8	0.265	2.51		
0.132	0.475	0.390	91	0.275	3.25		
0.176	0.70	0.417	236	0.285	4.44		
0.210	1.13			0.290	4.87		

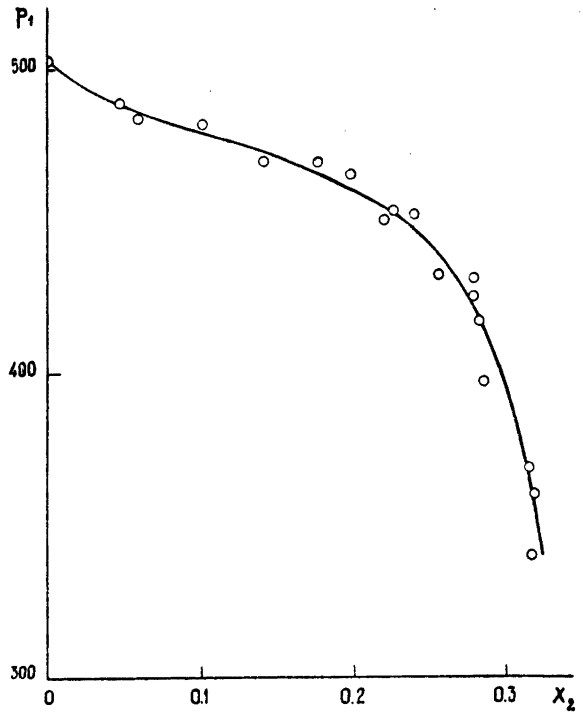

Fig. 4. Vapor pressure p_1 *(in torr) at 23.5° C and various* x_2.

One of Högfeldt's results is that the activity factor curves can be used for differentiating between the formation of A_uB_v ($(A_uB_v)_n$) on one hand and the formation of A_u or B_v on the other. If only associates of like molecules are formed, the activity factor curves will continually increase or decrease, and show no maxima or minima. Now, for the system diethyl ether (1)-lithium perchlorate (2), the activity factor of ether, $f_1 = p_1(x_1p_1^\circ)^{-1}$ goes from 1 to a maximum at about $x_2 = 0.25$, and then decreases, passing the value 1 at x_2 = about 0.33 (Fig. 5). The existence of the maximum seems to rule out explanations based on only $LiClO_4$-$LiClO_4$ or ether-ether associates.

We conclude that *lithium perchlorate - ether complexes* must also be formed. The simple assumption, that only one complex namely $(ether)_u(LiClO_4)_v$ is formed, was first tried. Then, according to Högfeldt,

$$x_{2(f_1=1)} = \frac{v-1}{u+v-1} \qquad x_{2(f_1=\max)} = \frac{1}{u+v}\left[v - \sqrt{\frac{uv}{u+v-1}}\right]$$

We made a diagram (Fig. 6) of v versus u, treating them as continuous variables, although of course they have a meaning only for integer values. In this diagram were drawn lines (broken) connecting points that would make $f_1 = 1$ at x_2

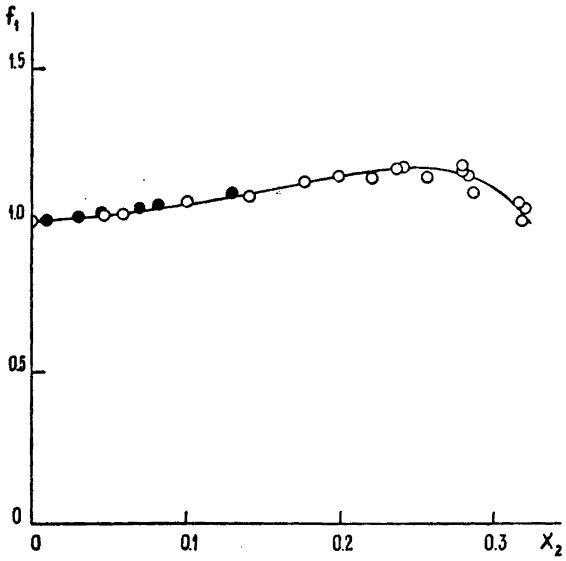

Fig. 5. Activity factor f_1 *of ether at various* x_2*: Circles* ○*:* f_1 *calculated from vapor pressure* p_1 *(present work); dots* ●*:* f_1 *calculated from the boiling point data of Chu and Fajans*[8].

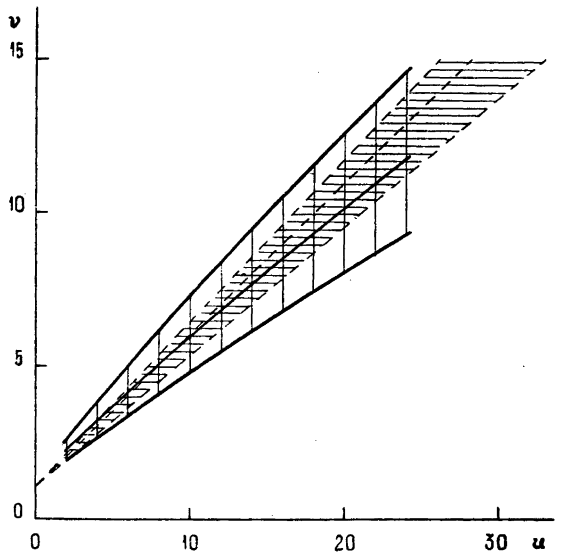

Fig. 6. Diagram for determining u *and* v*, assuming the complex formed to be exclusively* $(ether)_u(LiClO_4)_v$ *(see text).*

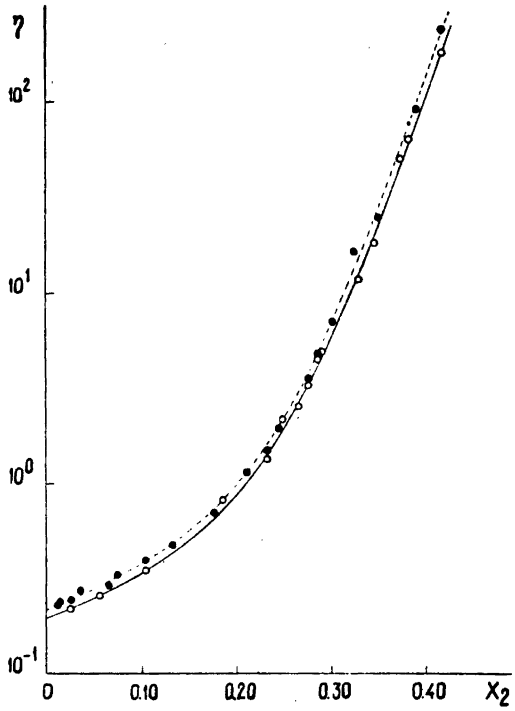

Fig. 7. Viscosity η in cP (logarithmic scale) at 25° C (circles) and 20° C (dots) at various x_2.

= 0.30, 0.333, and 0.35, which was the widest range we thought possible. Curves were also constructed for the points that would give f_1 a maximum at 0.20, 0.25, or 0.30 (full drawn). If only one complex $(\text{ether})_u(\text{LiClO}_4)_v$ is formed, its (u, v) should be in the area where the two bundles overlap (shaded in Fig. 6). Since the angle between the bundles of curves is small, the overlapping area is rather extended. Even if one might pick out some composition such as $(\text{ether})_{10}(\text{LiClO}_4)_6$, and work it into a geometrical model, one might object that it is neither proved, nor very likely that only this complex is formed and no other.

If there be a mechanism which can form a complex out of a certain number of ether molecules, Li^+ ions, and ClO_4^- ions, it seems likely that the same mechanism can build up still larger complexes, so that an infinite series of complexes are formed, perhaps of approximate formula $(A_uB_v)_n$.

Högfeldt has treated this general case too but, unfortunately, the calculations have so far given no explicit equation for calculating u and v from the $f_1(x_2)$ curve. However, it can be concluded that u is in this case greater than v and probably somewhat smaller than 2 v.

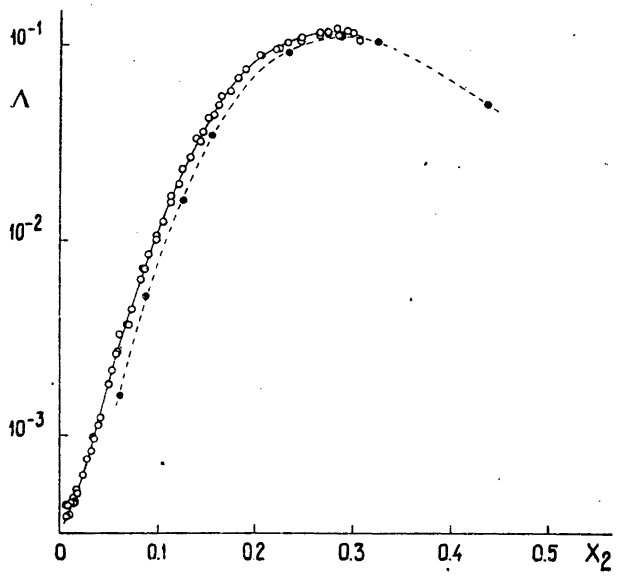

Fig. 8. Molar conductance Λ (logarithmic scale) of $LiClO_4$ at 23.5° C (circles, present work) and 25° C (dots, Chu [7]).

We thus conclude that ether-$LiClO_4$ complexes are formed, probably an infinite series of them, and that the ratio ether/$LiClO_4$ in the complexes is certainly greater than 1 and probably somewhat less than 2.

Conductivity and viscosity. Fig. 8 gives, for varying x_2, log Λ where Λ is the molar conductance of $LiClO_4$ at 25° C (circles, Chu [7]) and at 23.5° C (our data). The agreement is as good as can be expected; the temperature coefficient is negative, as has been observed in stray experiments by both Chu and ourselves.

It may seem surprising to some that Λ increases by a factor of several hundred with increasing concentration; for aqueous solutions a decrease by a few tenths of the value is the rule. However, as pointed out by Kraus [16], the increase of Λ with c (sometimes to a maximum) is the rule for electrolytes in solvents of low dielectric constant. A factor of increase as large as 10^4 has been observed. According to Kraus, no explanation of this behavior had been given in 1949; as for the views of Strong and Kraus (1950) [17], see below.

The increase in the molar conductance is still more surprising when one observes that for our solutions the viscosity, too, increases by several powers of ten; the most concentrated $LiClO_4$-ether solutions are as viscous as syrup.

One may well ask what the mechanism of conduction is. The ether-$LiClO_4$ complexes may well be charged, some containing excess of Li^+ and others excess of ClO_4^-. However, it seems extremely unlikely that electrical current is transported only by the movement of whole complexes. With increasing concentration, the contribution to the conductance from this mechanism must decrease since the complexes get bulkier and the solution more viscous.

It seems to us that the easier transport of current at high concentrations can only be explained by the *transport of single ions within the framework of the complexes*. This mechanism, of course, is favored by increased concentration; the complexes get larger and come closer together, and at the highest concentrations the complexes may have grown together throughout the solution, almost to a lattice, so that there are paths along which ions may move from one end to the other.

Fig. 9 gives a very schematic picture of the growth of the complexes with concentration. Of course, the figure is not intended to describe the geometrical arrangement of Li^+, ClO_4^-, and $(C_2H_5)_2O$ molecules in the complexes. In very dilute solutions, Li^+ may have as many as $4(C_2H_5)_2O$ molecules as closest neighbours, completely surrounding it. At the highest concentrations and especially in the complexes with less than $2(C_2H_5)_2O$ per $Li^+ClO_4^-$, the ether molecules can hardly suffice for shielding the ions from each other.

It seems likely that one kind of ion contributes more than the other to the conductivity, and one might imagine the small Li^+ jumping in the lattice of the ether and ClO_4^-. However, this is only a guess.

The mechanism proposed here resembles somewhat the "proton-jump" mechanism responsible for the high ionic conductance of H^+ in aqueous solutions according to Bernal and Fowler [18].

The negative T factor of Λ may be due to the breakdown of the complexes with increasing temperature.

Two liquid phases. Transition to fused salt. In some systems the attraction salt-solvent may cause the complexes to separate as a new liquid phase. For instance, in the system ether-$MgBr_2$ two separate liquid layers are formed, one of ether poor in $MgBr_2$, and one of composition around $MgBr_2(ether)_{2-4}$ (Doering and Noller [19]).

For some salt-solvent pairs the salt concentration may be raised beyond the point where the complexes coalesce and one may even have a gradual transition from concentrated solution to fused salt. It would be interesting to know whether in this concentration range the solvent molecules and ions are distributed completely at random in the liquid or whether there are solvent-rich and solvent-poor domains in this range too (cf Fig. 9 b and c).

Strong and Kraus [17] have suggested that concentrated solutions of salts in media of low dielectric constant (*e.g.* benzene) are best regarded as the fused salt, diluted with so much solvent. Whereas their general view is related to ours, we should like to object to some details of the mechanism proposed.

Strong and Kraus assume that the ions aggregate to ion pairs and larger complexes in an intermediate concentration range but are free at very low and very high concentrations. The main support for this assumption seems to be the "association numbers" which Kraus and coworkers have calculated from the freezing points of solutions of salts in benzene, assuming that the main process in the solution is the formation of $(salt)_n$ associates. These "association numbers" at first increase with increasing concentration, as could be expected. For salt of sufficient solubility, however, the "association number" goes through a maximum and then decreases with increasing salt concentration. This behavior seems hard to understand.

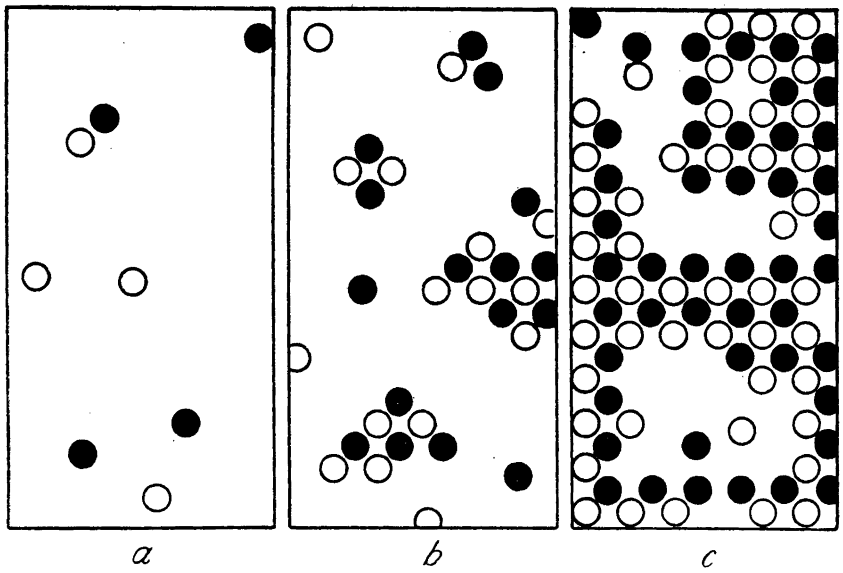

Fig. 9. Schematic picture of the proposed behavior of ions in $LiClO_4$-ether solutions of varying concentrations. The ions of opposite charges are pictured as circles and dots. The ether molecules, which are not shown here, should be figured as filling up the "empty" space between the ions and complexes and being intermixed with the ions in the complexes. a) In very dilute solutions, the ions occur separately or occasionally in pairs. b) In solutions of intermediate concentration, the ions form separate complexes of varying size. c) In solutions of high concentration, the complexes come closer together almost to a coherent lattice though the orderliness of the array is certainly less than drawn in the figure.

Let us, however, assume that solvent-salt complexes are formed — say, $(\text{solvent})_u$ $(\text{salt})_v$ and that "association numbers" are calculated with the incorrect assumption of solvent-free complexes. This "association number" will then be lower than the true average v, and the deviation will increase with increasing salt concentration (assuming ideal laws, as Kraus has done). One can see already intuitively that a maximum "association number" might arise by the combined effect of an increase in the true association number v and an increasing negative error. The question will be treated more fully by Högfeldt [10].

SUMMARY

$LiClO_4$ (2) is very soluble in ether (1). The vapor pressure, conductance and viscosity of $LiClO_4$-ether solutions have been studied at 23.5° C and for molar fractions x_2 up to about 0.35. The data are compared with densities and conductances determined by Johnson [6] and Chu [7,8].

The vapor pressure curves cannot be explained only by the formation of $(LiClO_4)_n$ associates; there must also be $LiClO_4$-ether complexes. Probably these complexes can form clusters of varying size with an average ether/$LiClO_4$ ratio certainly greater than 1 and probably somewhat smaller than 2.

With increasing concentration, both the molecular conductance and the viscosity increase by several powers of ten. It is concluded that the main mechanism of conduction must be the *transport of single ions within the complexes* and not the movement of the clusters as whole units.

The same explanation may hold true for other electrolyte solutions in media of low dielectric constant.

We wish to thank Miss Christina Mannerskantz, M.Sc., for carrying out the measurements, given in Table 4 and Figures 3b and 7; Dr. Erik Högfeldt for valuable discussions on the laws for compound formation; Lars Evers, fil.kand., and Berner Ålin, fil.kand., for valuable discussions and experimental aid. We are indebted to Professor Arne Ölander and Professor Paul Nylén for providing us with laboratory facilities.

Dr. Jean Chu, Professor Kasimir Fajans, and Dr. Oliver Johnson were kind enough to allow us to record some of their data. We have also had pleasant discussions with them.

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The Preparation of 8,11-Heptadecadienoic (Nor-Linoleic) Acid

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For metabolic studies concerning the specificity of essential fatty acids it was deemed of interest to prepare a close structural analogue of linoleic acid, *viz.*, one containing the same *cis-cis*-1,4-dienoic system but with one carbon atom less in the polymethylene chain carrying the carboxyl group. The sequence of reactions used in this synthesis is shown schematically I—VI.

Pure linoleic acid tetrabromide [1] I was used as starting material. Its silver salt (II) was treated with bromine and the resulting primary-tetrasecondary pentabromide (III) selectively solvolysed to form tetrabromoheptadecanol (IV). This primary alcohol was oxidised to the tetrabromo carboxylic acid (V). This acid after esterification and treatment with zinc gave the final product, methyl nor-linoleate (VI).

The scheme adopted and the procedures employed were not straightforward and require comment. The common alkali metal salts of acid (I) are insoluble in water so that the silver salt (II) could not be prepared by the usual technique [2].

$C_5H_{11}(CHBr)_2CH_2(CHBr)_2(CH_2)_7COOH$ (I)
$C_5H_{11}(CHBr)_2CH_2(CHBr)_2(CH_2)_7COOAg$ (II)
$C_5H_{11}(CHBr)_2CH_2(CHBr)_2(CH_2)_7Br$ (III)
$C_5H_{11}(CHBr)_2CH_2(CHBr)_2(CH_2)_7OH$ (IV)
$C_5H_{11}(CHBr)_2CH_2(CHBr)_2(CH_2)_6COOH$ (V)
$C_5H_{11}CH = CHCH_2CH = CH(CH_2)_6COOCH_3$ (VI)

A satisfactory procedure using the triethanolamine salt is described in the experimental part. A large number of individual silver salt-bromine decarboxylations have been carried out. The early experiments were run in carbon tetrachloride in the standard fashion [2] with yields ranging from 18 to 59 % of pure neutral pentabromide. Later on the procedure developed by Rottenberg for degrading steroid carboxylic acids [3] was applied which gave a substantial increase in yield (up to 90 %). One such run is described.

The partial debromination procedure as described by Silberman [4] appeared to be a promising means for converting the primary bromide (III) into a primary alcohol *via* the intermediate di-unsaturated bromide. On our substance,

however, this heterogeneous reaction gave very inconsistent results, and only in one experiment could a product be obtained which gave correct analytical values for halogen and unsaturation. Since this result could not be duplicated on subsequent attempts the method was abondoned. We next turned our attention to partial hydrolysis and solvolysis of the pentabromide. The polyhalide was treated with the solvolytic agent for different periods of time, the progress of the reaction was checked by mercurimetric titration of the liberated halide ion, and on appearance of one equivalent the reaction was stopped. The product was analysed for bromine and unsaturation. Stirring or shaking with potassium carbonate in methanol gave unchanged pentabromide. Potassium acetate in either ethanol or acetic acid, or silver acetate in acetic acid gave products showing considerable unsaturation after a one-equivalent stage of solvolysis. Sodium iodide in acetone turned out to be a highly selective agent which smoothly converted the pentabromide into an analytically pure tetrabromo iodide. No appreciable improvement in specificity could be achieved, however, when the solvolysis experiments were run on the iodide. It can be concluded that the solvolytic systems commonly used for transforming bromides into alcohols are too "basic", so that solvolysis is always accompanied by concurrent dehydrobromination. It was obvious therefore to try an alcohol-soluble salt of a very strong carboxylic acid as the solvolytic agent. The sodium or potassium salts of trifluoroacetic acid were indeed found to be the desired reagents. They are soluble in acetone or ethanol and turned out to be strictly selective toward the primary bromide group whereas the disecondary, vicinal dihalide groupings remained unattacked even after extended reaction periods. A disadvantage is that the replacement reaction is rather slow. Thus it was found by mercurimetric titration that it took 8 days boiling with sodium trifluoroacetate in ethanol to bring about a 90 % conversion. The titration values are shown in Table 1. No attempt was made to isolate the intermediate trifluoroacetic esters. They are little known as yet and stated to be extremely susceptible to alcoholysis [5]. Accordingly the tetrabromo alcohol (IV) was obtained directly on short after-treatment with methanolic HCl. The yields of material giving correct bromine analyses were almost quantitative but the amounts of crystallisable material ultimately obtained were much smaller. This point requires further investigation.

Table 1.

Time, hours	% Br^- liberated
0.6	2.0
1.5	2.5
4.3	4.0
22	15.0
46	28.7
124	58.7
166	82.5
190	92.7

The oxidation of the tetrabromo alcohol (IV) to the acid (V) had to be carried out under non-alkaline conditions. The excellent method developed by the Curtis, Heilbron *et al.* [8] for oxidising higher molecular primary alcohols in acetone-water-H_2SO_4 was not published at the time this problem was worked on. Among the methods then known the procedure described by Crombie and Harper [6] (oxidation with acid permanganate), which appeared most promising, gave only 46 % yield. We therefore approached the problem from the analytical side with the method that we have used earlier for the quantitative analytical oxidation of alcohols with chromium trioxide [9,7]. We then found that with chromium trioxide in 95 % acetic acid at 80° the tetrabromo acid was obtained directly from the alcohol in 97 % yield. The method described therefore compares favourably with that of Curtis and Heilbron [8]. The tetrabromo acid (V) was smoothly converted to methyl nor-linoleate IV by standard procedures, *cf.*[4] (VI) was obtained as a colourless oil, b.p. 139° at 0.7 mm Hg. The purity of (VI) was checked by comparison with the purest available specimen of authentic methyl linoleate (see Table 2), and by infrared spectroscopy.

Table 2.

	Methyl linoleate	Methyl nor-linoleate
B. p.	148°/0.5	139°/0.7
Unsat.	1.78	1.79
ε 231 mμ	34	516
% Conjug. diene *	0.1	1.67

* Calc. on the basis of $\varepsilon = 3.1 \times 10^4$ (J.F. Mead, *Science* **115** (1952) 471),

Dr. D. H. Wheeler, of General Mills, Inc., Minneapolis, Minn., U.S.A., has kindly co-operated with us by running infrared analysis on this material. He comments on the results of these two samples as follows: "Both seem to have about the same amount of *trans* double bond as is found in debromination linoleate. The band at 3.30 μ is very close in intensity to that of ordinary or debromination linoleate and falls in line with the expected intensity of the series oleate, linoleate, linolenate."

EXPERIMENTAL

All m.p.'s are corrected. Microanalysis by the Microanalytical Laboratory, Department of Medical Chemistry, Uppsala.

Silver salt. 24 g (= 40 millimoles) of linoleic acid tetrabromide (I) m.p. 114—115°, was dissolved in 100 ml of acetone, 40 ml of 1.0 *N* triethanolamine in water added followed by 3 liters of boiling water whereupon part of the acetone escaped. At *ca.* 90° there was then added, in small portions, 40 ml of 1.0 *N* $AgNO_3$ with swirling. The mixture was filtered hot and the white precipitate washed with 10 liters of boiling water. After drying, finally over P_2O_5, the yield was 25.2 g (89 %). Found: AgBr 24.5, 26.0 %. $C_{18}H_{31}O_2Br_4Ag$: AgBr 26.6 %.

1.8.9.11.12-pentabromo heptadecane (III) (silver salt bromine decarboxylation). 53 g of silver tetrabromostearate (75 millimoles) was combined with 12 g of silver acetate (72 millimoles); the mixture was finely ground and rigorously dried. After addition of 250 ml of pure, dry ethyl bromide it was treated with 7.8 ml (147 millimoles) of dry bromide at the b.p. of the solvent (38°), the operation taking ten minutes. The mixture was boiled for a further 90 min., then filtered and washed as described earlier [3]. The solution containing the total neutral plus acidic material was taken to dryness, the residue was taken up in petrol ether and filtered through a column of Al_2O_3 (200 g). Extraction of the column with petrol ether gave, after drying, 42.7 g (90 % yield) of white, crystalline neutral product that contained 62.9 % Br (calc. 62.93 %). A specimen was recrystallised from acetone-ethanol, m.p. 66—67°. Found: C 32.02, 32.01; H 5.01, 4.83; Br 63.50, 62.22. Calc. for $C_{17}H_{31}Br_5$ (635.00): C 32.15; H 4.92; Br 62.93.

Tetrabromo heptadecyl iodide. 3.85 g of pentabromoheptadecane (6.06 millimoles) in 10 ml of acetone was mixed with a solution of 1.1 g of sodium iodide in 10 ml of acetone. After 24 hours a crystalline precipitate had formed which after washing with acetone weighed 530 mg and on titration was equivalent to 52.1 ml of 0.1 *N* $Hg(NO_3)_2$. The orange-coloured filtrate was taken to dryness *in vacuo*, the residue in ether was washed with SO_2-water, $KHCO_3$, and water, dried over sodium sulphate, filtered, and the solvent removed. After drying, the residue weighed 4.05 g (= 98 % yield). A specimen after recrystallisation from petroleum ether had m.p. 57—60°. Found: C 29.58; H 4.49; Br 47.05, 47.08. Calc. for $C_{17}H_{31}Br_4$ I (682.00): C 29.94; H 4.58; Br 46.87 %.

Tetrabromoheptadecanol IV from pentabromide III.

Early experiment. 556 mg of pentabromide in 15 ml of acetone was added to a solution of sodium, potassium trifluoroacetates [8] in 5 ml of acetone and left in a glass-stoppered flask in the dark. After 22 months at room temperature a crust of colourless, cubic crystals had deposited which were soluble in water and gave a copious precipitate with acidified $AgNO_3$ solution. The acetone solution was concentrated, the residue which was sparingly soluble in petroleum ether was taken up in methylene chloride, washed several times with water, dried over Na_2SO_4, and filtered, yielding 0.55 g of waxy crystals. Crude product, found: Br 55.0; 54.7. Calc. for $C_{16}H_{29}Br_4CH_2OCOCF_3$: Br 46.7; for $C_{16}H_{29}Br_4CH_2OH$: Br 55.9 %.

Treatment with CH_3OH-HCl did not change the bromine content.

Preparative procedure. 1.725 g of pentabromide and 0.92 g of sodium potassium trifluoroacetate were dissolved in 99 per cent ethanol and made up to a total volume of 375 ml. The mixture was refluxed on a boiling water-bath. 5 ml aliquots were withdrawn at intervals listed in Table 1, and titrated for bromide ion using mercuric nitrate and diphenylcarbazide [10]. After 190 hours the titration value corresponded to 93 % conversion of primary halide. The alcohol solution was concentrated *in vacuo*, taken into ether, thoroughly washed with $KHCO_3$ and water, dried over Na_2SO_4 and the solvent distilled off. Drying *in vacuo* over KOH and paraffin gave 0.97 g of colourless crystalline wax, m.p. 76—78° after two recrystallisations from ethanol-petroleum ether. Found: C 35.9, 36.2; H 5.78, 5.59; Br 55.92. Calc. for $C_{17}H_{32}OBr_4$ (572.09): C 35.69; H 5.64; Br 55.87.

The following procedure has now been adopted: 6.2 g of pentabromide and 5.1 g of pure sodium trifluoroacetate were boiled under reflux with 10 ml of bromobenzene and 40 ml of 99 per cent ethanol during 12 days. 1 ml of methanol-HCl (ca. 8 *N*) was added and the mixture boiled for a further 4 hours. Working up gave 5.8 g of greasy colourless crystals. Recrystallisation from ether-ligroin furnished 2.2 g of needles, m.p. 83—85°. The highest observed melting point was 88°.

Tetrabromoheptadecanoic acid.

A. *Micro-quantitative oxidations.* Analytical oxidations were run exactly as described earlier [7]. The best and most clearcut results were obtained when the temperature was about 80° and the acetic acid concentration 95 % (Fig. 1). At lower temperatures and higher acid concentrations the oxidations usually stopped at an earlier stage.

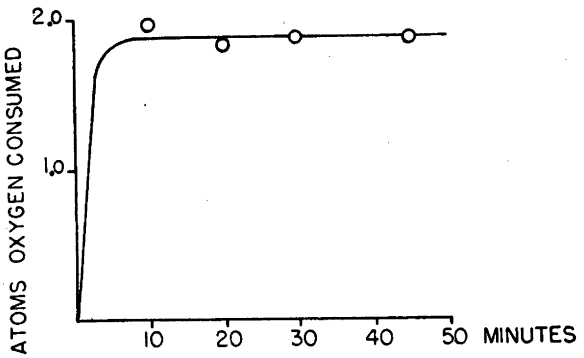

Fig. 1. Each point represents a sample of 1.543 mg tetrabromoheptadecanol dissolved in 1 ml of glacial acetic acid to which 0.794 mg of chromium trioxide in 0.1 ml of 95 per cent acetic acid had been added. Temperature 80°.

B. *Preparative run.* 3.2 g (5.6 millimoles) of tetrabromoheptadecanol in 342 ml of glacial acetic acid + 18 ml of water was placed in a water-bath maintained at 80—90°. At an inside temperature of 80—83° a solution of 2 g of chromium trioxide in 5 ml of water and 95 ml of acetic acid were added during five minutes and the mixture kept at 80—83°. After one hour 10 ml of methanol was added, followed by 500 ml of water and taken to dryness *in vacuo* in a water-bath at about 55°. The green residue was taken up in chloroform, the chloroform solution washed with HCl, water, NaH_2PO_4, and water, dried over Na_2SO_4, and filtered. Evaporation gave 3.34 g of slightly greenish crystalline wax, m.p. 101—102°, neut. eq. calc. 586; found 615, 606. One recrystallisation from ligroin gave 2.98 g of material, m.p. 99°, neut. eq. 601, 596, 599. The analytical specimen was obtained after two recrystallisations m.p. 105—106° (long needles). Found: C 35.09; H 5.09; Br 54.58, 54.74; neut.eq. 585. Calc. for $C_{17}H_{30}Br_4O_2$: C 34.84; H 5.16; Br 54.54; neut. eq. 586.08.

Methyl nor-linoleate VI. 1.5 g of tetrabromoheptanoic acid, m.p. 100—102° in 20 ml of methylene chloride was treated with ethereal diazomethane. Evaporation gave waxy crystals, m.p. 44—46° after one recrystallisation from acetone. 4 g of powdered zinc and 10 ml of 99 per cent ethanol were boiled in an atmosphere of CO_2. 0.15 ml of 48 % HBr were added and the mixture refluxed for 4 minutes. 1.4 g of tetrabromo ester in 5 ml of benzene and 20 ml of ethanol was added to the boiling mixture which was refluxed for one hour more. The mixture was filtered through "super-cel" and the filter extracted with petroleum ether. The combined filtrates were washed with HCl, $KHCO_3$, and water, dried over Na_2SO_4, and filtered. The solution was concentrated under CO_2 and distilled. Main fraction, 282 mg, b.p. 139° at 0.7 mm Hg. Unsat. calc. 2.00; found 1.79. (Best available linoleate 1.78).

The infra-red curve shows a band at 3.30 μ very close in intensity to that of linoleate. In the UV a weak absorption maximum (ε = 516) is observed at 231 mμ, corresponding to about 1.3 % of conjugated diene.

SUMMARY

Linoleic acid tetrabromide has been converted into methyl nor-linoleate by the following sequence of reactions: (a) silver salt-bromine decarboxylation, (b) selective solvolysis with trifluoroacetate of the primary bromine atom to tetrabromoheptadecanol, (c) chromic acid oxidation of this alcohol to the carboxylic acid in practically quantitative yield followed by debromination of the methyl ester.

We are most grateful to Dr. Wheeler, General Mills Inc., Minneapolis, and to Dr. W. Lundberg, Hormel Institute, Austin, Minn., U.S.A., for the help with the IR analysis. We are also indebted to Professor Maurice Stacey, F.R.S., Birmingham, for a gift of trifluoroacetate.

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Serum Protein Fractionation by Means of Ammonium Sulfate in the Presence of Phenolic Compounds

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Harms [1] described in 1946 a procedure, in which the fractional precipitation of horse serum proteins by means of ammonium sulfate was modified through the addition of phenolic compounds. The presence of 2 % phenol or 1 % tricresol caused a reversal of the order of precipitation of the different proteins, the albumin being precipitated first, thus making it possible to prepare a solution containing mainly β- and γ-globulins. The value of this procedure for the purification of antibodies was especially emphasized.

As this procedure might be more generally adaptable, it has now been studied by electrophoresis. Besides giving a more complete picture of the distribution of the different components, this method might disclose, whether the addition of the phenols caused an irreversible denaturation of the proteins, especially of the albumin, or whether it would be possible to recover the proteins unchanged.

Two kinds of horse serum were used. The first sample was prepared from fresh spontaneously coagulated blood and stored at −20° C, the other was a heat sterilized sample obtained from the State Serum Institute. The heat sterilization, which is generally applied to immune sera, is accompanied by profound changes in the electrophoretic pattern [2].

The experiments were performed as follows:

To a serum sample was added an equal volume of 4 per cent phenol or 2 % tricresol in water. This step as well as all the following were carried out in the cold room at 0—5° C. To the resulting only very slightly turbid solutions was added saturated ammonium sulfate and the amounts necessary to cause a suitable precipitation were estimated (Table 1). They may of cause be varied according to the special purpose of the fractionation. As already stated by Harms, much less ammonium sulfate is needed for this precipitation than in the absence of phenols. This is more marked in the case of the heat treated serum, indicating an increased lability caused by the heat sterilization. The samples were centrifuged, and the precipitates suspended in 0.9 % NaCl. These suspensions as well as the supernatants were dialyzed against phosphate-NaCl-buffer ($\mu_{\text{phosphate}}$ (ionic strength) = 0.075, μ_{NaCl} = 0.025, pH = 7.7)

Table 1.

Precipitant	Final $(NH_4)_2SO_4$ concentration in per cent saturation		Composition of sample in percentage		
Spontaneously coagulated serum:			Alb.	α-glob. (C-component)	$\beta+\gamma$-glob.
Control			53	8	39
2 % phenol	16	precipitate:	62	18	20
		supernatant:	10	16	74
1 % tricresol	20	precipitate:	69	8	23
		supernatant:	18	18	64
Heat treated serum:					
Control			24	39	37
2 % phenol	9	precipitate:	28	56	16
		supernatant:	9	17	74
1 % tricresol	14	precipitate:	25	61	14
		supernatant:	14	15	71

Distribution of the various proteins in the serum samples and in the fractions obtained by precipitation with ammonium sulfate in the presence of phenol and tricresol. Percentage calculated as the average from the ascending and descending pattern.

and studied by electrophoresis in our Tiselius apparatus [3]. Representative results are shown in Fig. 1 and Table 1.

It appears that the albumin is precipitated first, and that the β- and γ-globulins remain in solution in accordance with the findings of Harms. In the concentrations used, the α-globulin and the C-component of the heat treated serum appear in about equal amounts in both fractions. It is remarkable, that all components of the precipitates and supernatants showed completely unchanged mobilities as compared with the untreated samples. This indicates that the change in solubility of the albumin is not due to a simple denaturation. Probably the phenolic compounds form labile complexes with the proteins, the properties of which may be quite different from those of the free proteins. This behaviour is similar to that observed by Astrup and Birch-Andersen [4] in their experiments on protein fractionation by means of specific anions. The method may therefore probably find further applications than that suggested by Harms.

This investigation was aided by a grant to Dr. Tage Astrup from "*Teknisk-kemisk Fond*", Copenhagen.

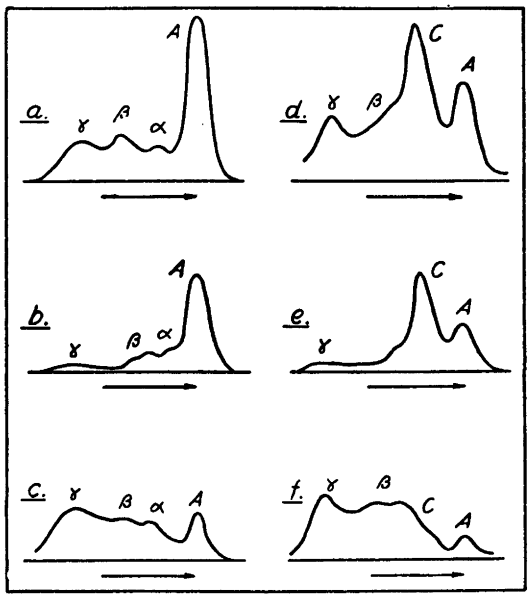

Fig. 1. Electrophoretic diagrams of the serum samples used and of some of the fractions (descending pattern).

a. Spontaneously coagulated serum.
b. Precipitate, and c. supernatant from precipitation of a. with 1 % tricresol and ammonium sulfate.
d. Heat treated serum.
e. Precipitate, and f. supernatant from precipitation of d. with 2 % phenol and ammonium sulfate.

SUMMARY

The precipitates obtained from horse serum by means of ammonium sulfate in the presence of phenol or tricresol were found by electrophoresis to contain mainly albumin and α-globulin. None of the components showed any sign of denaturation.

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Short Communications

An Inverted Osmometer and Osmotic Pressure of Some Specimens of Potassium Hyaluronate

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For the determination of the molecular weight of expensive substances the microosmometer formerly described by us [1] proved to be too clumsy.

We have therefore constructed an osmometer based on a new but quite obvious principle: The testtube-shaped membrane is placed on the upper end of a capillary which contains an air column, confined at the upper end by the inner liquid and at the lower end by the outer liquid. This "inverted osmometer" is completely immersed in the outer liquid. The resulting hydrostatic pressure-difference thus counteracts the osmotic pressure and moreover the capillary forces are very nearly compensated for. To determine the equilibrium pressure-difference, the total pressure on the whole system can be varied from just above one atmosphere to about a tenth of that amount. This causes a change in height of the air column and at each height the velocity with which the upper meniscus of the column moves up or down is determined. From a plot of heights versus velocities the equilibrium pressure is found graphically, compare [2].

The hydrostatic pressure-difference is of course nearly proportional to the height times the density of the outer solution, but there are two corrections: One for the (small) difference in density between outer and inner liquid and another for the (small) difference in surface tension of the two liquids. In the experiments reported below neither of these corrections have been applied.

As an example of the applicability of the method we quote in Table 1 the results of measurements on various specimens of potassium hyaluronate, the same which were described in a recent paper by one of us [3]. In the table the preparations are mentioned in the same order as that in which they occur in Table 1, p. 606—607 of the paper mentioned.

Table 1. Molecular weights of specimens of potassium hyaluronate. Temperature 20°. Outer liquid 0.2 M *solution of KCl. Density: 1.0078. Inner liquids about 10 mg dissolved in 1 ml of the outer liquid. Weighings accurate to about 0.01 mg. No buffer added. Estimated accuracy: Better than 5 %.*

% N	Rel. viscosity conc. 1g/l	Molecular weight times 10^{-3}
3.36	11.80	517
3.35	11.91	530
3.31	12.19	516
3.34	12.12	527
2.90	5.46	367
2.88	68.46	1 750
2.82	5.29	—
1.16	5.75	316
5.78	(4.62)	—
2.99	76.39	554

A more detailed report will be published later.

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*iso*Thiocyanates V. The Occurrence of *iso*Propyl *iso*Thiocyanate in Seeds and Fresh Plants of Various *Cruciferæ*

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We wish to report the occurrence of *iso*propyl *iso*thiocyanate in plants belonging to the *Cruciferæ*. To our knowledge this volatile mustard oil has not hitherto been reported as a constituent of any crucifer. The only previous indication of the natural occurrence of *iso*propyl *iso*thiocyanate is found in the paper by Puntambekar [1], who isolated a mixture of phenyl-, (+)-*sec*-butyl- and *iso*propyl *iso*thiocyanate from seed kernels of the Indian plant *Putranjiva roxburghii* Wall., belonging to the family *Euphorbiaceæ*.

During systematic studies of the volatile *iso*thiocyanates in seeds of numerous crucifers by a paper chromatographic technique [2], the appearance of spots with an R_{Ph}-value (*cf.* Ref. 2) of 0.41 was often noticed. Comparison with synthetic model thioureas showed that these spots were attributable to either *n*-propyl- or *iso*propyl-thiourea. Distillates of enzymatically cleaved glucosides from seeds of *Lunaria biennis* Mnch. yielded a single spot only, with an R_{Ph}-value of 0.40. Therefore, this plant material was selected for isolation of the *iso*thiocyanate.

The thoroughly ground seeds (97 g) were treated with a hot mixture of light petroleum and ethanol in order to denature the myrosinase, which otherwise acts on the glucoside during the following continuous and exhaustive extraction of residual fatty material with petroleum ether. The fat-free powder (71 g) was suspended in water (750 ml) and treated for 14 hours at room temperature with a cell-free myrosinase-preparation [3] (10 ml). At the end of this treatment an intensive odour of mustard oil was noticed. The reaction mixture was steam-distilled and the distillate collected in aqueous ammonia. After standing overnight at room temperature, the transformation into thiourea was virtually complete and the solution was concentrated to dryness *in vacuo*. The colourless, crystalline residue (370 mg) was recrystallised from water, m.p. 169—170°, alone or in admixture with a specimen of authentic N-*iso*propylthiourea. The identity was further confirmed by analysis (Calc. for $C_4H_{10}N_2S$: C 40.64; H 8.53; N 23.70. Found: C 40.64; H 8.54; N 23.67) and by infrared spectroscopy (Fig. 1).

So far, paper chromatographic studies have indicated the presence of *iso*propyl *iso*thiocyanate also in the seeds of *Cochlearia officinalis* L. and *Cochl. danica* L. In both, the simultaneous presence of *sec*-butyl *iso*thiocyanate was established. In seeds of *Matthiola annua*, a small amount of *iso*propyl *iso*thiocyanate was present,

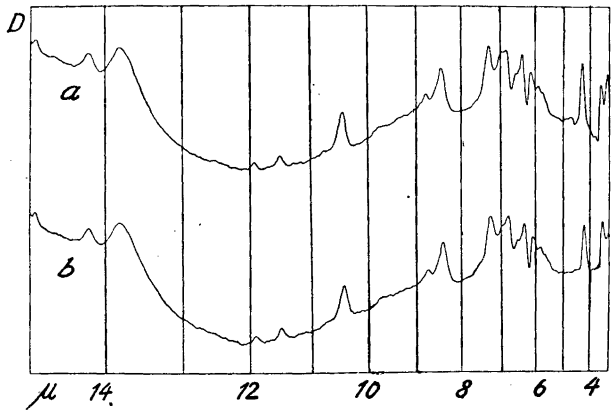

Fig. 1. Infrared spectra in Nujol mulls, showing the identity of N-isopropylthiourea from a natural source with a synthetic specimen. a. Synthetic N-isopropylthiourea. b. Thiourea derived from Lunaria biennis Mnch.

accompanied by a second mustard oil, presumably methyl *iso*thiocyanate. The latter combination was present also in seeds of *Cheiranthus cheiri* L.

Seeds of *Tropaeolum peregrinum (canariense)*, belonging to the family *Tropaeolaceæ*, were found to contain *sec*-butyl *iso*thiocyanate in addition to *iso*propyl *iso*thiocyanate.

That the occurrence of the new *iso*thiocyanate is not confined to seeds was demonstrated by the following example. Fresh plants (leaves, stems and flowers) of *Cochlearia anglica* (L.) Asch. & Grb. were homogenised in a Waring blendor and steam-distilled. Following the procedure above, analytically pure N-*iso*propylthiourea was isolated in amounts of 55 mg from 500 g of fresh plant material containing 91.5 % of water.

Further results of these studies will appear in forthcoming papers in this journal.

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1. Puntambekar, S. V. *Proc. Indian Acad. Sci.* **32 A** (1950) 114.
2. Kjær, A., and Rubinstein, K. *Acta Chem. Scand.* **7** (1953) 528.
3. Neuberg, C., and Wagner, J. *Biochem. Z.* **174** (1926) 457.

Received June 19, 1953.

Die Abwesenheit eines Austausches von Schwefelatomen zwischen Dithionit und Thiosulfat sowie zwischen Sulfat und Tetrathionat

HEINZ ELKELES

Institut für anorganische und physikalische Chemie der Universität, Stockholm, Schweden

Der Austausch von S^{35} zwischen *Dithionit* (Hyposulfit, $S_2O_4^{2-}$) und *Thiosulfat* ($S^*SO_3^{2-}$), welches in seinem äusseren Schwefelatom mit S^{35} markiert war, ist untersucht worden.

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Tabelle 1.

Nr.	$BaSO_4$ herstammend von:	Impulse/ Min.
1	Dithionit nach der Separation	109
2	Thiosulfat » » »	3 829
3	ursprünglichen Thiosulfat	4 061

Es wurde festgestellt, dass sich vor der Jodoxydation kein Sulfid nach der Reaktion von Binz und Sondag[1]

$$S_2O_4^{2-} + S_2O_3^{2-} + 4\,OH^- \rightarrow 3\,SO_3^{2-} + S^{2-} + 2\,H_2O$$

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Tabelle 2.

Nr.	$SrSO_4$ aus:	Impulse/ Min.
1	Dithionit nach der Separation	3
2	Thiosulfat » » »	3 529
3	ursprünglichem Thiosulfat	4 526

Aus den Versuchen geht hervor, dass bei 18° C in einer Lösung vom pH = 13 nach 44 Stunden und in einer Lösung vom pH = 6 nach einer Stunde kein Schwefelaustausch zwischen $S_2O_3^{2-}$ und $S^*SO_3^{2-}$ nachgewiesen werden konnte. In einer kommenden Arbeit soll über das experimentelle Verfahren dieser Arbeit und die Resultate der Untersuchungen über den Austausch zwischen $SS^*O_3^{2-}$ und $S_2O_4^{2-}$ sowie zwischen $O_3S^*SSS^*O_3^{2-}$ und SO_4^{2-} ausführlicher berichtet werden.

1. Binz, A., und Sondag, W. *Ber.* **38** (1905) 3830.
2. Kurtenacker, A., Mutschin, A., und Stastny, F. *Z. anorg. u. allgem. Chem.* **224** (1935) 410.
3. van der Heijde, H. B. *Rec. trav. chim.* **72** (1953) 95.

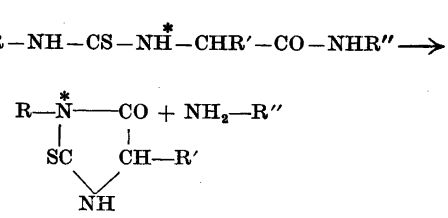
Eingegangen am 7. Juli 1953.

Rate of Ringclosure in the Edman Method

B. DAHLERUP-PETERSEN

Chemical Department, Carlsberg Laboratory, Copenhagen, Denmark

In the course of investigations carried out at the Carlsberg Laboratory on the Edman method for stepwise breakdown of peptides, the rate of ringclosure with formation of thiohydantoin was studied for a series of different compounds:



R being methyl-, *a*-naphthyl-, *p*-methoxyphenyl-, phenyl-, *o*-, *m*-, *p*-nitrophenyl. The method used was that described by Dahlerup-Petersen, Linderstrøm-Lang, and Ottesen [1], this method having the advantage that the thiohydantoin, which is relatively unstable in aqueous solutions, is removed continuously by benzene, in which it is very soluble. Experiments have shown that the rate of hydantoin formation is slow as compared to that of extraction under the given conditions [1] and that therefore the rate at which the hydantoin appears in the benzene is a reasonably quantitative measure of the rate of ringclosure. Table 1 shows the monomolecular rate constants obtained for the reaction at 70° C ± 1° in pH 4.5 (at 20°) 0.05 *N* citrate buffer, concentration of thiourea compound 0.02 molar.

Table 1.

R	$R' = CH_3$ $R'' =$ $CH_2CONHCH_2COOH$ K_1	$R' = H$ $R'' =$ CH_2COOH K_2
Methyl	0.09	
p-Methoxyphenyl	0.13	
a-Naphthyl	0.18	
Phenyl	0.19	0.11
p-Nitrophenyl	0.18	0.15
m-Nitrophenyl	0.76	0.25
o-Nitrophenyl	0.36	0.21

It will appear from this table that the rate constants increase with increasing electronegative influence exerted by R upon the nitrogen marked with an asterisk, the only definite deviation from this rule being the constant for the *o*-nitrocompound. Steric hindrance may play a role here.

1. Dahlerup-Petersen, B., Linderstrøm-Lang, K., and Ottesen, M., *Acta Chem. Scand.* **6** (1952) 1135.

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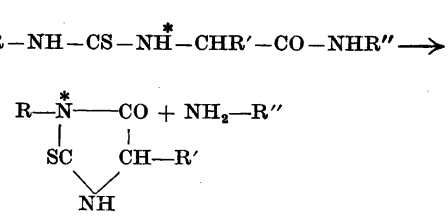

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Received July 11, 1953.

The Demonstration of a Liver Factor Stimulating the Sulphate Exhange of Chondroitin Sulphuric Acid

HARRY BOSTRÖM and
BENGT MÅNSSON

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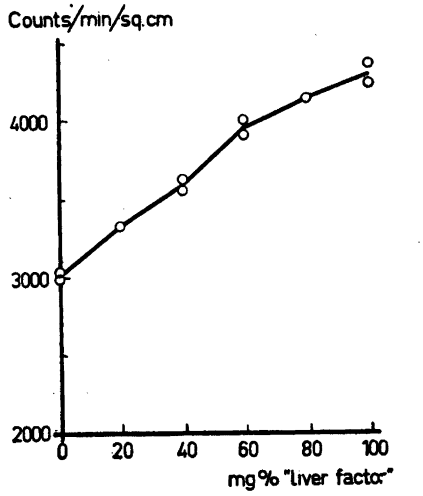
Recently, we discussed some problems concerning the sulphate exchange in chondroitin sulphuric acid of cartilage on the basis of a series of experiments with a slicing technique [1,2]. Slices of cartilage suspended in a Krebs-Ringer-bicarbonate solution containing S^{35}-labelled sulphate were incubated at 37° C for some hours. A considerable S^{35}-incorporation in the ester sulphate group of chondroitin sulphuric acid occurred, as demonstrated after isolation of this compound and splitting off its sulphate group through acid hydrolysis. This *in vitro* sulphate fixation did not occur in slices heated to 47° C, in frozen and subsequently thawed slices or in homogenized cartilage. Moreover, this reaction could be inhibited by different enzyme inhibitors, amongst which the SH inhibitors were found to be the most active.

In subsequent experiments we found that the reaction studied was strongly stimulated by the presence of small amounts of a liver homogenate. It was also found that the active "liver factor" was thermostable and could easily be extracted from fresh liver by water. In the present paper, a preliminary report is given of these observations.

The liver from a newly killed suckling calf was washed in tap water, freed from the large vessels and cut in ½ – 1 cm thick slices. The slices were gently stirred in 1 liter of distilled water for 1 hour at room temperature. After removal of the liver slices by pouring the mixture on a Büchner funnel (without filter paper) the fluid was filtered through hyflo supercel. The clear, red-coloured filtrate was then freeze dried and yielded 3 g of a reddish, light powder.

The ability of this powder to stimulate the sulphate exchange of chondroitin sulphuric acid of costal cartilage of young calves *in vitro* was tested by means of the slicing technique previously described [2].



In one series of experiments, different amounts of the powder (0 – 100 mg %) were added to the Krebs-Ringer-bicarbonate solution, in which the slices of cartilage were suspended, 30 minutes before the addition of S^{35}-labelled sodium sulphate. Incubation at 37° C for 2 hours. The result of these experiments is recorded in Fig. 1. It is seen from the course of the curve that there was a successive increase in the incorporation of S^{35} in the ester sulphate group of chondroitinsulphuric acid with an increase in the amount of liver powder added to the slices. In those samples incubated in the presence of 100 mg % of the liver powder, the S^{35} uptake was about 45 % higher than in the control samples.

In another series of experiments, it was shown that heating of the liver powder suspended in a small volume of water to 100° C did not destroy its ability to stimulate the S^{35} incorporation in sulphuric acid.

As shown previously, the reproducibility of the *in vitro* technique is extremely good [2]. Thus, the response obtained in the present experiments is highly significant. The true nature of the stimulating liver factor is unknown. However, the fact that it is thermostable may indicate that it represents some kind of co-factor interacting with the esterifying enzyme system.

1. Boström, H. *Arkiv Kemi* **6** (1953) 43.
2. Boström, H., and Månsson, B. *Arkiv Kemi* **6** (1953) 23.

Received August 5, 1953.

Activity of Cosmene and Alloöcimene as Nutritional Coagulation Factors

OYVIND SÖRBYE, INGER KRUSE and HENRIK DAM

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In a previous communication [1] it was reported that oxalated chicken plasma contains several different coagulation factors which cannot be adsorbed by $SrCO_3$ or $BaCO_3$. Most of these factors are labile on storage. At present it is possible to distinguish between 7 different coagulation factors in oxalated $SrCO_3$-treated plasma, because of their different behaviour on treatment with selective adsorbents. It was also briefly mentioned that a quantitative "titration" of each factor with its selective adsorbent seemed possible, thus offering a method for the study of the relation between the concentration of each factor and nutritional coagulation factors (NC-factors).

A number of such factors have been shown to exist; they must be considered necessary for the formation of the coagulation factors in the body of chick[2]. Early work along these lines indicated that the formation of the π-factor (selectively adsorbed from $SrCO_3$-treated plasma by $PbCO_3$ and some other adsorbents) was dependent on a lipo-soluble factor present in cod liver oil, lard and corn oil, in addition to other water-soluble factors.

This factor has been concentrated from corn oil. It is non-saponifiable and its behaviour during chromatography and partition between solvents suggests that it might be a hydrocarbon. Spectrography suggests a diene or triene structure.

Through the courtesy of Professor, Dr. N. A. Sørensen, Trondheim, Norway, it has become possible to test some hydrocarbons, prepared in his laboratory, from animal and plant material. We have examined their ability to raise the π-level in chickens having a low level of this factor due to lack of the corn oil factor.

The basal diet is indicated in Table 1, and the results are presented in Table 2. Samples were given on 3 following days and the π-level was determined before feeding and on the 4th day.

Table 1. Composition of diet no. S—11. 2A.

Casein, vitamin low	150
Pancreas powder, defatted	80
Gelatine	80
Salt mixture *	50
Choline chloride	2
Vitamin mixture **	2
Inositol	1
p-amino benzoic acid	0.3
Cystine	2
Vitamin E acetate ("Ephynal", Roche)	0.1
Vitamin K substitute ("Synkavit", Roche)	0.01
Sugar	633
Alfalfa	80
Refined peanut oil	30

* McCollum-Simmond's Salt Mixture no. 185 supplemented with 0.013 % KJ, 0.13 % $CuSO_4$, $5H_2O$ and 0.52 % $MnSO_4$, $4H_2O$.

** Thiamine HCl: 3, riboflavin: 4, nicotinic acid: 50, Ca-pantothenate: 12, pyridoxine: 3.5, biotin: 2, sugar: 925.4.

Table 2.

Supplement	Amount fed per g body weight	π-factor levels in % of maximal: Start	End
Squalene	3.1 μg	23	<17
Pristan	3.3 »	30	<17
Farnesene	15 »	<17	<17
	30 »	<17	33
Cosmene *	0.35 »	<17	33
	0.40 »	<17	60
	0.41 »	<17	70
	0.42 »	<17	77
	0.43 »	<17	87
	0.45 »	<17	100
	0.50 »	<17	100
	0.70 »	<17	100
Alloöcimene	0.10 »	30	47
	0.12 »	<17	46
	0.12 »	<17	43
	0.13 »	<17	73
	0.14 »	20	100
	0.14 »	<17	100
	0.16 »	<17	100
	0.18 »	<17	100
	0.94 »	20	100

* 2,6-dimethyloctatetraene (1 : 3 : 5 : 7).

The results indicate that alloöcimene has considerable activity, the minimum dose for maximal action being 0.14 μ g per g body weight. The cosmene [3] preparation has about 1/3 of the activity of alloöcimene. The activity of the preparation rich in farnesene is very much lower. Squalene and pristan had no effect in the quantities tested. The factor in corn oil which can be replaced by cosmene and alloöcimene is being studied further.

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Reactions between Imino Esters and α-Amino Acid Esters

I. The Structure of Finger and Zeh's Benzylimidazolones

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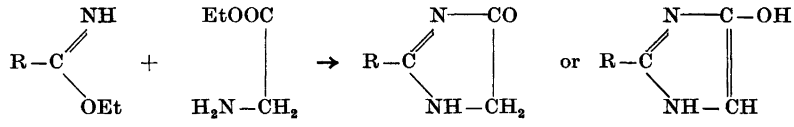
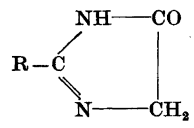
The reaction between imino esters and α-amino acid esters was first studied by Finger [1,2]. He reported the formation of 5(4)-imidazolones (I, R = CH_3 or $C_6H_5CH_2$) when the reactants in molecular proportions were brought into contact at room temperature with or without ether as a diluent.

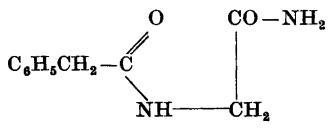


I

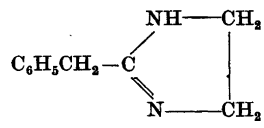
The formulation of the reaction products (I) was supported by their chemical reactions. In the case of R representing benzyl, however, two different products were isolated according to the experimental conditions selected [2]. They were considered to be isomerides and were formulated as (I) and (II), respectively.



II



III



IV

Our present knowledge of tautomerism in amidines and imidazoles (*cf.* Ref. 3,4) renders the correctness of the proposed formulations highly questionable. This fact, in conjunction with a general interest in the chemistry of 5(4)-imidazolones, led to the present reinvestigation of the "isomerides" of Finger and Zeh [2].

Attention was first directed to the reaction between molecular proportions of ethyl phenylacetimidate and glycine ethyl ester in ether solution at room temperature. Trituration of the crude, brown reaction product with hot

benzene gave a filtrate which deposited crystalline material on cooling. After purification, the pale yellow crystals melted at 138—139° (dec.) and gave analytical figures confirming the formula $C_{10}H_{10}ON_2$ of Finger and Zeh. A cryoscopical determination of the molecular weight gave values which agreed reasonably well with the empirical formula. Most of the chemical reactions previously reported were repeated and the results confirmed. Treatment of the imidazolone with boiling water resulted in hydrolysis to a crystalline compound, definitely proved to be phenaceturamide (III) on comparison with authentic material. This facile hydrolytic cleavage of the imidazole nucleus is paralleled in *e.g.* the work of Miescher *et al.*[5] who found the salts of 2-benzyl-imidazoline (IV) very stable towards hydrolysis, whereas the free base was rapidly opened on boiling with water. The formulation of the compound above as 2-benzyl-5(4)-imidazolone (II, R = $C_6H_5CH_2$) is furthermore consistent with its solubility in alkali, its weakly basic properties, its ability to give condensation products with aldehydes and ketones as well as its ultraviolet absorption spectrum (Fig. 1). The latter may be considered as the spectrum of the imidazolone-ring showing only end-absorption, which has superimposed upon it the typical benzyl bands at *ca.* 250 mμ. The similarity with the absorption spectra of benzylpenillic acid and various benzylimidazolines is obvious [6]. The flat and low maximum at about 340 mμ may be attributable to traces of the higher melting reaction product discussed below. Benzoylation experiments in pyridine yielded a dibenzoyl-derivative, m.p. 138°, in accord with the previous results of Finger and Zeh. The molecular weight determination gave figures indicating the presence of two benzoyl groupings in the monomeric 2-benzylimidazolone.

The higher melting "isomeride" — Finger and Zeh's "Isoglyoxalidon" — was produced in rather poor yield on mixing ethyl phenylacetimidate and glycine ethyl ester in molecular proportions and initiating the reaction by local heating. An exothermic reaction took place, accompanied by extensive browning of the contents of the vessel. Treatment with hot benzene removed simultaneously formed 2-benzylimidazolone and left a crystalline residue which melted at 218—220° (vigorous decomposition) after repeated recrystallisations. The very pale yellow needles gave analytical figures indicating the composition $C_{20}H_{20}O_2N_4$, H_2O, apparently a monohydrate of a dimeride of 2-benzylimidazolone. Cryoscopical determinations of the molecular weight gave erratic and inconsistent results and hence provided no confirmatory evidence of the above empirical formula. The degradation experiments reported below, however, left little doubt as to the composition. The compound was sparingly soluble in water, cold ethanol and benzene and could conveniently be recrystallised from aqueous pyridine. Contrary to 2-benzylimidazolone, the dimeride proved stable in boiling water, while hydrolysis with hydrochloric acid at elevated temperature afforded ammonia, glycine and phenylacetic acid. Furthermore, the ability to condense with carbonyl-compounds was lost after dimerisation. Spectroscopically it could be shown that cold aqueous alkali caused a rather rapid and irreversible structural change. The dimeride produced a characteristic deep-blue colour with ferric chloride, while 2-benzylimidazolone gave only a weak brownish colour with this reagent.

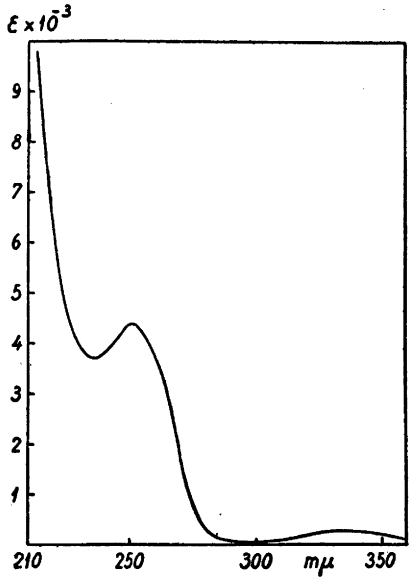


Fig. 1. Ultraviolet absorption spectrum of 2-benzyl-5(4)-imidazolone in water.

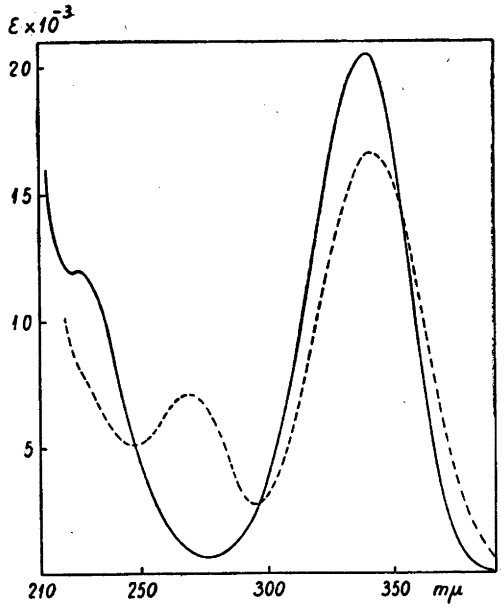
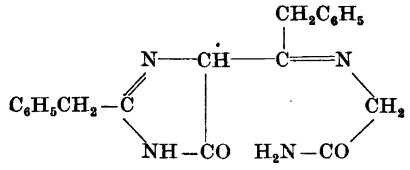


Fig. 2. Ultraviolet absorption curves of the dimeride (V or Va), ———— in 96 % ethanol; - - - - -: in 0.05 N NaOH in 20 % ethanol, measured immediately after dissolution.

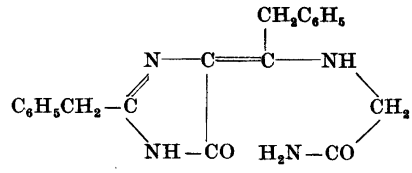
An important clue to the nature of the dimeride was provided by treating it with cold 4 *N* hydrochloric acid, when a high-melting colourless compound, $C_{18}H_{16}O_2N_2$, resulted. The latter was soluble in sodium hydroxide and gave a pink colour with ferric chloride in neutral solution. It proved identical with an unidentified compound, described by Finger and Zeh, resulting from the rather drastic treatment of their "Isoglyoxalidon" with alkali. Inspection of the empirical formula indicated that glycine and ammonia had been lost during the mild acid hydrolysis of the dimeride. The C_{18}-formula of the remaining molecular fragment pointed to 2-benzylimidazolone, substituted by a phenylacetyl-grouping, as a likely structural possibility. No condensations were noticed between the acid degradation product and carbonyl compounds indicating the attachment of the phenacetyl-grouping to the reactive 4(5)-position of the intact imidazolone ring.

It will be recalled that the linkage in 1—2-position of the 2-benzylimidazolone (II, R = $C_6H_5CH_2$) was readily broken to give (III) upon boiling of its aqueous solution. Therefore, it seemed likely that the dimerisation reaction involved attaching the 2-carbon atom of one imidazolone nucleus, under simultaneous ring fission, to the active methylene group of another, intact 2-benzylimidazolone molecule. Thus the expression (V), possibly in equilibrium with (Va) for reasons to be discussed below, is presented as a tentative structu-

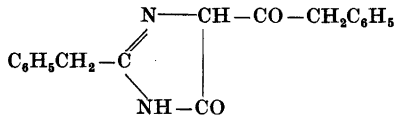
ral formulation of the "Isoglyoxalidon" of Finger and Zeh. This structure seems to accomodate satisfactorily the known facts and offers (VI), or possibly a tautomeride, as a likely formulation of the acid degradation product.



V



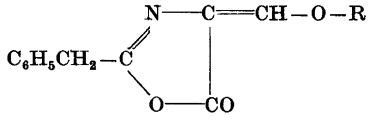
Va



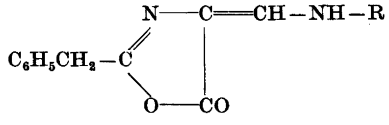
VI

It should be noted that other structures have been considered for the dimeride. Although admittedly tentative, the formulae presented here seem to account better than any alternative ones for the experimental results. The possible participation of the methylene group of the benzyl substituent as a site of reaction could be ruled out, when it was found that 2-methyl-5(4)-imidazolone gave an analogous isomeride. The latter was prepared under different conditions and will be described in the following paper.

The above chemical evidence of the structures was strongly supported by studies of the ultraviolet absorption spectra. In ethanol solution the dimeride, (V) or (Va), exhibited a very strong absorption maximum at 340 mμ (ε 20 600); a secondary band at 226 mμ (ε 12 600) and a low minimum at 276 mμ (ε 720) were also apparent (Fig. 2). Whereas acidification resulted in no pronounced alteration of the spectrum, except for some hyperchromic effect, addition of alkali changed the absorption curve rather profoundly. At 270 mμ a new band appeared which, however, was destroyed when the alkaline solution was kept at ordinary temperature. The spectroscopical data are strikingly similar to the absorption spectra, determined during the penicillin work [7], of the penicillenates and various model benzyloxazolones carrying a substituted hydroxy- or aminomethylene-grouping in 4-position (VII and VIII), (Fig. 3).



VII



VIII

The bathochromic shift in the absorption spectra of the imidazolones compared with the corresponding azlactones is not surprising. It merely reflects the generally experienced progression of the absorption bands to longer wavelengths upon passing from heterocyclic oxygen-rings to the

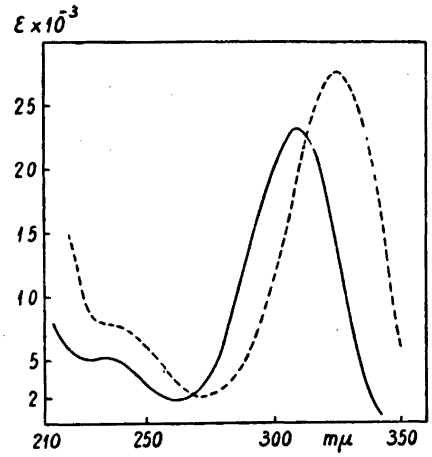
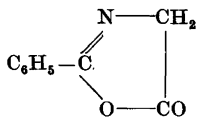


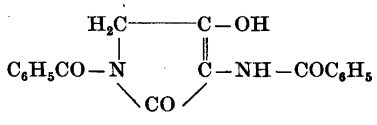
Fig. 3. Ultraviolet absorption curves of: ——————: 2-benzyl-4(5)-aminomethylene-5(4)-oxazolone (VIII, R = H) in ethanol; - - - - -: S-benzyl benzylpenicillenic acid (VIII, R = CH(COOH)C(CH₃)₂-SCH₂C₆H₅) in ethanol.

isosteric nitrogen-analogues. The spectroscopical studies therefore favor the substituted aminomethylene-structure (Va) while the acid hydrolysis to (VI) is more readily explained on basis of the tautomeric structure (V). An equilibrium between the two forms may conceivably exist. The acid degradation product gave an intense absorption band at 340 mμ. This fact, in conjunction with the pink colour produced with ferric chloride, indicates the formula (VI), enolised in the side chain, as the most correct expression.

The above dimerisation reaction presents no essential novelty, being paralleled in other heterocyclic series. Thus it recalls a somewhat similar change which takes place when 2-phenyloxazolone (IX) is exposed to Grignard reagents or a mixture of triethylamine and hydrogen cyanide [8]. The intricate structural problem of this dimeride was eventually solved by Cornforth and Huang [9], when they proved the formula (X) to be the correct expression.

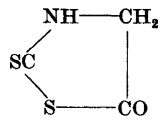


IX

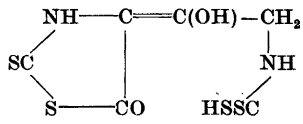


X

A formally similar expression for the present dimeride would be incompatible with its chemical properties and therefore can be excluded. Cook and Levy [10] found that 2-thio-5-thiazolidone (XI) underwent dimerisation under the influence of triethylamine. They established the expression (XII), formally similar to the one here proposed, as the most likely structure of their dimeride.



XI



XII

EXPERIMENTAL *

Ethyl phenylacetimidate. The hydrochloride was prepared from benzyl cyanide and ethanol in the usual way [11]. The free ester was liberated on addition of $NaHCO_3$ and distilled. B.p. 87—88° at 1.8 mm, m.p. 5°. Contrary to the literature [11] it was observed that the free ester did not keep well at room temperature. In the following experiments freshly distilled ester or material which had been kept at dry ice temperature was used throughout.

2-Benzyl-5(4)-imidazolone (I, II, R = $C_6H_5CH_2$). Freshly distilled glycine ethyl ester [12] (4.0 g) was dissolved in anhydrous ether (20 ml). To this solution was added another, containing ethyl phenylacetimidate (6.4 g) in ether (10 ml), and the mixture was kept at room temperature. After a few hours, the separation of a crystalline solid started. Next day the crystals (4.8 g) were separated from the brown filtrate and thoroughly triturated with ether, when they appeared as a light brown powder. This was treated with hot benzene and an insoluble residue removed by filtration. On cooling, the filtrate deposited yellow crystals (1.5 g), m.p. 137—140°.

An analytical sample was prepared by an additional recrystallisation from methanol, containing ether and petroleum ether. Tiny, yellowish needles separated. M.p. 138—139° (dec.), (Finger and Zeh report the m.p. 143°).

$C_{10}H_{10}ON_2$ (174.2)	Calc.	C	68.94	H	5.79	N	16.08
	Found	»	68.95	»	5.81	»	16.02

Cryoscopical determinations of the molecular weight (camphor) gave the values 166, 158, 188.

Addition of ferric chloride to an ethanolic solution produced an uncharacteristic, brownish colour.

Hydrolysis of 2-benzyl-5(4)-imidazolone. The above product (237 mg) was refluxed with water (10 ml) for one hour. Filtration of the hot solution removed traces of insoluble material. On cooling, the filtrate deposited crystals, which after recrystallisation from water with addition of a little charcoal, appeared as colourless plates. M.p. 178—179° alone or mixed with authentic phenaceturamide, prepared by ammonolysis of methyl phenaceturate.

Dibenzoyl derivative of 2-benzyl-5(4)-imidazolone. The benzoylation of 2-benzyl-5(4)-imidazolone in pyridine solution was performed essentially as described by Finger and Zeh. The derivative separated from ethanol in clusters of colourless needles. M.p. 137—138°.

$C_{24}H_{18}O_3N_2$ (382.4)	Calc.	C	75.37	H	4.74	N	7.33
	Found	»	75.50	»	4.91	»	7.55

Cryoscopical determinations of the molecular weight (camphor) gave the values 351, 362.

Preparation of the dimeride (V, Va). Ethyl phenylacetimidate (6.4 g) and glycine ethyl ester (4.0 g) were mixed and heated with a small free flame until the formation of a slightly yellow colour was observed. The flame was removed and a vigorous exothermic reaction took place, accompanied by a colour change to dark brown. After *ca.* 2 minutes, the reaction ceased and after cooling, ether (*ca.* 50 ml) was added when a light brown crystalline powder remained (5.0 g). This was refluxed with benzene (50 ml) for 0.5 hour, the benzene removed by decantation and the operation repeated with a portion of fresh benzene (20 ml). On concentration and cooling, crude 2-benzylimidazolone (1.0 g) separated from the benzene filtrates. The residue was recrystallised twice from aqueous ethanol, with addition of decolourising charcoal, to give slightly yellow needles (1.4 g), m.p. 211—213° (dec.). An analytical sample was recrystallised once more from aqueous pyridine and separated in small colourless needles. The m.p. values depended on the rate of heating. When rapidly heated, the sample melted at 216—220° under vigorous decomposition. The air-dried sample gave analytical figures indicating a monohydrate.

$C_{20}H_{20}O_2N_4$, H_2O (366.4)	Calc.	C	65.56	H	6.05	N	15.29
	Found	»	65.50	»	6.20	»	15.35

* All melting points are uncorrected and determined in capillary tubes in an electrically heated block.

The hydrate was dried at room temperature over P_2O_5 for 40 hours and the weight loss determined.

Calc.	for 1 H_2O	4.92 %	H_2O
Found		4.70 %	»

The anhydrous substance was analysed for nitrogen.

$C_{20}H_{20}O_2N_4$ (348.4)	Calc.	N	16.08
	Found	»	16.01

On keeping in the open atmosphere, the anhydrous material rapidly reconverted into the monohydrate.

No reproducible results were obtained in the cryoscopical molecular weight determinations, neither in camphor nor in phenol.

Acid hydrolysis of the dimeride to (VI). When the dimeride (1.0 g) was added to 4 *N* hydrochloric acid (50 ml) a clear solution was obtained. Within less than one minute the separation of colourless crystals started. The product was isolated and carefully recrystallised from glacial acetic acid; prolonged heating or too high a temperature must be avoided. Otherwise the solution took on yellow and red colours indicating unwanted secondary transformations taking place. The crystalline powder was washed with ethanol and ether. M.p. 298—299° (dec.).

$C_{18}H_{16}O_2N_2$ (292.3)	Calc.	C	73.96	H	5.52	N	9.59
	Found	»	73.70	»	5.27	»	9.82

Ultraviolet absorption spectra. The absorption spectra were determined in 1 cm cells with a Beckman model DU quartz spectrophotometer.

SUMMARY

The structures of two compounds, reported in the literature as isomeric 2-benzyl-5(4)-imidazolones, have been reinvestigated and the dimeric nature of the higher melting isomeride established.

Chemical and spectroscopical studies provide evidence for the proposed structural expressions (II) and (V), or their tautomeric forms.

Microanalyses have been performed in this laboratory by Mr. A. Grossmann.

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Reactions between Imino Esters and α-Amino Acid Esters

II. The Reaction of N-Substituted Imino Esters with Amines

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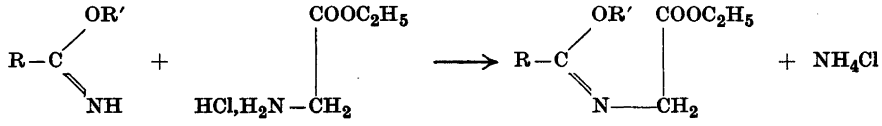
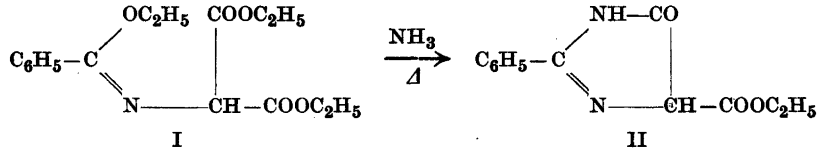
The preceding paper described an example of the preparation of 5(4)-imidazolones by condensation of iminoesters and α-amino acid esters. Although this preparative method promised to be of extensive application, several cases were noted where diminished reactivity (*e.g.* in case of tertiary α-amino acid esters, *cf.* Ref. 1), complications due to dimerisation (*cf.* the preceding paper), and the production of undesired coloured by-products, made an alternative route desirable.

In 1914 Schmidt [2] made the interesting observation that if an imino ester as the free base in an organic solvent was shaken with an aqueous solution of the hydrochloride of glycine ethyl ester, a substituted iminoester was formed according to the following scheme.



It was not until the penicillin programme that the synthetic potentialities of this reaction were recognised, when it was found that ethyl α-ethoxybenzylideneaminomalonate (I) upon treatment with ammonia and subsequent heating was transformed into 2-phenyl-4(5)-carbethoxy-5(4)-imidazolone (II) [3].

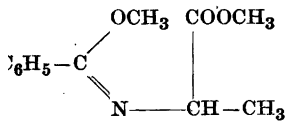


I II

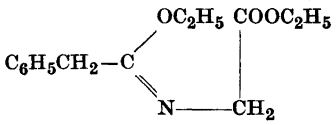
This represents the only example to be found in the literature of ammonolysis of Schmidt-esters, although the latter have been utilised for other synthetic purposes, *e.g.* in the elegant oxazole-syntheses of Cornforth and Cornforth [4].

After the conclusion of the present work a paper by Miller, Gurin and Wilson [5] appeared in which they reported the preparation of two 4(5)-carboxamido-5(4)-imidazolones in a one-step operation from an iminoester and aminomalonamide hydrochloride.

The first attempts to cyclise a substituted iminoester were made with methyl α-methoxybenzylideneaminopropionate (III), readily prepared from methyl benzimidate and DL-alanine methyl ester hydrochloride by the ordinary Schmidt-reaction. When (III) was kept at room temperature in ethanol, saturated with ammonia, no reaction was observed.



III



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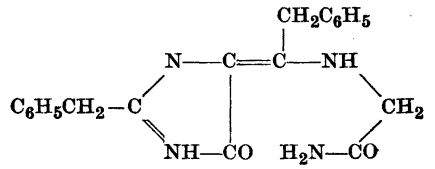
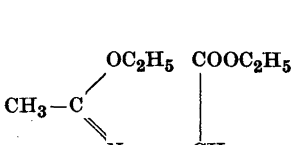


V

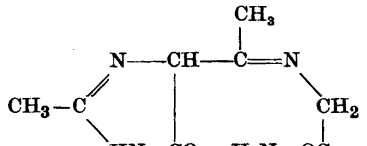
The application of higher temperature led to an intractable dark reaction mixture. Recalling the otherwise established anormal reactivity of benzimino-ester derivatives (*cf.* the following paper), it was thought that esters containing a benzyl grouping might provide a more attractive starting material.

Ethyl phenylacetimidate was condensed with the hydrochloride of glycine ethyl ester to give ethyl α-ethoxyphenylethylideneaminoacetate (IV) as a distillable base. When the latter was kept at 0° with ethanolic ammonia, the solution rapidly turned brown and after a few days deposited a colourless, crystalline powder, m.p. 214—218° (dec.). Quite unexpected, the product proved to be identical with the dimeride (V), discussed in the preceding communication. The appearance of this compound in the present reaction may be considered as resulting from dimerisation of primarily formed 2-benzyl-5(4)-imidazolone under the influence of excess ammonia. This suggestion was further supported by the observation that alkali or tertiary amines would bring about dimerisation. When (IV) was treated with excess methylamine in ethanol, extensive browning occurred and no well-defined crystalline material could be isolated.

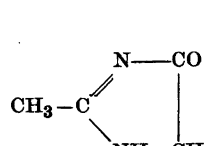
Ethyl α-ethoxyethylideneaminoacetate (VI) was prepared by the Schmidt-reaction with minor modifications [4]. Treatment of (VI) with alcoholic ammonia afforded a crystalline solid $C_8H_{12}O_2N_4$, 2 H_2O, m.p. 240—242° (dec.). In analogy with the result above the structure (VII), or possibly a tautomeride, suggested itself for this compound.



VI



VII

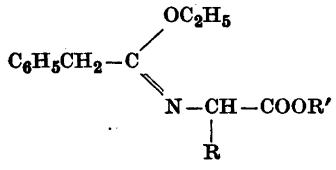


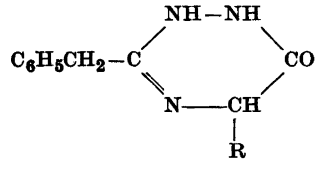
VIII

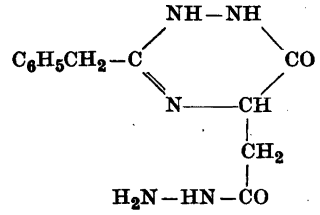
The dimeric nature of (VII) was deduced from its high melting point, the blue colour produced with ferric chloride and the absorption spectrum which exhibited a high peak at 335 mμ and was formally similar to that of (V), reported in the preceding paper. The auxiliary band at 225 mμ had disappeared but otherwise the absorption curve was so similar to that of (V) that it can be excluded that the benzene ring of the latter is part of the chromophoric system. This fact further strengthens the formula (V) proposed in the preceding paper.

Finger [6] formerly prepared 2-methyl-5(4)-imidazolone (VIII) by condensation of ethyl acetimidate with glycine ester. No indication of dimerisation is found in his paper, except maybe for the isolation of a benzoyl-derivative containing one benzoyl grouping per two imidazolone rings. This benzoylated product is probably a derivative of the dimeride (VII), the latter being spontaneously formed in the alkaline solution employed in the Schotten-Baumann reaction.

In order to study the scope of the above dimerisation reaction, ethyl phenylacetimidate was condensed with the hydrochlorides of DL-alanine methyl ester, ethyl L-glutamate, ethyl L-aspartate and DL-phenylalanine ethyl ester to give the corresponding substituted iminoesters (IX). These were colourless liquids which could be distilled at low pressure. They were best stored at dry ice temperature. The phenylalanine-derivative (IX d) crystallised on keeping, m.p. 30—31°.







a. $R = CH_3$; $R' = CH_3$
b. $R = CH_2CH_2COOC_2H_5$; $R' = C_2H_5$
c. $R = CH_2COOC_2H_5$; $R' = C_2H_5$
d. $R = CH_2C_6H_5$; $R' = C_2H_5$

IX

$R = H$
$R = CH_3$
$R = CH_2C_6H_5$

X

XI

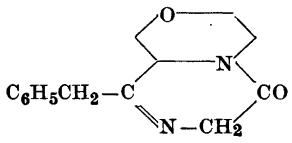
When the esters (IX) were exposed to dry ammonia dissolved in ethanol, the solutions gradually took on colours changing from yellow, through red, to dark brown. Traces of crystalline material were obtained from the solutions but many attempts to isolate sufficient material for a closer investigation were not rewarded. Similar experiments with *n*-butylamine and *cyclo*hexylamine instead of ammonia gave no promising results.

The Schmidt-esters were next studied in their behaviour towards hydrazine. When ethyl α-ethoxyphenylethylideneaminoacetate (IV) was mixed with a molecular proportion of hydrazine hydrate in ethanol, an exothermic reaction took place followed by the separation of a highly crystalline solid, $C_{10}H_{11}ON_3$. Again, (IXa) and (IXd) similarly treated afforded colourless products of the compositions $C_{11}H_{13}ON_3$ and $C_{17}H_{17}ON_3$, respectively. None of the compounds

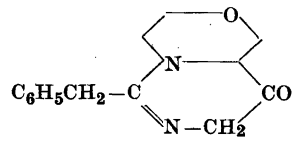
gave any colour with ferric chloride and they proved unreactive in the presence of Fehling's and Tollen's reagents. These facts and the empirical formulæ above support the formulation of the products as 3-benzyl-1,2,4-dihydrotriazinones (X), probably resulting from spontaneous cyclisation of primarily formed amidrazones or hydrazides. When (IXc) was treated with excess hydrazine at slightly increased temperature, a colourless solid, $C_{12}H_{15}O_2N_5$, resulted which smoothly reduced Fehling's solution and gave a crystalline benzylidene-derivative with benzaldehyde. The expression (XI) accounts well for these facts and is suggested as the most probable structure. Condensation of (IXb) with hydrazine did not yield any crystalline reaction product.

A few experiments were made in which the esters, (IV) and (IXa), were treated with phenylhydrazine. Crystalline solids were isolated in poor yields, but analyses of the purified reaction products indicated their complicated composition. No structural suggestions can be given at present.

With morpholine as a model secondary amine, the Schmidt-ester (IV) afforded a crystalline solid $C_{14}H_{15}O_2N_2$. This composition suggests that (IV) and morpholine have combined in the ratio 1 : 1 with the elimination of the elements of two molecules of ethanol. The bicyclic structures (XII) or (XIII) therefore present themselves as possibilities. The tentative character of these postulations should be emphasized, not least because the Dieckmann type of condensation involved seems unprecedented in such systems.



XII



XIII

EXPERIMENTAL *

Methyl α-methoxybenzylideneaminopropionate (III). The heterogenous mixture of methyl benzimidate (9.7 g) in ether (10 ml) and DL-alanine methyl ester hydrochloride (10.1 g) in water (5 ml) was shaken mechanically for 3 hours. The ethereal phase was removed by decantation and the aqueous layer, containing crystalline ammonium chloride, thoroughly washed with small portions of fresh ether. The combined ether solutions were washed with water and dried. The ether was removed and the residue fractionated at 0.7 mm pressure. The first fraction (2.4 g), b.p. 67—68°, consisted of unreacted methyl benzimidate. Then came a mixture (3.0 g), b.p. 69—106°, and finally the Schmidt-ester (4.5 g), b.p. 106—107°. The analytical sample was redistilled and appeared as a colourless oil.

$C_{12}H_{15}O_3N$ (221.3)	Calc.	C	65.11	H	6.83	N	6.33
	Found	»	64.89	»	6.91	»	6.40

No reaction was noticed on keeping the ester in ethanol, saturated with ammonia, for two days at room temperature. Heating at 60° of a similar mixture in a closed vessel gave a viscous, dark brown residue from which no crystalline material could be isolated.

Ethyl α-ethoxyphenylethylideneaminoacetate (IV). This substituted iminoester was prepared from ethyl phenylacetimidate and glycine ethyl ester hydrochloride, essentially as described above. After a small fore-run of unchanged iminoester, the Schmidt-ester distilled as a colourless oil at 125—128° and 0.5 mm. The yield was 70 %, calculated on

* The melting points are uncorrected and determined in capillary tubes in an electrically heated block; those below 80° though in a water bath.

unrecovered iminoester. The product remained colourless at dry ice temperature, whereas it rapidly turned yellow at ordinary temperature.

$C_{14}H_{19}O_3N$ (249.3)	Calc.	C	67.44	H	7.68	N	5.62
	Found	»	67.64	»	7.76	»	5.86

Ammonolysis of (IV) to (V). When the above ester (10.6 g) was kept at 0° in 96 % ethanol (50 ml), previously saturated at 0° with dry ammonia, the solution rapidly took on yellow and later brown colours. Next day, a crystalline solid had separated, and after 4 days the crystals (2.2 g) were filtered off. The product recrystallised from aqueous pyridine as a colourless crystalline powder, developing a deep-blue colour with ferric chloride in alcohol solution. M.p. 214—218° (dec.). Its identity with the dimeride (V), discussed in the preceding paper, was definitely established on comparison.

The analogous treatment with methylamine afforded no crystalline material.

Dimerisation of 2-benzyl-5(4)-imidazolone to (V). When the monomeride was dissolved in ethanol, containing 2 *N* sodium hydroxide or excess triethylamine, colouration rapidly occurred followed by gradual separation of a colourless product in varying yields. After purification, its identity as the dimeride (V) was secured upon comparison.

Ammonolysis of (VI) to (VII). Ethyl α-ethoxyethylideneaminoacetate (VI)[4], treated with ethanolic ammonia as described above, gave a 30 % yield of the dimeride (VII). This separated as colourless needles from aqueous ethanol, m.p. 240—242° (dec.). The air-dried sample gave analytical results indicating a dihydrate.

$C_8H_{12}O_2N_4$, $2H_2O$ (232.2)	Calc.	C	41.37	H	6.95	N	24.13
	Found	»	41.60	»	6.71	»	24.10

The water was lost on drying over P_2O_5 at ordinary temperature for 48 hours.

Calc. for 2 H_2O	15.5 % H_2O
Found	15.2 % »

The dimeride gave qualitative reactions similar to those previously noted for the analogous benzyl-derivative (V).

Methyl α-ethoxyphenylethylideneaminopropionate (IXa). This substituted iminoester was prepared from ethyl phenylacetimidate and DL-alanine methyl ester hydrochloride in the usual way. The ester distilled as a colourless, viscous oil at 90—110° and 0.1 mm. The yield was 42 %, calculated on unrecovered iminoester. The ester was stored at low temperature.

$C_{14}H_{19}O_3N$ (249.3)	Calc.	C	67.44	H	7.68	N	5.62
	Found	»	67.54	»	7.58	»	5.72

Diethyl α-(α-ethoxyphenylethylideneamino)glutarate (IXb). This was obtained in a similar way from ethyl phenylacetimidate and diethyl L-glutamate hydrochloride. The ester distilled as a very viscous, colourless oil, b.p. 159° at 0.2 mm. Yield 35 %, calculated on unrecovered ester.

$C_{19}H_{27}O_5N$ (349.4)	Calc.	C	65.31	H	7.79	N	4.01
	Found	»	65.54	»	7.68	»	3.85

Diethyl α-ethoxyphenylethylideneaminosuccinate (IXc). Ethyl phenylacetimidate and ethyl L-aspartate hydrochloride, treated in the usual way, gave the Schmidt-ester in 67 % yield, calculated on unrecovered iminoester. B.p. 153—157° at 0.2 mm. The viscous, slightly yellow oil was kept at dry ice temperature.

$C_{18}H_{25}O_5N$ (335.4)	Calc.	C	64.46	H	7.52	N	4.18
	Found	»	64.65	»	7.56	»	4.34

Ethyl α-(α-ethoxyphenylethylideneamino)-β-phenylpropionate (IXd). This was prepared from ethyl phenylacetimidate and DL-phenylalanine ethyl ester hydrochloride in the usual way. The yield of distilled, colourless ester was 73 %, calculated on unrecovered iminoester. B.p. 163° at 0.2 mm. The ester crystallised on keeping at low temperature, m.p. 30—31°.

$C_{21}H_{25}O_3N$ (339.4)	Calc.	C	74.31	H	7.42	N	4.13
	Found	»	74.59	»	7.45	»	4.13

3-Benzyl-6-oxo-1,2,5,6-tetrahydro-1,2,4-triazine (X, R = H). The substituted iminoester (IV) (603 mg) was dissolved in ethanol (1 ml) and hydrazine hydrate (125 mg) added, when an exothermic reaction occurred. Next day, ether was added and colourless prisms (398 mg) separated on scratching. They recrystallised from ethanol-ether in rhombic plates. M.p. 184—186°.

$C_{10}H_{11}ON_3$ (189.2)	Calc.	C	63.64	H	5.86	N	22.20
	Found	»	63.22	»	5.84	»	22.27

3-Benzyl-5-methyl-6-oxo-1,2,5,6-tetrahydro-1,2,4-triazine (X, R = CH_3). This compound was prepared in 84 % yield from (IXa) and hydrazine in a similar way. It separated in dense, rhombic plates from ethanol, m.p. 189—190°.

$C_{11}H_{13}ON_3$ (203.2)	Calc.	C	65.01	H	6.45	N 20.68
	Found	»	64.70	»	6.78	» 20.88

3,5-Dibenzyl-6-oxo-1,2,5,6-tetrahydro-1,2,4-triazine (X, R = $C_6H_5CH_2$). When (IXd) was treated with hydrazine as above, a 90 % yield of the condensation product was obtained. It crystallised from absolute ethanol in colourless, rhombic plates, m.p. 171°.

$C_{17}H_{17}ON_3$ (279.3)	Calc.	C	73.10	H	6.14	N 15.05
	Found	»	73.38	»	6.24	» 14.91

3-Benzyl-5-carboxhydrazidomethyl-6-oxo-1,2,5,6-tetrahydro-1,2,4-triazine (XI). The ester (IXc) (695 mg) and hydrazine hydrate (609 mg) were dissolved in ethanol (2 ml) and the solution refluxed for 30 minutes. On cooling, the crystalline reaction product separated (464 mg). It recrystallised from hot water in small needles, m.p. 197—198°.

$C_{12}H_{15}O_2N_5$ (261.3)	Calc.	C	55.15	H	5.79	N 26.81
	Found	»	55.08	»	5.76	» 26.78

The compound reduced Fehling's reagent in the cold.

Benzylidene-derivative of (XI). The hydrazone (196 mg) was readily obtained, when a solution of (XI) (212 mg) and benzaldehyde (106 mg) in 50 % ethanol (6 ml) was briefly heated. It separated in colourless needles from ethanol. The derivative sintered from *ca.* 140° but did not melt completely until 200° (dec.).

$C_{19}H_{19}O_2N_5$ (349.4)	Calc.	C	65.30	H	5.48	N 20.05
	Found	»	65.04	»	5.75	» 20.02

The reaction product of (IV) and morpholine, (XII) or (XIII)? When (IV) (890 mg) and morpholine (622 mg) were mixed and heated at 100° for 30 minutes, crystallisation sat in spontaneously. The product was repeatedly recrystallised from ethanol, and finally once from chloroform-petroleum ether. It appeared as colourless needles, m.p. 219°, soluble in dilute acid.

$C_{14}H_{16}O_2N_2$ (244.3)	Calc.	C	68.82	H	6.60	N 11.47
	Found	»	69.06	»	6.29	» 11.49

The above composition was supported by a molecular weight determination in phenol giving the values 218, 229 and 241.

SUMMARY

Ammonolysis of the substituted iminoesters, (IV) and (VI), has been shown to give compounds possessing the dimeric structures (V) and (VII).

Several N-substituted iminoesters (IX) have been prepared and their behaviour towards ammonia and hydrazine studied. With the latter they afford crystalline compounds, formulated as benzyldihydrotriazinones, (X) and (XI).

The ester (IV) reacts with morpholine at higher temperature to a crystalline compound, tentatively formulated as (XII) or (XIII).

Microanalyses have been performed in this laboratory by Mr. A. Grossmann.

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Reactions between Imino Esters and α-Amino Acid Esters

III. 2-Phenyl-5(4)-imidazolone and its Reactions

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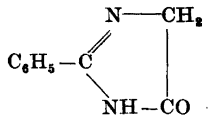
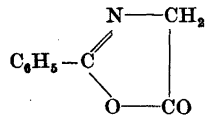
The reaction between iminoesters and α-amino acid esters as the free bases to give 5(4)-imidazolones was first reported from Finger's laboratory. The two documented examples of the reaction [1,2] were supplemented by additional ones in a thesis by Zeh [3]. He demonstrated the applicability of ring-substituted alkyl benzimidates in the above reaction but gave no notice to the simple 2-phenyl-5(4)-imidazolone (I). Karrer and Gränacher [4] later reported a compound, m.p. 141—143°, prepared by treating hippuramide with phosphorus pentachloride, to which they ascribed the structure (I). In a previous communication [5] we showed their product to be hippuronitrile.



I



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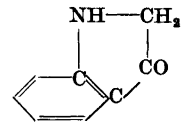
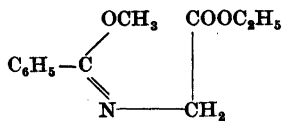


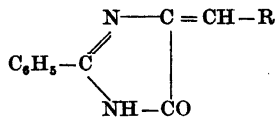
III

For several reasons a special interest was attached to the imidazolone (I) in the current studies. First, its formal resemblance to the highly reactive and synthetically important hippuric acid azlactone (II) made its synthesis and a closer investigation of its properties and possible preparative applications desirable. Secondly, the apparent structural analogy of (I) to indoxyl (III) allowed one to expect interesting indigoid properties of 2-phenyl-5(4)-imidazolone, an assumption which has been experimentally borne out.

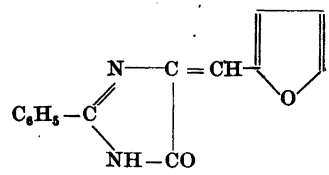
In the preceding paper [6] the failure to achieve ammonolysis of methyl α-methoxybenzylideneaminopropionate to a crystalline imidazolone was reported. No better results attended attempts to ring-close the corresponding glycine-derivative (IV) on treatment with ammonia. The high reactivity of the expected reaction products may explain these negative results, secondary reactions leading to complex by-products being predominant under the experimental conditions employed.



IV



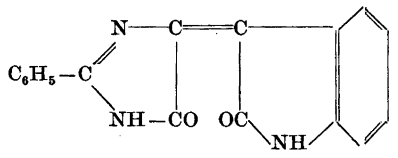
V



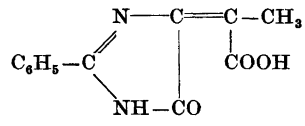
VI

When ethyl benzimidate in ether solution was allowed to react with a molecular proportion of glycine ethyl ester, preferably in a nitrogen atmosphere, the mixture gradually deposited a light brown crystalline solid. The reaction was accompanied by the formation of an intensely red substance, the amount of which could be somewhat diminished by careful exclusion of oxygen. Crystallisation from benzene, followed by sublimation in high-vacuum, finally afforded a crystalline substance $C_9H_8ON_2$, m.p. 165—167° (dec.). That this compound was the desired 2-phenyl-5(4)-imidazolone (I) was proved from its reactions.

Upon treatment with benzaldehyde and a trace of morpholine in glacial acetic acid, (I) gave 2-phenyl-4(5)-benzylidene-5(4)-imidazolone (V, R = C_6H_5), identical with a specimen prepared by the Erlenmeyer synthesis [7] (*cf.* Ref. 8). With furfural under similar conditions, the analogous 2-phenyl-4(5)-furfurylidene-5(4)-imidazolone (VI), m.p. 268—269° (dec.), was obtained. Its m.p. agreed with the value (266—267°) reported by Cornforth and Huang [9] for the same compound, while Ekeley and Ronzio [10] found the m.p. 293.5° and Erlenmeyer and Stadlin [11] reported the value 241° (dec.). This divergency may be attributable to the presence of varying amounts of the two geometrical isomerides in the various preparations. Again, on reaction with 1-naphthaldehyde the unknown 4(5)-naphthylidene-5(4)-imidazolone (V, R = $C_{10}H_7$) was obtained. Condensation with isatin afforded a dark-red, high-melting condensation product. In view of the well-established reactivity of the β-position in isatin towards reactive methylene groups [12], the structure (VII), or a tautomeride, is proposed for the condensation product. Pyruvic acid and (I) yielded a dark-violet reaction product possessing the structure (VIII).



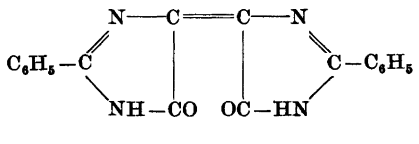
VII



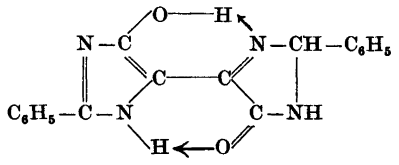
VIII

2-Phenyl-5(4)-imidazolone proved to be a highly reactive substance, particularly sensitive to oxygen. Even in the crystalline state it was transformed into a yellow substance, while its solutions in various solvents rapidly turned cherry-red, apparently as a result of oxidation. This phenomenon drew our attention to an older observation by Ruhemann and Stapleton [13]. They isolated from the reaction between benzamidine and acetylenedicarboxylic acid diethyl

ester a product, forming "ruby-red crystals", which they named "glyoxaline red" and to which they ascribed the bicyclic structure (IX), formally similar to pyrazole blue and indigotin. Ekeley and Ronzio [14] discussed the complex reactions between benzamidine and glyoxal in alkaline solution, yielding a series of differently composed red substances, one of which was "glyoxaline red".



IX



X

Therefore, it was not surprising to find that the red by-product in the condensation above consisted of essentially pure "glyoxaline red". In view of modern concepts of the indigoid structures, the formulation (X) is proposed for the red pigment. The latter was best prepared upon treatment of a solution of (I) in glacial acetic acid with oxygen. The identity with Ruhemann and Stapleton's pigment followed from the coincidence in absorption spectrum with that previously reported [14] and from the characteristic transformation into a yellow substance of the hydrated composition $C_{18}H_{14}O_3N_4$ upon heating in glacial acetic acid [13].

The reaction between aromatic aldehydes, glyoxal and amidines, studied by Ekeley *et al.* in a series of papers was a subject of discussion for many years, until it was eventually shown [15] that the reaction products possessed the imidazolone-structures (V). It was further suggested that (I) was intermediately formed from benzamidine and glyoxal and then in turn condensed with the aldehydes. The above preparation of 2-phenyl-5(4)-imidazolone (I) and its established reactivity towards aromatic aldehydes lend strong support to this assumption.

That the reaction between a benziminoester and glycine ethyl ester is by no means a simple reaction appeared from an experiment, in which a chromatographic purification of the crude reaction product was attempted. Using aluminium oxide as an adsorbent and chloroform with progressively increasing amounts of ethanol as the eluting solvent, a great number of differently coloured bands were noticed. Besides a small amount of 2-phenyl-5(4)-imidazolone, a considerable quantity of a secondary product, $C_{25}H_{21}O_2N_5$, was isolated. It appeared as intensely yellow needles, m.p. 128—130°, soluble in acid and yielding a picrate in ethanolic solution. No reaction was noticed with benzaldehyde, and addition of ferric chloride produced only a weak, greenish colouration. The above empirical formula was confirmed through analysis of a well-crystallising, yellow mono-acetyl derivative. The composition suggests that the compound is a result of reaction between two molecules of α-amino ester and three molecules of iminoester, with the elimination of five molecules of ethanol. Therefore, the intermediate formation of some dimeric structure (*cf.* the preceding papers of this series), followed by further combina-

tion with an additional molecule of iminoester, seems an attractive possibility. Owing to the complex nature of the product, no satisfactory structural expression has yet been established. It should be noted in this connection that Zeh [3] demonstrated reaction to take place between substituted 2-phenyl-5(4)-imidazolones and iminoesters to give products where the latter have combined with glycine ester in the ratio 2 : 1.

EXPERIMENTAL *

Ethyl benzimidate. The ester was liberated from its hydrochloride by means of sodium bicarbonate, as described by Glickman and Cope [16] for the analogous ethyl acetimidate. B.p. 85—86° at 2.9 mm.

2-Phenyl-5(4)-imidazolone (I). Glycine ethyl ester (7.9 g) and ethyl benzimidate (11.4 g) were dissolved in anhydrous ether. The solution was covered with nitrogen and kept at room temperature for 19 hours. The crystalline, light-brown solid (2.3 g) formed was isolated by filtration in a nitrogen atmosphere and thoroughly washed with ether. It was rapidly crystallised from anhydrous benzene, care being taken to exclude oxygen. The product was finally sublimed at a bath-temperature of 140° and 0.5 mm, when it appeared as colourless crystals, m.p. 165—167° (dec.), rapidly turning yellow on exposure to air.

$C_9H_8ON_2$ (160.2)	Calc.	C	67.48	H	5.03	N	17.50
	Found	»	67.35	»	4.89	»	17.72

The mother liquor on further keeping deposited a mixture of a higher melting brown compound and an intensely red substance. In addition, the crude product contained additional amounts of (I), which could be isolated upon extraction with benzene.

Experiments conducted under no special precautions resulted in the production of a more impure product, contaminated with large amounts of the red pigment.

2-Phenyl-4(5)-benzylidene-5(4)-imidazolone (V, R = C_6H_5). Pure (I) (0.5 g) was dissolved in glacial acetic acid (5 ml) and benzaldehyde (0.5 ml) and a drop of morpholine added. The mixture was briefly heated, whereupon the separation of an orange solid (0.6 g) started. The product recrystallised from amyl acetate as small, golden needles. M.p. 278—279°.

$C_{16}H_{12}ON_2$ (248.3)	Calc.	C	77.38	H	4.84	N	11.28
	Found	»	77.41	»	4.80	»	11.45

No depression of the m.p. was noticed on admixture with a specimen prepared by cyclodehydration of *a*-benzamidocinnamamide [7].

2-Phenyl-4(5)-furfurylidene-5(4)-imidazolone (VI). This was prepared from (I) (0.5 g) and furfural (0.4 ml) in a similar way. The product separated from amyl acetate in greenish-yellow needles. M.p. 268—269° (dec.).

$C_{14}H_{10}O_2N_2$ (238.2)	Calc.	C	70.57	H	4.23	N	11.76
	Found	»	70.89	»	4.09	»	11.56

2-Phenyl-4(5)-1'-naphthylidene-5(4)-imidazolone (V, R = 1-$C_{10}H_7$). (I) (0.5 g), 1-naphthaldehyde (0.65 ml) and a few drops of morpholine were briefly heated in glacial acetic acid (5 ml). The red, crude material (0.82 g) recrystallised from amyl acetate in thin, golden needles. M.p. 273—274°.

$C_{20}H_{14}ON_2$ (298.3)	Calc.	C	80.54	H	4.73	N	9.40
	Found	»	80.58	»	4.74	»	9.36

2-Phenyl-4(5)-3'-oxindolylidene-5(4)-imidazolone (VII). When a hot solution of isatin (450 mg) in acetic acid (4 ml) was mixed with a solution of (I) (450 mg) in acetic acid (4 ml), a dark-purple, crystalline solid separated at once. The product was soluble in NaOH and could be reprecipitated unchanged on addition of acid. It was recrystallised

* The melting points are uncorrected and determined in capillary tubes in an electrically heated block.

from a large volume of amyl acetate and appeared as a dark-red, crystalline powder which melted above 300°.

$C_{17}H_{11}O_2N_3$ (289.3)	Calc.	C	70.57	H	3.82	N	14.52
	Found	»	70.82	»	3.70	»	14.55

2-Phenyl-4(5)-1'-carboxyethylidene-5(4)-imidazolone (VIII). A solution of (I) (200 mg), pyruvic acid (0.25 ml of a 53 % solution) and a few drops of morpholine in acetic acid (2 ml), rapidly deposited the crystalline, dark-violet condensation product. This recrystallised from amyl acetate in dense, pointed prisms which melted completely above 300°.

$C_{12}H_{10}O_3N_2$ (230.2)	Calc.	C	62.63	H	4.38	N	12.18
	Found	»	62.90	»	4.52	»	12.18

The formation of "glyoxaline red" (X). When a solution of (I) in glacial acetic acid was treated with a stream of oxygen, the solution rapidly turned cherry-red. After some hours it was concentrated *in vacuo* to half its volume, when a dark-red, crystalline solid separated. It was filtered off and thoroughly washed with ethanol and ether. Then it was dissolved in NaOH and reprecipitated on addition of acid; this operation was repeated several times. The appearance of the product did not change during these treatments. The m.p. was found to be higher than 300°.

The ultra-violet absorption spectrum of a saturated solution in dioxane agreed well with the one previously reported [14].

When heated to boiling in anhydrous acetic acid a colour-shift to yellow took place, and the yellow reaction product gave analytical figures agreeing with the composition, $C_{18}H_{14}O_3N_4$, previously stated.

$C_{18}H_{14}O_3N_4$ (334.3)	Calc.	C	64.68	H	4.22	N	16.76
	Found	»	64.90	»	4.01	»	17.04

Chromatography of a crude reaction product from the interaction of ethyl benzimidate and glycine ethyl ester. The yellow reaction product (9.1 g), resulting from a condensation between ethyl benzimidate and glycine ester, was dissolved in 250 ml of chloroform. A trace of insoluble material was removed by filtration, and the solution poured on to a column (200 × 40 mm), packed with active aluminium oxide.

After development with fresh chloroform, eight different bands were noticed. The elution proceeded as shown in Table 1.

Table 1. Elution of adsorbed material.

Fraction No.	Eluting solvent	ml	mg	Appearance
1–6	$CHCl_3$	300	120	brown oil
7	»	250	450	yellow crystals
8	$CHCl_3$/EtOH 10/1	200	1 950	brownish crystals
9	»	30	600	red crystals
10	»	100	400	red crystals
11	»	100	220	red oil
12	$CHCl_3$/EtOH 10/2	300	290	dark-red oil
13	»	100	80	red oil

The remaining material was very firmly bound to the adsorbent and could not be effectively eluted, even with pure ethanol.

The crystalline material from fraction 7 was repeatedly recrystallised from methanol. It separated in clusters of thin, yellow needles, m.p. 128–130° (dec.).

$C_{25}H_{21}O_2N_5$ (423.5)	Calc.	C	70.91	H	5.00	N	16.54
	Found	»	70.69	»	5.23	»	16.60

The compound was soluble in acid and gave a crystalline picrate, m.p. 260–261°, in ethanol solution.

When the yellow crystals were briefly heated with acetic anhydride, a mono-acetyl derivative crystallised on cooling. It separated in hair-fine, yellow needles from ethanol, m.p. 280—281°.

$C_{27}H_{23}O_3N_5$ (465.5)	Calc.	C	69.65	H	4.98	N	15.05
	Found	»	69.53	»	4.73	»	14.96

Treatment of fraction 8 with cold benzene removed *ca.* 400 mg of 2-phenyl-5(4)-imidazolone (I), while the residue after purification proved identical with the yellow compound from fraction 7.

The fractions 9 and 10 consisted of 2-phenyl-5(4)-imidazolone, contaminated with the red pigment discussed above. Rather surprisingly, acetylation afforded a yellow product, identical with the mono-acetyl derivative mentioned above.

SUMMARY

The previously unknown and rather unstable 2-phenyl-5(4)-imidazolone (I) has been prepared by condensation of ethyl benzimidate with glycine ethyl ester. Its reactivity towards aromatic aldehydes, furfural, isatin and pyruvic acid has been demonstrated.

Its close relationship to "glyoxaline red" has been established.

The complex nature of the condensation between benziminoesters and glycine ester has been studied and discussed.

Microanalyses were performed in this laboratory by Mr. A. Grossmann.

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Vitamin B_{12}, Folic Acid, and Folinic Acid Factors in Digested Municipal Sludge

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Many bacteria and streptomycetes are able to produce vitamin B_{12} * when cultivated under aerobic conditions as reported by many workers. It has also been found that facultative and obligate anaerobic bacteria can produce the vitamin in the absence of oxygen. Pfiffner *et al.*[1] found two vitamin B_{12}-like substances, which they named pseudo-vitamin B_{12} and pseudo-vitamin B_{12b}, in the fermentation broth of an anaerobic bacteria isolated from rumen contents. Lewis *et al.*[2] isolated a similar substance, named vitamin B_{12f}, from rat faeces. Ford *et al.*[3] have isolated three vitamin B_{12}-like factors, named factors A, B, and C, from calf rumen contents and faeces. Leviton *et al.*[4] found that propionic acid bacteria synthesized vitamin B_{12} in very high amounts when cultivated under aerobic or anaerobic conditions. Finally, Hodge *et al.*[5] have reported the production of vitamin B_{12} by certain anaerobic bacteria in pure and mixed cultures.

Hoover *et al.*[6] found significant amounts of vitamin B_{12} in "activated" sludge from municipal sewage treatment plants. They did not show, however, if the vitamin found had been formed during aerobic or anaerobic digestion. In the following it will be shown that vitamin B_{12} is produced in the anaerobic digestion of municipal sewage sludge.

The vitamin B_{12} content of digested sludge from three sewage treatment plants was estimated. The nature of the vitamin B_{12} factors present was determined by chromatography and ionophoresis in combination with bioautography. Crystalline cyanocobalamin was isolated from the sludge and its identity confirmed spectrophotometrically. The nature and amount of desoxyribosides, folic acid and folinic acid factors in fresh and digested sludge were also investigated.

* In this paper the term "vitamin B_{12}" is used as the collective name for cyanocobalamin and other, vitamin B_{12}-like substances.

EXPERIMENTAL

Samples of sludge from the different stages of the digestion process were taken at three municipal sewage treatment plants in Stockholm, *viz.* the Henriksdal, Loudden, and Åkeshov plants, hereafter referred to as plants 1, 2, and 3 respectively. The samples were stored at −20° C.

More than 98 % of the vitamin B_{12} amount present was found to be adsorbed on particles suspended in the sludge. In order to release the vitamin into the water phase before analysing, the samples were autoclaved. The maximum amount of vitamin B_{12} was released by autoclaving at pH 6.0 and 120° C for 15 min. in the presence of 0.01 % potassium cyanide. After centrifugation the clear solution was diluted to an appropriate concentration and applied to the agar cup plate method with *E. coli* 113-3 [7] using the medium described by Diding [8]. Crystalline cyanocobalamin (Merck) was used as a standard.

The same solution was analysed for desoxyribosides, folinic acid and folic acid factors with the agar cup plate method using *Lactobacillus lactis* Dorner (ATCC 8 000), *Leuconostoc citrovorum* (ATCC 8 081), and *Streptococcus faecalis* (ATCC 8 043) as test bacteria. The media used were those described by Bánhidi *et al.* [9] (for *L. lactis* and *L. citrovorum*) and Capps *et al.* [10] (for *S. faecalis*). The last mentioned medium was prepared from Difco folic acid assay medium with 1.6 % agar. Thymidine, synthetic folinic acid (Leucovorin, Lederle), and folic acid (Folvite, Lederle) were used as standards.

The nature of the different growth factors was determined by chromatography or ionophoresis in combination with the bioautographic technique first introduced by Winsten and Eigen [11]. The bacteria and media were the same as in the agar cup plate methods. The paper chromatograms were run at 20° C on Whatman No. 1 filter paper and developed with *sec.* butanol saturated with water and containing 3 % acetic acid and 0.0025 % potassium cyanide. The R_F-values of some of the growth factors showed to be sensitive to the pH and the salt concentration of the samples. The comparison of the growth factors with known substances was, therefore, always performed by chromatography of samples and of known factors on the same strip.

The electrophoretic separation of the vitamin B_{12} factors was made according to Ericson *et al.*[12].

RESULTS

The vitamin B_{12} content of the digested sludge from plant 1 was followed from Jan. 1952 to Febr. 1953. No regular seasonal variations were observed. The concentration of the vitamin varied from 1.2—2.4 μg/ml; the mean value was 1.7 μg/ml or 22 μg/g dry matter. Digested sludge from plants 2 and 3 was analysed on two occasions and was found to contain 1.5 and 1.6 μg/ml vitamin B_{12}, respectively.

In order to investigate whether the vitamin B_{12} found had been synthesized during the digestion process or whether it had been brought into the plant with the sewage, samples from different stages of the sewage treatment process in plant 1 were collected and analysed. It was found that 82 % of the vitamin B_{12} content of the digested sludge was produced during the digestion process; 18 %, already present in the fresh sludge (0.13 μg/ml), possibly originates from human faecal matter.

Paper chromatography with bioautography revealed the presence of four growth factors (called factors I, II, III, IV) for *E. coli* 113-3 in fresh and digested sludge with the R_F-values 0.08, 0.14, 0.20, and 0.28. Factors I, II, III were also found in human faeces. Factor IV had the same R_F-value as factor B [3], factor III the same as cyanocobalamin, and factor II the same as factor A [3], pseudo-vitamin B_{12} [1], and vitamin B_{12f} [2]. Factors I, II, and III

Table 1. L. citrovorum and S. faecalis factors in fresh and digested sludge.

Factor	R_F-value	Fresh sludge		Digested sludge	
		L. c.	*S. f.*	*L. c.*	*S. f.*
Pteroic acid	0.10		+		(+)
Unidentified	0.18	+		(+)	
Unidentified	0.22	++		+	
Pteroyltriglutamic a	0.22		+		(+)
Folic acid	0.27		++		++
Unidentified	0.28	+		+	
Formylpteroylglutamic a.	0.40		+		(+)
Folinic acid	0.47	++	+++	+	+
Formylpteroic acid	0.57		(+)		(+)
Thymidine	0.66	+	+	(+)	(+)

The number of plus signs indicates the intensity of growth of the different factors as observed after chromatographic separation.

were also active towards *L. lactis* Dorner (ATCC 8 000) and *Lactobacillus leichmannii* 313 (ATCC 7 830). By autoclaving samples at 120° C and at different pH for 15 min. it was shown that the four factors were stable in the pH range 3—9. At pH 13.3 the vitamin B_{12} activity of the crude samples was fully destroyed. This is in agreement with the properties of vitamin B_{12} in liver extracts [14].

Paper electrophoresis of the samples showed the presence of six factors; three moving towards the cathode, one immobile, and two moving towards the anode. By comparison with known vitamin B_{12} factors it was found that the three positively charged factors migrated with the same speed as factor B, factor A, and pseudo-vitamin B_{12}, respectively. The immobile factor behaved as cyanocobalamin. Paper electrophoresis of a preparation, which contained mainly factor I, showed that this factor was one of the factors moving towards the anode. This behaviour is typical for factor C [3], as reported by Holdsworth [15].

From the above it is probable that the vitamin B_{12} activity of fresh and digested sludge derives from a mixture of cyanocobalamin *, factor A, pseudo-vitamin B_{12}, factor B, factor C, and a sixth factor (possibly the same as factor C_2 [16]). Factor I (C) seems to be rather labile and is either destroyed or converted to factor II (A and ψ-B_{12}), or factor IV (B), or both on standing in a water solution at room temperature. The bioautograms indicated that more than 50 % of the *E. coli* activity found was due to factor III (cyanocobalamin), 25 % to factor II and 25 % to factors I and IV.

In order to get a better proof for the identity of factors I, II, III, IV with the above mentioned vitamin B_{12} factors an attempt was made to isolate the different factors in a crystalline form from digested sludge.

* Chromatographic analysis without the addition of cyanide showed the presence in fresh and digested sludge of hydroxocobalamin and very little cyanocobalamin.

30 l digested sludge were boiled at pH 6.0 for 15 min. in the presence of 100 mg/l KCN. After centrifugation the solution was acidified to pH 3.0 and filtered with 2 % filter aid. The vitamin B_{12} activity of the filtrate was adsorbed on IRC50 [17] (Rohm & Haas) and thereafter eluted with *iso*propanol-water (60 : 40). The *iso*propanol of the eluate was distilled off *in vacuo* and the remaining liquid treated according to Schindler and Reichstein [21]. Factors I and IV were partly lost in the water phase during the phenol-chloroform extraction and in the methanol-insoluble material. Chromatography of the purified extracts on alumina column separated factors III and II. Factor III was eluted with methanol in a rather pure form while factor II left the column in impure form only after elution with water containing cyanide. The methanol eluate was evaporated and the red material purified by repeated crystallizations in acetone-water. Under the microscope the product was found to consist of red, needleshaped crystals.

A water solution of the pure factor III showed absorption maxima at 278, 323, 361, 523, and 550 mμ. The ratio E_{361}/E_{550} was 3.22. Cyanocobalamin gives the same absorption maxima and ratio [18] and it can therefore be concluded that factor III is identical with cyanocobalamin.

The digestion of municipal sewage sludge, which is carried out at 30° C at the three plants investigated, is believed to be accomplished in two stages by two types of bacteria. In the first stage the sludge is decomposed into simple organic compounds by bacteria with highly developed enzyme systems, *inter alia* cellulose fermenting bacteria. In the second step the degradation products formed are "mineralized" to methane, carbon dioxide and ammonia by methane bacteria. Whether the vitamin B_{12} is formed by one or both of these two types of bacteria has not been investigated. A cellulose thermophile, *Clostridium thermocellulaseum*, produced small amounts of vitamin B_{12} (mostly factor A [3]) when cultivated according to Enebo [13]. This indicates that at least some bacteria of the first type are able to synthesize vitamin B_{12}.

Other growth factors. Chromatography with *L. lactis* Dorner showed the presence of small amounts (less than 200 μg/g dry matter) of three desoxyribosides in fresh and digested sludge. They were identified as the desoxyribosides of thymine, adenine, and guanine by comparison with the corresponding pure compounds.

Fresh and digested sludge possessed 3.8 and 0.9 μg/g dry matter folic acid activity resp., and 7.7 and 1.3 μg/g folinic acid activity resp. It was evident that none of these growth factors was produced during the digestion process. Chromatographic studies revealed that the experimentally determined *L. citrovorum* and *S. faecalis* activities derived from five and seven different growth factors resp. (listed in Table 1). Some of the factors were identified with pure substances. The three unknown are probably identical with factors found in algae and lichens [19,20].

SUMMARY

Considerable amounts of vitamin B_{12} are formed during the anaerobic digestion of municipal sewage sludge. The vitamin B_{12} activity derives from hydroxocobalamin (50 %), factor A and pseudovitamin B_{12} (25 %), and factor B, factor C and factor C_2 (together 25 %).

Desoxyribosides and factors belonging to the folic acid and folinic acid groups have been found in fresh and digested sludge.

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Darzens' Glycidic Ester Condensation. The Isolation of an Aldol Intermediate *

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The aldol-halohydrin type of product, which is generally postulated as an intermediate in the Darzens condensation process, has been isolated in the condensation of benzaldehyde with ethyl chloroacetate (and ethyl α-chloropropionate), by using di*iso*propylaminomagnesium bromide as the basic condensing agent.

For the Darzens glycidic ester condensation [1] of an aldehyde or a ketone and an α-haloester ** in the presence of a basic reagent to form a glycidic ester, *e.g.* benzaldehyde and ethyl chloroacetate, with sodium ethoxide as the condensing agent, the following mechanism appears to be generally accepted***:

$$CH_2ClCOOEt + \bar{O}Et \rightleftharpoons \bar{C}HClCOOEt + HOEt$$

$$ArCH{=}O + \bar{C}HClCOOEt \rightleftharpoons Ar\overset{O^-}{\overset{|}{C}}H\text{-}CHClCOOEt \rightarrow Ar\overset{O}{\diagup\diagdown}CH\text{—}CHCOOEt + Cl^-$$

The base converts the ester partially into its anion which attacks the carbonyl carbon of the aldehyde to form an aldol-anion. This anion undergoes an intramolecular nucleophilic displacement of the halogen by the negatively charged oxygen. The aldol, whose anion is thus postulated as an intermediate, would be a halohydrin, and it appears quite reasonable that such a compound, in the basic medium, splits off hydrogen halide forming an epoxy compound. Furthermore, Darzens and Lévy [5] prepared the aldol ester, ethyl α-chloro-β-

* An abstract of this work was presented at The 8th Scandinavian Chemical Meeting in Oslo, June 1953.

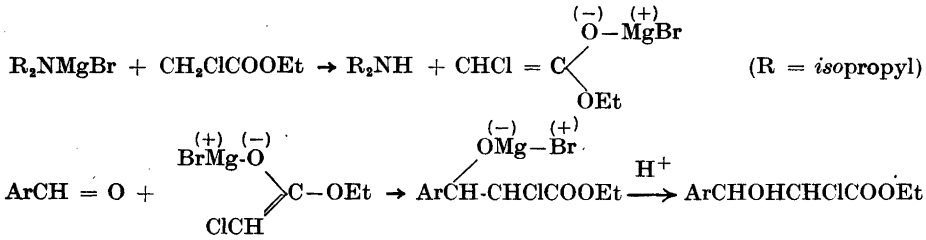
** The α-haloester may be replaced by α-haloketones, acylethylene-oxides being formed [2,3]. Preliminary attempts during the present work to obtain an aldol intermediate in the reaction between benzaldehyde or 2,4-dichlorobenzaldehyde (m.p. 72°) and phenacyl chloride failed as no crystalline product could be isolated and the reaction products decomposed on attempted distillation.

*** In reference 1 further references concerning mechanisms and applications are given. A review of various mechanisms suggested is given by Fourneau and Billeter[4] who, however, themselves favor a different mechanism.

hydroxy-β-phenylpropionate, independently, by a Grignard type of reaction from ethyl dichloroacetate and benzaldehyde, and they found that, by treatment with sodium ethoxide, it was transformed into phenylglycidic ester. Claisen[6], using sodium amide as the condensing agent, observed that some ethyl α-chlorocinnamate was formed in addition to phenylglycidic ester. This corresponds to the usual Claisen-Schmidt cinnamic ester synthesis and is consistent with the intermediate formation of an aldol:

$$ArCH = O + CH_2ClCOOEt \rightarrow ArCHOHCHClCOOEt \rightarrow ArCH = CClCOOEt + H_2O$$

The aldol itself, however, has hitherto not been isolated from the reaction products. This has now been accomplished by using di*iso*propylaminomagnesium bromide as the basic reagent. This reagent was introduced by Frostick and Hauser[7], and Hauser and Walker[8] have suggested the mechanism for the action of this type of reagent. In the present investigation di*iso*propylaminomagnesium bromide was chosen, because it might be expected that the largely covalent magnesium derivative of the aldol would be less apt to undergo nucleophilic displacement than the highly ionic sodium derivative *:



The condensations were generally carried out as described below for the case of benzaldehyde and ethyl chloroacetate:

To 0.2 equivalents of di*iso*propylaminomagnesium bromide suspended in dry ether and prepared as previously described [12] was added, during 20—30 minutes, with stirring and frequent cooling in ice-water, an ether solution of a mixture of 0.2 moles each of the aldehyde and the ester. An oily precipitate was formed, and, after the addition of about half of the solution the precipitate became doughy and stirring was hindered. The lumps were broken up with a spatula to render further stirring possible. The inefficient stirring may, in part, explain the poor yield **. After stirring for about two hours at room

* While this work was in progress a report has been published[9] describing the use of diethylaminomagnesium bromide and certain similar reagents for the condensation of ketones with esters to form β-hydroxyesters. Methylanilinomagnesium bromide has been used for the condensation of aldehydes with ketones[10] and for the mixed condensation af ketones[11], to form ketols.

** Several variations in the procedure were tried in order to improve the yield. The basic reagent was isolated by filtration and then added in small portions to the mixture of the starting materials. By this procedure the obstacle of the doughy precipitate was avoided as the reaction mixture remained a suspension of solid material. The yield, however, was still less than that described. Excess of basic reagent, longer reaction time and refluxing for some hours was tried. The recovery of starting materials was decreased, but the amount of non-distillable residue was increased, and the yield of desired product was negligible. In order to decrease the attack on the carbonyl group of the ester, *t*-butyl chloroacetate was tried, but the product obtained decomposed completely on distillation.

temperature the mixture was cooled in ice-salt; then an excess of glacial acetic acid (0.5 mole) and water were added. Stirring was continued until all solid material dissolved. The organic layer was separated, washed with sodium bicarbonate solution and water, dried over anhydrous sodium sulfate and the ether distilled. By fractional distillation *in vacuo* (2—3 mm) a fore-run, consisting of a mixture of the starting materials, was obtained below 80°. This amounted to 20—30 % of the total weight. By further distillation some fumes were evolved while material boiling at 130—160° passed over. The yield of this product was about 50 %. Then strong decomposition of the residue took place. This residue probably consists partially of products formed by attack of the base on the carbonyl groups of the esters. Di*iso*propylammonium chloride generally separated in the distillate. The distillate was, therefore, dissolved in ether, the ether solution successively washed with diluted hydrochloric acid, water, sodium bicarbonate solution and water, and dried. The solvent was distilled and fractional distillation now gave a product boiling at 145—150° at 3 mm, the over-all yield being about 40 %. Further redistillation rendered a fraction boiling at 148—150° at 3 mm, the over-all yield now amounting to 30 %. This fraction had a chlorine content of 14.5—15.5 % (calc. for the pure aldol Cl = 15.51) corresponding to an aldol content of 93—100 %. It is here assumed that the impurity is ethyl phenylglycidate. Partial crystallization was generally observed after 2—3 weeks; on seeding, crystallization started immediately. After standing, the crystals from several runs were collected by filtration, washed with ice-cold ethanol and recrystallized from ligroin (b.p. 60—100°), m.p. 72° (uncorr.).

$C_{11}H_{13}O_3Cl$ (228.69) Calc. C 57.77 H 5.72 Cl 15.51
Found » 57.91 » 5.95 » 15.44

In the case of ethyl α-chloropropionate, similar results were obtained. However, no crystallization took place, but two further redistillations gave a colorless oil, ,b.p. 158—60° at 5 mm, $n_D^{25} = 1.5256$, which analysed correctly for chlorine:

$C_{12}H_{15}O_3Cl$ (242.72) Calc. Cl 14.61
Found » 14.65

Compared with Claisen's observation [6] of the formation of ethyl α-chlorocinnamate in addition to ethyl phenylglycidate, the chlorine content in the above mentioned condensation product could, of course, be due also to that ester. Claisen's experiment was repeated and his observations verified. 50 % yield of a water-white product boiling sharply at 131° at 5 mm was obtained. No higher boiling material and only very little distillation residue was encountered. Its chlorine content corresponded to about 20 % of ethyl α-chlorocinnamate *. The boiling point of this product was, however, about 20° lower than that of the aldol. Another possibility would be that the aldol obtained in the condensation is contaminated also with ethyl α-chlorocinnamate. In that case the yield of aldol in the product could not be calculated from its chlorine content. However, in the case of ethyl α-chloropropionate, no cinnamic-type of product could be formed, and the assumption seems to be justified that the main product is the aldol, which is contaminated with small amounts of glycidic ester, whose boiling point is so near to that of the aldol that separation by distillation is difficult. The product also has a faint but characteristic smell of phenylglycidic ester, whereas the pure aldol isolated from it is odorless. Darzens and Lévy, in their preparation of the aldol ester, apparently obtained the compound as a yellowish liquid, and their

* The conclusion that the chlorine containing material is this ester is, according to Claisen, drawn from the fact that α-chlorocinnamic acid, m.p. 137°, neutr. equiv. 183.1 (calc. 182.63) is obtained after saponification, acidification and steam-distillation, by which latter process the glycidic acid is decarboxylated into phenylacetaldehyde; the chlorocinnamic acid crystallizes from the residue.

product may also have been contaminated with glycidic ester. During the present investigation this procedure was repeated. The product was a yellow oil which, according to its chlorine content, contained about 85 % aldol; the pure aldol did, however, after long standing, crystallize also from this product. The aldol was, furthermore, prepared in a second independent way, *viz.* by esterification of α-chloro-β-hydroxy-β-phenylpropionic acid, and was found to be identical with the aldol obtained in the condensation process and with that obtained according to the Darzens-Lévy procedure.

α-Chloro-β-hydroxy-β-phenylpropionic acid was prepared according to Rassow and Burmeister [13] by the addition of hypochlorous acid to cinnamic acid. The yield was 20 % (18 g). The esterification was effected by dissolving the crystalline acid in absolute ethanol and refluxing for ten hours while passing dry hydrogen chloride through the solution. On pouring into ice-water a viscous oil, which soon crystallized partially, was obtained. The product was distilled *in vacuo*, b.p. 180—190° at 15 mm. After recrystallization from ligroin (b.p. 60—100°) the melting point was 72° (uncorr.). The yield was 50 % (10 g).

$C_{11}H_{13}O_3Cl$ (228.69)	Calc.	C 57.77	H 5.72	Cl 15.51
	Found	» 57.68	» 5.82	» 15.48

Mixed melting points with the products obtained by the condensation and by the Darzens-Lèvy procedure showed no depression.

The analogous preparation of the aldol ester corresponding to ethyl α-chloropropionate was attempted. However, α-chloro-α-methyl-β-hydroxy-β-phenylpropionic acid is a liquid, which apparently dissolves some unchanged α-methylcinnamic acid, and by the subsequent esterification, the ester becomes contaminated with ethyl α-methylcinnamate. The chlorine content of the product (b.p. 115—117° at 0.5 mm, $n_D^{25} = 1.5145$) was therefore too low (13.2 %; calc. Cl = 14.61).

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Structure and Vapor Pressure of Aqueous Acid Solutions

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Heats of vaporization and relative partial vapor pressures for a few aqueous acid solutions have been calculated from available experimental data. Empirical relations have been established between those quantities and the molecular composition and the structure of the liquid. Those relations are discussed in terms of statistical methods developed for solutions which possess a lattice. Owing to lack of structure determinations on aqueous solutions, this discussion is limited to the dependence upon the number of nearest neighbors, while the dependence upon their geometrical arrangement cannot be treated.

The maximum number of nearest water molecule neighbors which can form bonds with an acid molecule is determined from the heat of vaporization of water. This number is 28 for H_2SO_4, H_2SeO_4 and $HClO_4$, 15 for HNO_3, and 10 for HCl.

The relations found are compared with the degrees of dissociation of the acids which have been determined by independent methods.

In the classical theory of liquids, the liquids, like gases, have been treated as statistically disordered, even as continua. However, it has been shown experimentally that liquids possess a structure in which the constituent atoms or molecules occupy spatially more or less well-defined sites. Although this structural nature of liquids is now almost universally accepted, this has not led to the revision of the classical theory of aqueous solutions required by this new concept.

The object of this paper is to draw attention to some empirical relations of a bearing upon the structural properties of aqueous acid solutions.

Our knowledge of structures of aqueous acid solutions is largely due to the experimental work done by Finbak and coworkers [1-2]. The structure of aqueous solutions is characterized by three main features:

1. Each constituent molecule is surrounded by a characteristic number of nearest neighbors.
2. Those nearest neighbors are located at almost fixed coordinates relative to the molecule considered, and in a characteristic geometrical arrangement.
3. The structure is "ordered" in the sense that solute and solvent molecules are distributed on the lattice sites in a characteristic order.

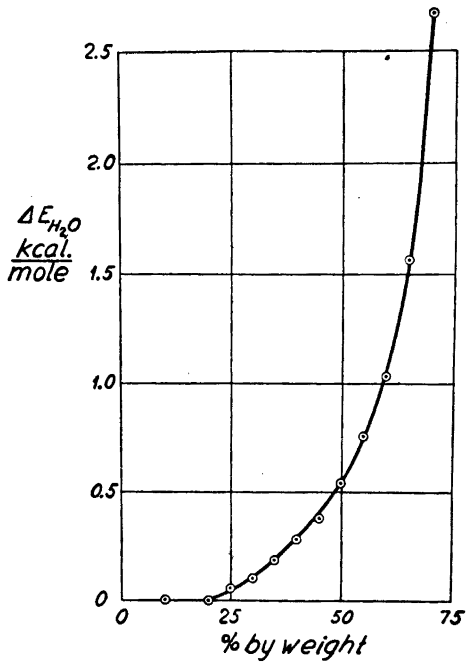


Fig. 1. ΔE_{H_2O} *for* H_2SO_4 *solutions.*

The liquid structure differs, thus, from the solid structure by lacking a "long range order", the liquid structure being essentially confined to the nearest neighbors only. Further, the structure seems to be less rigidly defined than that of solids.

In a recent series of papers [3] the author has shown that the dissociation of an acid in aqueous solution is a structural property which is very sharply quantitatively defined. It was concluded from those investigations that, within the experimental accuracy,

4. when the solution contains m molecules of water per molecule of acid on the average (and at not too great dilution), each single individual acid molecule is surrounded by m molecules of water;
5. this structural property is independent of the temperature.

A structure characterized by those five features should make itself particularly conspicuous in the partial vapor pressures of the solution. For that reason we will in the following discuss partial vapor pressures of some acid solutions on which experimental data are available.

A. HEATS OF VAPORIZATION

The heat of vaporization E of a constituent in a solution can be determined from its partial vapor pressure P by plotting $\ln P$ against $\frac{1}{T}$. The author has determined E and its dependence upon concentration for a few aqueous acid

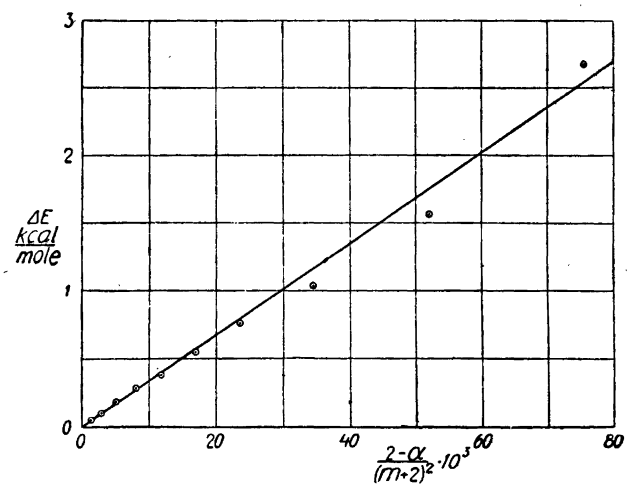


Fig. 2. ΔE_{H_2O} for H_2SO_4 solutions.

solutions on which experimental data are available. Data employed and results arrived at are listed in Table 1. For water we have written

$$E_{H_2O} = E_W + \Delta E_{H_2O} \quad (1)$$

with $E_W = 10.54$ kcal/mole, the value of E for pure water.

Table 1.

Solute	Experimental data by	Range of $\Delta E_{H_2O} = 0$	ΔE_{H_2O}	E_{solute}
HNO_3	Taylor [4]	all concentrations		
H_2SO_4	Collins [4]	$m > 28$	Figs. 1, 2; eq. (2)	
HCl	Yannakis [4]	$m > 10$	Figs. 3, 4; eq. (3)	
HCl	Zeisberg [4]			Figs. 5, 6; eq. (4)
NaCl	Olnyk & Gordon [5]	below saturation		
NaOH	Stock & Seelig [4]	<30 % by weight	at 50 %, $\Delta E_{H_2O} = 6$	

Fig. 3 shows particularly conspicuously the sudden rise of ΔE_{H_2O} at a certain concentration, $c = 5\ N$; $m = 10$.

From the plots in Figs. 2 and 4 we derive the empirical formulae for ΔE_{H_2O} in H_2SO_4 and HCl solutions, respectively,

$$\Delta E_{H_2O} = 3\ \frac{2 - \alpha}{(m + 2)^2}\ E_W \quad (2)$$

$$\Delta E_{H_2O} = 4.4\ \frac{10 - m}{(m + 1)^2}\ \text{kcal/mole} \quad (3)$$

The symbol α in (2) stands for the degree of dissociation [6] given in Table 3.

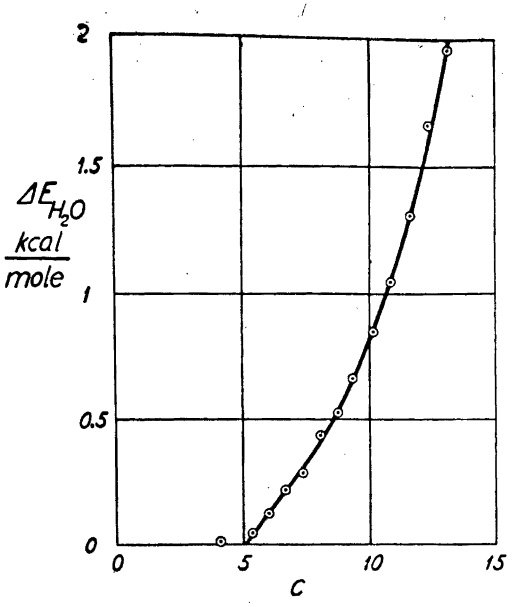


Fig. 3. ΔE_{H_2O} *for HCl solutions.*

For E_{HCl} we find from Fig. 5 the formula

$$E_{HCl} = 19.97 - \frac{46.27}{m+1} \text{ kcal/mole} \tag{4}$$

in the range $m \leqq 10$. The difference

$$\Delta E_{HCl} = E_{HCl} - (19.97 - \frac{46.27}{m+1}) \tag{5}$$

is shown in Fig. 6.

In a sufficiently dilute solution there are H_2O molecules which are bound to other H_2O molecules only, *i.e.* exactly as in pure water. Hence, $\Delta E_{H_2O} = 0$. At sufficiently high concentrations, however, there are no such H_2O molecules, all H_2O molecules present being bound to solute molecules too. Hence, ΔE_{H_2O} may or may not deviate from zero, depending upon the nature of the solute molecule.

According to Table 1 all solutions on which data are available show the first region $\Delta E_{H_2O} = 0$, more or less extended. From the data on H_2SO_4 and HCl solutions we may conclude that the H_2SO_4 molecule can form bonds with 28 H_2O molecules, and that the HCl molecule can form bonds with 10 H_2O molecules. At $m = 28$ the H_2SO_4, and at $m = 10$ the HCl molecules are "saturated" with water. When $m < 28$ and $m < 10$, there are H_2O molecules missing which may be replaced by H_2O molecules already bound to another acid molecule or by acid molecules. We may, for convenience, speak of $28-m$ or $10-m$ holes in the structure.

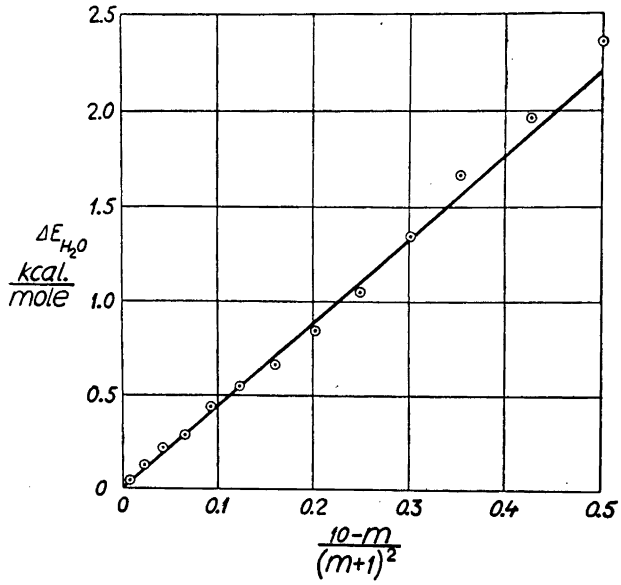


Fig. 4. ΔE_{H_2O} *for HCl solutions.*

As a first step in the discussion of the empirical formulae we will consider the significance of the various empirical constants.

The HCl molecule is bound to H_2O molecules by Cl—H—O hydrogen bonds. The energy of such a bond in o—C_6H_4OHCl is $E_{Cl-H-O} = 3.9$ kcal/mole [7]. It is also bound with the van der Waals energy $E_{v.d.W.}$ which we may estimate to the order of 0.5 kcal/mole. From considerations of the degree of dissociation as determined from Raman measurements [8], from electrolytic conductivity [9], and from the rates of dissolution of iron [10] and aluminum [11] in HCl solutions it may be concluded that the HCl molecule can form 5 hydrogen bonds with neighbor H_2O molecules in the region where (4) is valid. Hence, we should have $E_{HCl} = E_{v.d.W.} + 5\ E_{Cl-H-O}$. Putting this expression equal to the value given by (4) at infinite dilution we find $E_{v.d.W.} = 0.47$ kcal/mole. In H_2O (ice) and H_2O_2, $E_{O-H-O} = 4.5$ kcal/mole [7]. Assuming that $E_{O-H-O} = 4.627$ kcal/mole in HCl solutions we have, thus, from (4)

$$E_{HCl} = E_{v.d.W.} + 5\ E_{Cl-H-O} - \frac{10}{m+1} E_{O-H-O} \qquad (6)$$

According to Finbak the structures of HCl and NaOH solutions are very similar. Hemily has determined the structure of solid $NaOH \cdot 4\ H_2O$ and found the coordination number 5 [12]. At higher H_2O contents the coordination number increases to 6 [13]. The same increase of the number of nearest neighbors in HCl solutions would lead to

$$E_{HCl} = \tfrac{6}{5}\,(E_{v.d.W.} + 5\ E_{Cl-H-O}) = 23.96 \text{ kcal/mole} \qquad (7)$$

in good agreement with a reasonable extrapolation of observed values (Fig. 5).

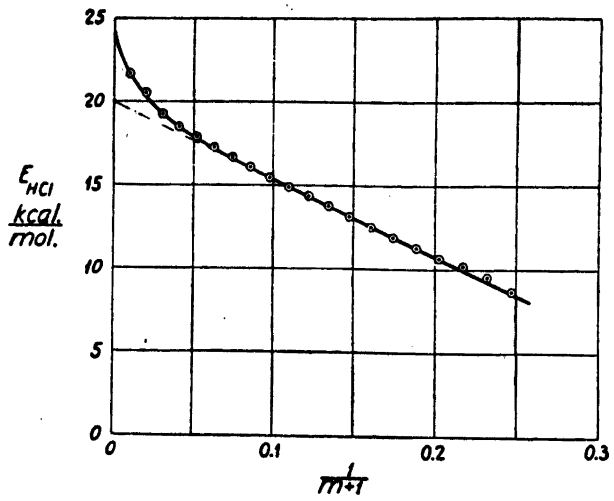


Fig. 5. E_{HCl} *for HCl solutions.*

The same values of $E_{v.d.W.}$ and E_{Cl-H-O} inserted in (3) give

$$\Delta E_{H_2O} = \frac{10 - m}{(m + 1)^2} (E_{v.d.W.} + E_{Cl-H-O}) \qquad (8)$$

In HCl solutions the total number of molecules, or sites occupied by molecules, is $m + 1$. The probabilities of finding an HCl molecule, an H_2O molecule, or a hole on a given site are $\frac{1}{m+1}$, $\frac{m}{m+1}$, and $\frac{10-m}{m+1}$, respectively. The prolability of the coincidence of an H_2O molecule and a hole is $\frac{m}{m+1} \cdot \frac{10-m}{m+1}$. It follows from the nature of the holes that an H_2O molecule filling a hole will form bonds with an HCl molecule with a corresponding change in its total binding energy. Dividing this change in bond energy equally between the m H_2O molecules we find an energy change per H_2O molecule proportional to $\frac{10-m}{(m+1)^2}$ as required by the empirical formula (3). However, if the number of nearest neighbors of the H_2O molecule filling the hole were unchanged, it would have to drop a bond with an H_2O molecule in order to form one with the HCl molecule. We find from (8) on the contrary that the energy of the H_2O—HCl bond is simply added to the energy of the bonds with the 4 H_2O neighbor molecules. Hence, the HCl molecule is simply added to the nearest neighbors and the number of nearest neighbors is effectively increased by $\frac{10-m}{(m+1)^2}$.

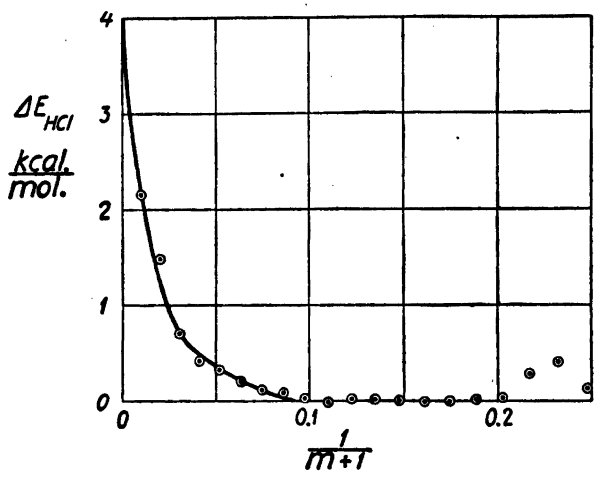


Fig. 6. ΔE_{HCl} for HCl solutions.

The same argument applied to the empirical formula (2) for H_2SO_4 solutions gives an effective increase of the number of nearest neighbors by

$$\Delta z = 4 \cdot 3 \ (2-\alpha). \tag{9}$$

Since any one of the 10 H_2O molecules may be missing, the probability of the formation of a hole is $\frac{10}{m+1}$. We find thus from (6) that the formation of a hole leads to a decrease in the binding energy of the HCl molecule by the energy of an O—H—O bond. The total increase of E_{H_2O} corresponds to an almost equal total decrease of E_{HCl}

$$\frac{10}{m+1} E_{O-H-O} - m\,\Delta E_{H_2O} = \frac{10}{m+1}\ 4.6 - m\frac{10-m}{(m+1)^2}\ 4.4 = \sim 4.5 \text{ kcal/mole}$$

We find thus a redistribution of bond energies which is difficult to interpret without an intimate knowledge of the structure.

B. VAPOR PRESSURE.

The relative partial vapor pressure p of a constituent in a solution is its actual partial vapor pressure divided by that of the pure substance. In the following we will consider water vapor pressures only.

For an ideal solution, *i.e.* when the molecules are nearly equal and no hydrates are formed, p is equal to the mole fraction (Raoult's law). Thus,

$$p = \frac{m}{m+1}. \tag{10}$$

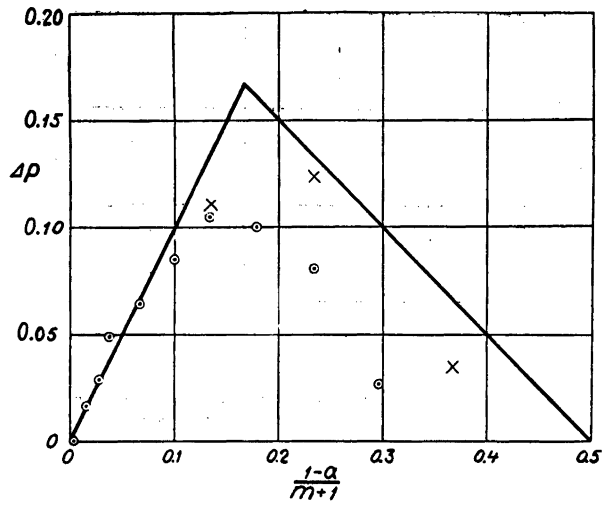


Fig. 7. Δp for HNO_3 solutions at 20° on the data of Taylor (⊙) and Wilson and Miles (×).

We will consider this simple case in some detail. Vaporization and condensation may be looked upon as rate processes at equilibrium. If the vapor is an ideal gas, which is a good approximation at low pressures, the rate of condensation is independent of the solute. Hence, this process does not appear in the relative partial pressure p. The activation energy of vaporization is E. The activation entropy of vaporization is composed of a term which is independent of the solute and which therefore cancels, and the entropy of mixing ΔS. Hence,

$$p = e^{\frac{\Delta S}{R}} e^{-\frac{\Delta E}{RT}}. \tag{11}$$

For an ideal solution we have $\Delta E = 0$ and

$$\begin{cases} \Delta S = k \ln W \\ W = (\frac{m}{m+1})^N. \end{cases} \tag{12}$$

As already pointed out we may consider a system of N identical aggregates consisting of one acid molecule and m water molecules. Hence, the probability of finding an H_2O molecule on a given site is $\frac{m}{m+1}$ and the total probability is $W = (\frac{m}{m+1})^N$. With $\Delta E = 0$ and ΔS from (12), (11) gives (10).

The condition $\Delta E = 0$ is fulfilled for HNO_3 solutions. Although HNO_3 and H_2O molecules are far from equal, we may expect (10) to be valid with the modifications required by the differences in shape and size of the two species.

From a plot of p against $\frac{1}{m+1}$ we find, in fact, at low concentrations,

$$p = \frac{m-1}{m+1}, \; m > 15. \tag{13}$$

Although $\Delta E = 0$, the simple formula (13) does not hold at higher concentrations.

For other acids $\Delta E = 0$ at low concentrations only. However, for the time being we will disregard this fact and merely present the data and the empirical formulae derived for p as a function of m at constant temperature, *viz.* room temperature. The data are listed in Table 2.

Table 2

Eq.	Fig.	Solute	Empirical formula	Experimental data by
(14)	7	HNO_3	$p = \frac{m-1}{m+1}(1-\frac{1-a}{m-1})$; $m > 3$	Taylor [4], Wilson & Miles [14]
(15)	8	H_2SO_4	$p = \frac{m}{m+2}(1-3\frac{2-a}{m})$; $m > 4$	Collins [4], Shankman & Gordon [15], Harned & Hamer [16]
(16)	8	H_2SO_4	$p = \frac{m}{m+2}(1-\frac{m+2}{2m})$; $2<m<4$	
(15)		H_2SeO_4	$p = \frac{m}{m+2}(1-3\frac{2-a}{m})$; $m > 4$	
(16)	9	H_2SeO_4	$p = \frac{m}{m+2}(1-\frac{m+2}{2m})$; $2<m<4$	Macalpine & Sayce [4]
(17)		H_2SeO_4	$p = 0$; $m < 2$	
(15)	10	$HClO_4$	$p = \frac{m}{m+2}(1-3\frac{2-a}{m})$; $m > 4$	Robinson & Baker [17], Pearce & Nelson [4]
(18)	11	HCl	$p = \frac{m-2}{m+1}$; $m > 26$	Yannakis [4], Wrewsky, Savaritsky & Scharloff [4]
(19)	11	HCl	$p = \frac{m-2}{m+1}(1-\frac{1}{2}\frac{26-m}{m}\frac{a}{m+1})$; $m < 26$	Yannakis [4], Wrewsky, Savaritsky & Scharloff [4]

The symbols a are defined in Table 3.

Table 3

HNO_3	H_2SO_4, H_2SeO_4, $HClO_4$	HCl
$a = \frac{m}{9}$; $3 \leqq m \leqq 6$	$a = \frac{m}{4}$; $m \leqq 4$	$a = \frac{m}{5}$; $m \leqq 5$
$a = \frac{12+m}{27}$; $6 \leqq m \leqq 15$	$a = 1 + \frac{m-4}{24}$; $4 \leqq m \leqq 28$	$a = 1$; $m \geqq 5$
$a = 1$; $m \geqq 15$	$a = 2$; $m \geqq 28$	

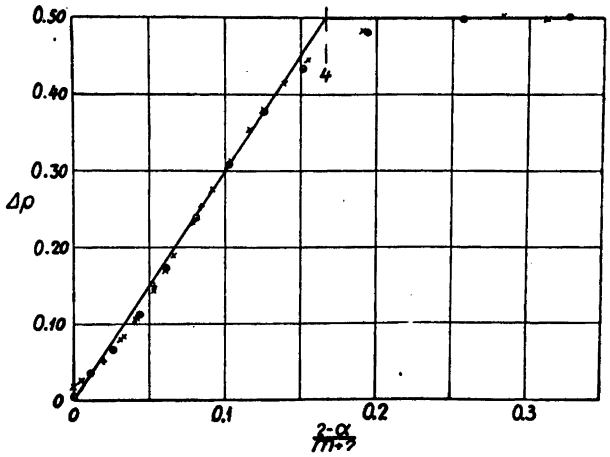


Fig. 8. Δp for H_2SO_4 solutions at 20° on the data of Collins (⊙), Shankman and Gordon (×), and Hanred and Hamer (+).

For HNO_3[18] and H_2SO_4[6] and H_2SeO_4, α is the degree of dissociation. For $HClO_4$[9] the degree of dissociation is $\alpha = \frac{m}{4}$, $m \leqq 4$; $\alpha = 1$, $m \geqq 4$; thus, in this case α is merely a structure variable for $m > 4$. For HCl no direct measurements of the degree of dissociation have been made. From the considerations mentioned earlier in this paper we feel safe in concluding that $\alpha = 1$ for $m > 5$. The rate of dissolution of aluminum[11] in HCl solutions seems to indicate that for $m < 5$, $\alpha = \frac{m}{5}$ is the degree of dissociation of HCl.

The figures listed in Table 2 show the functions Δp defined as

$$\Delta p = \frac{m-1}{m+1} - p \text{ for } HNO_3 \tag{20}$$

$$\Delta p = \frac{m}{m+2} - p \text{ for } H_2SO_4,\ H_2SeO_4,\ HClO_4 \tag{21}$$

$$\Delta p = \frac{m-2}{m+1} - p \text{ for HCl} \tag{22}$$

Before turning to a discussion of the details of those empirical formulae we will consider the role of ΔE in the basic formula (11).

The empirical formulae are all of the same general form whether $\Delta E = 0$ (HNO_3) or $\Delta E \neq 0$. In the case of H_2SO_4, $\Delta p = 0$ and $\Delta E = 0$ in the same range, $m > 28$, in the case of HCl, however, $\Delta p = 0$ for $m > 26$ and $\Delta E = 0$ for $m > 10$, whereas in the case of HNO_3 $\Delta p = 0$ for $m > 15$ and $\Delta E = 0$ for all m. Hence, there is no obvious connection between Δp and ΔE, *i.e.* the deviations from the limiting formulae (m large) do not follow ΔE.

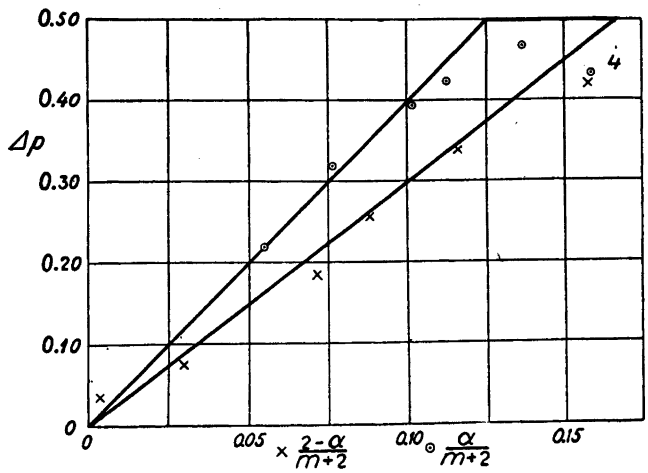


Fig. 9. Δ p for H_2SeO_4 solutions at 25°. $\times\ \Delta p = f\left(\frac{2-a}{m+2}\right), \odot \Delta p = f\left(\frac{a}{m+2}\right)$.

The activation entropy ΔS consists of two parts, the configurational entropy $\Delta S_{\text{conf.}}$ and the thermal entropy $\Delta S_{\text{th.}}$. If we put

$$\Delta S_{\text{th.}} = \frac{\Delta E}{T_0} \tag{23}$$

in (11), we obtain

$$p = e^{\frac{\Delta S_{\text{conf.}}}{R}} e^{\frac{\Delta E}{RT_0}} e^{-\frac{\Delta E}{RT}} \tag{24}$$

Hence, at $T = T_0$ and $\Delta E \neq 0$

$$p = e^{\frac{\Delta S_{\text{conf.}}}{R}} \tag{25}$$

Thus, at $T = T_0$ or $\Delta E = 0$, p depends on the configurational entropy $\Delta S_{\text{conf.}}$ only. We may then determine $\Delta S_{\text{conf.}}$ from (25).

Formulae similar to (24) have been derived previously from empirical and theoretical considerations. For the splitting of the entropy into $\Delta S_{\text{conf.}}$ and $\Delta S_{\text{th.}}$ reference is made to Gurney [19]. Moelwyn-Hughes [20] and Guggenheim [21] arrive at a $\Delta S_{\text{th.}}$ for reaction rates in solution and vapor pressure, respectively, by way of a temperature-dependent activation energy. Cremer [22] and Barrer [23] report that the logarithm of the entropy factor A in the Arrhenius rate expression is a linear function of the activation energy E. For catalysis, Cremer finds that E and log A are linear functions of the temperature at which the catalyst was prepared which intersect at a certain temperature. Barrer [23] finds that, for diffusion, viscosity, and gas reactions, T_0 is the average temperature in the temperature range of the experiment. Dienes [24,25] finds that,

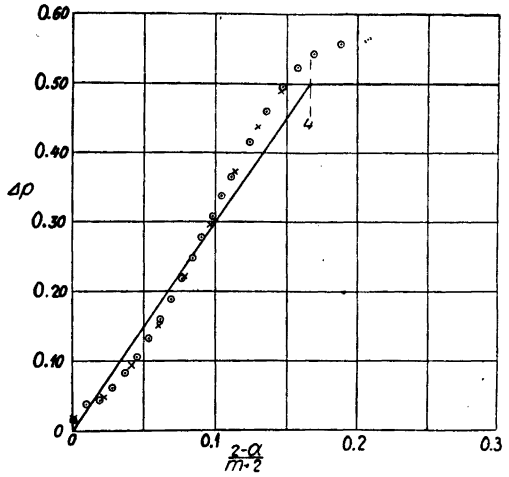
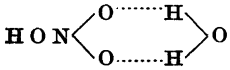


Fig. 10. Δp for $HClO_4$ solutions at 25° on the data of Robinson and Baker (⊙) and Pearce and Nelson (×).

in diffusion in metals, log A is a linear function of $\frac{E}{T_m}$, T_m being the melting point; $\frac{E}{T_m}$ is identified with the entropy of activation.

It is interesting to observe that the formula (25) is valid at room temperature when $\Delta E \neq 0$. This would seem to imply that T_0 is the temperature at which the solution was prepared. The same result has been obtained for electrolytic conductivities [9]. Preliminary measurements of conductivities of solutions prepared at different temperatures do not support that view.

The thermodynamic interpretation of (23) seems to be a theoretical problem which still remains to be solved.

At sufficiently low concentrations the formulae of Table 2 are of the type (10). Denoting by m^* the effective number of H_2O molecules we have $m^* = m-1$ for HNO_3, $m^* = m$ for H_2SO_4, and $m^* = m-2$ for HCl. According to Chédin and coworkers [26,27], when H_2O is added to HNO_3, all H_2O molecules added are bound within the HNO_3 molecules to form the monohydrate  as long as $m \leqq 1$. A H_2O molecule bound in this way may not be expected to contribute to the vapor pressure of H_2O. It was also mainly from the very low value of p in the region $m < 1$ as well as from spectroscopic evidence that Chédin concluded on this structure. It has been shown by Luzzati that this structure does not occur in the solid mono-[28] and trihydrates [29]. However, it is difficult to conclude from the structure of the solid hydrate upon that of the liquid hydrate. In the X-ray investigations by Finbak and Lunde this structure was not found at $m = 3$ and $m = 6$, nor do structural considerations on the degree of dissociation admit of this structure in that region.

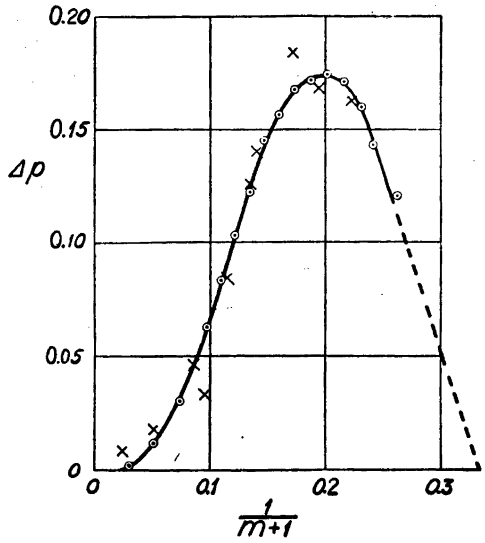


Fig. 11. Δp for HCl solutions at 20° on the data of Yannakis (⊙) and Wrewsky, Savaritsky and Scharloff (×).

In order to account for all this evidence we are led to assume that the monohydrate of Chédin is not actually present in the dilute solution but that there is such a strong tendency towards its formation that one of the m H_2O molecules should be excluded when computing the probability of H_2O vaporization. The limiting formulae thus lead us to conclude upon the ultimate formation of the hydrates $HNO_3 \cdot H_2O$ and $HCl \cdot 2H_2O$.

Applying the same argument to the range of incomplete dissociation we find $m^* = m-1-(1-\alpha)$ for HNO_3 and $m^* = m-3(2-\alpha)$ for H_2SO_4. The latter formula should be compared with the formula (9) for the number of nearest neighbors. When $\Delta m = m-m^* = 3(2-\alpha)$ is the decrease of the number of nearest neighbors, the theory of regular solutions [21] gives a decrease of E by the amount

$$\Delta E = \frac{3(2-\alpha)}{m} \frac{m}{m+2} \frac{1}{m+2} \cdot \frac{1}{4} E_w \tag{26}$$

Comparing (2) and (26) we have

$$\Delta E_{H_2O} = -4\ \Delta E. \tag{27}$$

Hence, the vapor pressure shows an effective decrease of the number of H_2O molecules by $\Delta m = 3(2-\alpha)$ whereas the heat of vaporization shows an effective increase of the number of nearest neighbors by $\Delta z = 4 \cdot 3(2-\alpha)$, or, inserting $2-\alpha = \frac{28-m}{24}$,

$$\begin{cases} \Delta m = -\frac{1}{8}(28-m) \\ \Delta z = \frac{1}{2}(28-m), \end{cases} \tag{28}$$

$28-m$ being the number of missing H_2O molecules.

The curves of Δp for HNO_3 and for H_2SO_4 and H_2SeO_4 are fairly well approximated by the formulae given in Table 2. In particular, the curves drawn pass through the origin, *i.e.* $\Delta p = 0$ for $\alpha = 1$ and $\alpha = 2$, respectively. In the case of HNO_3 the data are not too reliable though, as shown by the great difference between the data of Taylor and of Wilson and Miles. At high concentrations the HNO_3 molecule is likely to decompose into N_2O_5 and H_2O. This would tend to increase p and reduce Δp.

The values of Δp for H_2SO_4 fall close to a straight line. However, the experimental data show a tendency towards a S-shaped curve. This is particularly conspicuous in the case of $HClO_4$. The formulae for those acids give therefore a first approximation only. We may interpret the deviation as a random deviation from the structure.

The formula (19) for HCl has been derived as follows: Inserting for α the expressions given in Table 3 we have

$$\Delta p = \frac{1}{27}\frac{15-m}{m+1};\ m > 3,\ \text{for } HNO_3 \tag{29}$$

$$\Delta p = \frac{1}{8}\frac{28-m}{m}\frac{m}{m+2};\ m > 4 \text{ for } H_2SO_4 \tag{30}$$

Trying for HCl an expression of a similar form

$$p = \frac{m-2}{m+1}\left[1-A\frac{B-m}{m}\frac{1}{m+1}\right];\ m > 5 \tag{31}$$

we find $A = \frac{1}{2}$ and $B = 25$ or $B = 26$. At higher concentrations we have to add a factor $\frac{m}{5}$ to the second term.

Introducing a quantity α defined in Table 3 we have thus, at all concentrations,

$$p = \frac{m-2}{m+1}\left[1-A\frac{B-m}{m}\frac{\alpha}{m+1}\right] \tag{32}$$

Trying various values of B we find that $B = 25$ and $B = 26$ give constant values of A, *viz.* $A = 0.517 \pm 0.004$ and $A = 0.491 \pm 0.004$ respectively. The constancy of A is, thus, the same for both values of B. We have taken in (19), somewhat arbitrarily, $B = 26$. The values of A calculated from (32) are given in Table 8. It is difficult to see the significance of $m = 26$. No other property of HCl solutions is known to show any change at that particular concentration.

According to Finbak [30] the structure of HCl solutions is not well defined. It is true that certain values of m, such as $m = 5$ and $m = 10$, are as conspicuously manifested in physical and chemical properties of the HCl solutions as are the corresponding values of m for HNO_3 and H_2SO_4 solutions. The comparatively complicated formula for p with

$$\Delta m = \tfrac{1}{2}\frac{m-2}{m+1}\frac{26-m}{m}\frac{\alpha}{m+1} \tag{33}$$

seems to indicate a certain lack of definition of the structure.

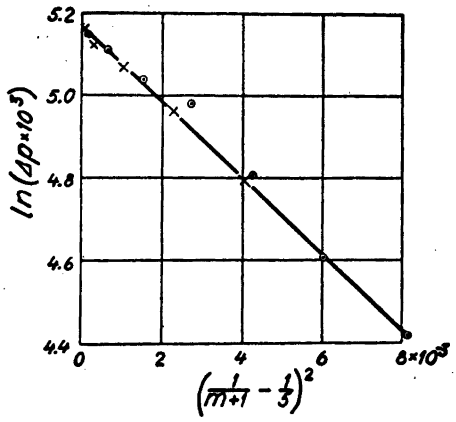


Fig. 12. $ln(\Delta p \cdot 10^3) = f\left[(\frac{1}{m+1} - \frac{1}{5})^2\right]$ *for HCl solutions at 20° on the data of Yaninakis,* ⊙ $m > 4$, × $m < 4$.

Fig. 11 shows Δp as a function of $\frac{1}{m+1}$. The form of the curve suggests an error function for Δp. Fig. 12 shows $\ln(\Delta p \cdot 10^3) = f\left[(\frac{1}{m+1} - \frac{1}{5})^2\right]$. This gives us

$$\Delta p = 0.175\ e^{-3.6(\frac{m-4}{m+1})^2} \tag{34}$$

and

$$\Delta m = \frac{1}{\sqrt{\pi\ h}}\ e^{-\frac{x^2}{h}} \tag{35}$$

with

$$\begin{cases} h = \dfrac{10}{(m+1)^2} \\ x = 6\ \dfrac{m-4}{(m+1)^2} \end{cases} \tag{36}$$

It should be added that the representation (4) of E_{HCl} as a function of the molecular composition may as well, disregarding any physical significance of the formula, be substituted by a function of the normality of the acid. In fact, the formula

$$E_{HCl} = 20.3 - 0.89\ c \text{ kcal/mole} \tag{37}$$

is valid in a still wider range of concentrations, *viz.* $c > 3\ N$; $m < 17.4$, than is (4).

We may now look at the denominators of the empirical formulae from the same point of view as previously applied to the nominators. From the formulae for H_2SO_4 we may conclude that the H_2SO_4 molecule effectively occupies two sites. This is consistent with the formula for HNO_3, because if one H_2O molecule is taken up by the HNO_3 molecule, this hydrate would be similar to the H_2SO_4

molecule and therefore cover two sites as does the H_2SO_4 molecule. Hence, the total number of occupied sites should be $(m-1) + 2 = m + 1$.

This result is somewhat surprising. We should, in fact, expect the H_2SO_4 molecule to occupy 5 and the HNO_3 molecule to occupy 4 times as many sites as does the H_2O molecule. However, it should be recalled that the atomic distances in the H_2SO_4 and HNO_3 molecules are much smaller than, about half, the distance between adjacent H_2O molecules. The result is therefore altogether reasonable.

From the data and from the discussion given above it may be concluded that the structural properties of aqueous acid solutions are conspicuously manifested in their heats of vaporization and vapor pressures. It may also be concluded that a simple approach to the theory of liquids may be based upon statistical methods developed for solid solutions. However, the structures are not sufficiently known as yet for a rigorous treatment along those lines. From the empirical data and relations presented in this paper we may only conclude upon the number of nearest neighbors but not upon their geometry. Due to this lack of data we can not interpret the significance of the change of "effective" number of neighbors. For that purpose we need not only the structural positions of the O, S, N etc. atoms but also those of the H atoms. Another difficulty pointed out is the significance of the constant temperature T_0 found in various connections. This seems to be a problem of theoretical thermodynamics.

Although it is hard to believe that the vapor pressure should depend upon the degree of dissociation, those two quantities are undoubtedly related, probably owing to their both being dependent upon the structure. For that reason experimental determinations of degrees of dissociation may be expected to contribute to our knowledge of the structure and, therefore, facilitate the study of vapor pressures.

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APPENDIX

The calculated values are given in the following Tables 4—11. At low concentrations where $\Delta p = 0$ and $\Delta E_{H_2O} = 0$, the small deviations actually observed and which are of the order of experimental errors, can not be represented to the scale of the curves of Δp and ΔE_{H_2O}. This is apparent from an examination of the data given in the tables. However, in some cases, notably for $HClO_4$, there is a tendency towards increasing values of Δp in the range where, according to the text, Δp should be zero. This tendency would not show up properly in the graphical representation of Δp.

In calculating ΔE_{H_2O} for HCl, we have not employed the data given by Yannakis at 6 and 10 % HCl by weight because the spread of the data is so large that no estimate of ΔE_{H_2O} can be made. At 14 % HCl by weight the data seemed to allow the determination of ΔE_{H_2O}; the value given in Table 11

Table 4. Relative partial vapor pressure of water in HNO_3 solutions at 20°

% HNO_3 by weight	m	a	$\frac{m-1}{m+1}$	$\frac{1-a}{m+1}$	p	Δp
	Taylor		%	%	%	%
20	13.9911	0.9626	86.66	0.25	86.66	0.00
25	10.4933	0.8331	82.60	1.45	80.96	1.64
30	8.1615	0.7467	78.16	2.77	75.26	2.90
35	6.4958	0.7218	73.32	3.71	68.42	4.90
40	5.2467	0.3830	67.98	6.68	61.57	6.41
45	4.2751	0.4750	62.08	9.96	53.59	8.49
50	3.4978	0.3886	55.54	13.59	45.04	10.50
55	2.8618	0.3103	48.21	17.85	38.20	10.01
60	2.3319	0.2220	39.98	23.35	31.93	8.05
65	1.8834	0.1472	30.64	29.57	27.94	2.70
70	1.4991	0.0832	19.98	36.68	23.38	
	Wilson and Miles					
50	3.4978	0.3886	55.54	13.59	44.47	11.07
60	2.3319	0.2220	39.98	23.35	27.65	12.33
70	1.4991	0.0832	19.98	36.68	16.53	3.45

Table 5. Relative partial vapor pressure of water in H_2SO_4 solutions at 25°.

% H_2SO_4 by weight	m	a	$\frac{m}{m+2}$	$\frac{2-a}{2+m}$	p	Δp
	Collins		%	%	%	%
10	49.00	2	96.08	0.00	95.6	0.5
20	21.776	1.7407	91.59	1.09	88.0	3.6
25	16.3322	1.5318	89.09	2.65	82.5	6.6
30	12.7026	1.3628	86.40	4.33	75.2	11.2
35	10.1104	1.2546	83.49	6.15	66.3	17.2
40	8.1561	1.1732	80.31	8.14	56.5	23.8
45	6.6539	1.1106	76.89	10.28	46.1	30.8
50	5.4440	1.0602	73.13	12.63	35.7	37.6
55	4.4542	1.0189	69.01	15.20	25.8	43.2
60	3.6294	0.9074	64.47	19.41	16.6	47.9
65	2.9314	0.7329	59.44	25.70	9.7	49.7
70	2.3331	0.5833	53.84	32.70	3.7	50.1
	Shankman and Gordon					
15.835	28.9346	2	93.535		91.74	1.79
18.005	24.7912	1.8663	92.535	0.499	89.95	2.58
26.410	15.1689	1.4654	88.351	3.114	80.48	7.87
27.025	14.6998	1.4458	88.024	3.319	79.64	8.38
29.265	13.1580	1.3816	86.806	4.080	76.27	10.54
29.560	12.9724	1.3739	86.642	4.182	75.86	10.78
29.850	12.7935	1.3664	86.481	4.283	75.39	11.09
32.67	11.2192	1.3008	84.870	5.289	70.62	14.25
34.85	10.1769	1.2574	83.575	6.098	66.79	16.78
36.39	9.5159	1.2298	82.633	6.688	63.79	18.84
39.55	8.3206	1.1800	80.621	7.945	57.43	23.19
42.51	7.3622	1.1401	78.637	9.185	51.10	27.54
45.12	6.6214	1.1092	76.802	10.332	45.42	31.38
47.97	5.9046	1.0794	74.698	11.646	39.34	35.36
49.85	5.4766	1.0615	73.250	12.552	35.37	37.88
52.42	4.9412	1.0392	71.187	13.842	29.93	41.26
55.51	4.3631	1.0151	68.569	15.478	24.04	44.53
59.73	3.6702	0.9176	64.728	19.089	16.67	48.16
66.95	2.6873	0.6718	57.332	28.336	7.20	50.13
68.94	2.4527	0.6107	55.083	31.201	5.38	49.70

	Harned and Hamer					
0.5 *	111.012	2	98.230		98.21	0.02
1	55.506	2	96.522		96.20	0.32
2	27.753	1.9897	93.278	0.035	91.36	1.92
3	18.502	1.6043	90.245	1.930	85.06	5.18
5	11.1012	1.2959	84.734	5.374	69.80	14.93
7	7.9295	1.1637	79.858	8.422	54.53	25.33

*) moles per 1 000 g of H_2O.

Table 6. Relative partial vapor pressure of water in H_2SeO_4 solutions at 25° according to Macalpine and Saycer.

% H_2SeO_4 by weight	m	a	$\frac{m}{m+2}$ %	$\frac{2-a}{2+m}$ %	p %	Δp %
23.93	25.580	1.8992	92.75	0.36	89.27	3.58
34.13	15.5306	1.4804	88.59	2.97	81.02	7.57
47.08	9.0452	1.2102	81.89	7.15	63.59	18.30
51.34	7.6270	1.1511	79.23	8.81	53.83	25.40
57.58	5.9284	1.0804	74.77	11.60	41.04	33.73
65.30	4.2761	1.0115	68.13	15.75	26.18	41.95
67.58	3.8604	0.9289	65.87	18.28	22.64	43.21
76.69	2.4459	0.6077	55.01	31.32	8.25	46.76
83.18	1.6272	0.4068	44.86	43.92	2.61	42.25
85.48	1.3669	0.3417	40.60	49.25	1.35	39.25
89.29	0.9652	0.2413	32.55	59.31	0.76	31.79
93.50	0.5594	0.1399	21.86	72.67	0.13	21.73

Table 7. Relative partial vapor pressure of water in $HClO_4$ solutions at 25°.

Moles of $HClO_4$ per 1 000 g of H_2O	m	a	$\frac{m}{m+2}$ %	$\frac{2-a}{m+2}$ %	p %	Δp %
			Robinson and Baker			
0.1	555.06	2	99.641	0	99.658	0.02
0.2	277.53	2	99.285	0	99.317	0.03
0.3	185.02	2	98.931	0	98.970	0.04
0.4	138.77	2	98.579	0	98.618	0.04
0.5	111.01	2	98.230	0	98.258	0.03
0.6	92.510	2	97.884	0	97.888	0.00
0.7	72.295	2	97.308	0	97.510	0.20
0.8	69.438	2	97.200	0	97.124	0.08
0.9	61.723	2	96.861	0	96.727	0.13
1.0	55.506	2	96.522	0	96.319	0.20
1.2	46.255	2	95.855	0	95.471	0.38
1.4	39.647	2	95.198	0	94.572	0.63
1.6	34.691	2	94.549	0	93.637	0.91
1.8	30.837	2	93.909	0	92.660	1.25
2.0	27.753	1.9897	93.278	0.0346	91.650	1.63
2.5	22.203	1.7585	91.737	0.9978	88.915	3.82
3.0	18.502	1.6043	90.245	1.9303	85.900	4.35
3.5	15.859	1.4941	88.801	2.8327	82.650	6.15

4.0	13.877	1.4115	87.403	3.7066	79.152	8.25
4.5	12.335	1.3473	86.048	4.5532	75.479	10.57
5.0	11.101	1.2959	84.734	5.3744	71.530	13.20
5.5	10.092	1.2538	83.460	6.1710	67.528	15.93
6.0	9.2510	1.2188	82.224	6.9434	63.430	18.79
6.5	8.5394	1.1891	81.024	7.6940	59.271	21.75
7.0	7.9295	1.1637	79.858	8.4224	55.081	24.78
7.5	7.4008	1.1417	78.725	9.0237	50.972	27.75
8.0	6.9438	1.1227	77.638	9.8090	46.872	30.77
8.5	6.5301	1.1054	76.554	10.487	42.888	33.67
9.0	6.1723	1.0905	75.527	11.129	39.043	36.48
10.0	5.5506	1.0646	73.512	12.388	31.950	41.56
11.0	5.0460	1.0436	71.615	13.574	25.651	45.96
12.0	4.6255	1.0261	69.814	14.699	20.296	49.52
13.0	4.2697	1.0112	68.101	15.771	15.825	52.28
14.0	3.9647	1.9912	66.469	16.913	12.234	54.24
15.0	3.7004	1.9251	64.915	18.857	9.310	55.61
16.0	3.4691	1.8673	63.431	20.711	7.024	56.41
			Pearce and Nelson			
0.10016	554.18	2	99.640	0	99.663	0.02
0.20064	276.646	2	99.282	0	99.331	0.05
0.40257	137.890	2	98.570	0	98.640	0.08
0.60655	91.511	2	97.861	0	97.903	0.04
0.81037	68.495	2	97.163	0	97.116	0.04
1.01589	54.638	2	96.469	0	96.287	0.18
2.0661	26.865	1.9527	93.071	0.1639	91.331	1.74
3.1512	17.614	1.5673	89.803	2.2051	85.012	4.79
4.2734	12.989	1.3745	86.657	4.1731	77.412	9.25
5.4347	10.2133	1.2589	83.624	6.0680	68.659	14.97
6.6372	8.3629	1.1818	80.700	7.8955	58.669	22.03
7.8719	7.0512	1.1271	77.903	9.6440	48.375	29.53
9.1723	6.0515	1.0855	75.360	11.358	37.959	37.20
10.513	5.2798	1.0533	72.527	13.005	28.789	43.74
11.905	4.6624	1.0276	69.981	14.595	20.975	49.01

Table 8. Relative partial vapor pressure of water in HCl solutions at 20°

% HCl by weight	m	$\frac{1}{m+1}$ %	$P^{\circ}_{H_2O}$ mm Hg	p %	$\frac{m-2}{m+1}$ %	Δp %	$\frac{A}{m=25}$	$\frac{A}{m=26}$
	Yannakis							
6	31.72	3.06	15.9	90.65	90.82	0.17	—	—
10	18.22	5.20	14.6	83.24	84.40	1.16	(0.710)	(0.619)
14	12.44	7.44	13.1	74.69	77.68	2.99	0.512	0.474
18	9.223	9.78	11.3	64.42	70.66	6.24	0.528	0.496
20	8.098	10.99	10.3	58.72	67.03	8.31	0.540	0.510
22	7.178	12.23	9.30	53.02	63.31	10.29	0.535	0.507
24	6.411	13.49	8.30	47.32	59.53	12.21	0.524	0.498
26	5.762	14.79	7.21	41.11	55.63	14.52	0.529	0.502
28	5.206	16.11	6.32	36.03	51.67	15.44	0.488	0.464
30	4.724	17.47	5.41	30.84	47.59	16.75	0.497	0.473
32	4.302	18.86	4.55	25.94	43.42	17.18	0.507	0.483
34	3.930	20.28	3.81	21.72	39.16	17.44	0.521	0.498
36	3.599	21.74	3.10	17.67	34.78	17.11	0.529	0.505
38	3.303	23.24	2.51	14.31	30.28	15.97	0.523	0.498
40	3.037	24.77	2.00	11.40	25.69	14.29	0.511	0.489
42	2.796	26.35	1.56	8.89	20.95	12.06	0.492	0.471

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5.05	38.06	2.56	16.05	91.51	0.81		
10.00	18.22	5.20	14.5	82.67	1.73	1.060	0.924
16.03	10.60	8.62	12.2	69.56	4.59	0.529	0.495
18.05	9.192	9.81	11.8	67.27	3.29	0.276	0.256
20.9	7.662	11.54	10.0	57.01	8.36	0.490	0.463
24.1	6.376	13.56	8.2	46.75	12.58	0.536	0.509
24.98	6.080	14.12	7.65	43.61	14.02	0.554	0.526
30.05	4.713	17.51	5.1	29.08	18.40	0.546	0.520
32.97	4.116	19.55	4.3	24.52	16.84	0.499	0.476
36.89	3.463	22.40	2.9	16.53	16.26	0.514	0.491

Table 9. Relative partial vapor pressure (in %) of water in HNO_3 solutions at various temperatures according to Taylor.

t°C % HNO_3 by weight	0	5	10	15	20	25	30	35	40	45	50
20	89.52	87.16	86.86	85.22	86.66	86.70	86.74	86.53	85.86	86.26	86.48
25	82.97	82.57	82.52	80.53	80.96	80.81	80.77	80.13	79.54	79.99	81.07
30	78.60	76.45	77.09	75.84	75.26	74.92	74.80	73.73	74.11	73.73	74.59
35	72.05	70.34	70.58	69.59	68.42	66.18	68.20	67.09	68.15	66.78	68.10
40	65.50	64.22	62.98	62.55	61.57	61.45	61.28	60.46	60.56	59.82	60.53
45	56.77	55.05	54.29	53.95	53.59	53.45	53.11	52.87	52.96	52.87	53.51
50	45.85	45.87	45.60	45.35	45.04	45.03	45.25	45.05	45.19	45.21	45.94
55	39.30	38.23	38.00	38.31	38.20	38.30	38.34	38.17	38.50	38.95	39.24
60	32.75	32.11	32.57	32.06	31.93	32.41	32.37	32.24	32.72	32.97	33.51
65	28.38	27.52	28.23	27.37	27.94	27.78	27.66	27.50	28.08	27.82	28.10
70	24.02	24.46	23.89	23.46	23.38	23.15	23.26	23.23	23.14	23.23	23.56
80	—	—	13.03	13.29	13.68	13.47	12.57	13.04	12.65	13.22	12.97
90	—	—	—	—	—	4.21	4.09	4.27	4.34	4.17	4.32

Table 10. ΔE_{H_2O} for H_2SO_4 solutions calculated from data by Collins.

% H_2SO_4 by weight	m	a	$\frac{2-a}{(m+2)^2} \cdot 10^3$	ΔE_{H_2O} kcal/mole	$\Delta E_{H_2O} \frac{(m+2)^2}{2-a}$
10	49.000	2	0	0	0
20	21.776	1.7407	0.4587	0	0
25	16.3322	1.5318	1.3932	0.0549	39.41
30	12.7026	1.3628	2.9477	0.101	34.26
35	10.1104	1.2546	5.0824	0.185	36.40
40	8.1561	1.1732	8.0158	0.281	35.06
45	6.6539	1.1106	11.8761	0.379	31.91
50	5.4440	1.0602	16.960	0.543	32.02
55	4.4542	1.0189	23.552	0.759	32.23
60	3.6294	0.9074	34.478	1.037	30.08
65	2.9314	0.7329	52.104	1.564	30.02
70	2.3331	0.5833	75.454	2.676	35.47
				Mean value	33.69

Table 11. ΔE_{H_2O} and E_{HCl} for HCl solutions from data by Yannakis and Zeisberg, respectively.

% HCl by weight	m	$\frac{1}{m+1}$ %	ΔE_{H_2O} kcal per mole	ΔE_{H_2O} kcal per mole from (3)	$\frac{10-m}{(m+2)^2}$	E_{HCl} kcal per mole	E_{HCl} kcal per mole from (5)	ΔE_{HCl} kcal per mole
2	99.19	0.998				21.66	19.51	2.15
4	45.58	2.02				20.51	19.03	1.48
6	31.72	3.06				19.25	18.55	0.70
8	23.28	4.11				18.48	18.07	0.41
10	18.22	5.20				17.88	17.56	0.32
12	14.85	6.31				17.25	17.05	0.20
14	12.44	7.44	0.01*			16.64	16.53	0.11
16	10.63	8.60				16.07	15.99	0.08
18	9.223	9.78	0.0433	0.0328	0.00745	15.46	15.44	0.02
20	8.098	10.99	0.124	0.101	0.0230	14.86	14.88	−0.02
22	7.178	12.23	0.219	0.186	0.0422	14.33	14.31	0.02
24	6.411	13.49	0.284	0.288	0.0654	13.74	13.73	0.01
26	5.762	14.79	0.439	0.408	0.0928	13.12	13.12	0.00
28	5.206	16.11	0.525	0.547	0.1233	12.50	12.51	−0.01
30	4.724	17.47	0.662	0.709	0.1611	11.87	11.88	−0.01
32	4.302	18.86	0.842	0.893	0.203	11.25	11.24	0.01
34	3.930	20.28	1.048	1.10	0.250	10.61	10.58	0.03
36	3.599	21.74	1.341	1.33	0.302	10.19*	9.91	0.28
38	3.303	23.24	1.663	1.60	0.354	9.52*	9.22	0.30
40	3.037	24.77	1.962	1.88	0.427	8.63*	8.51	0.12
42	2.796	26.35	2.356	2.20	0.500			

* less accurate values.

is very rough, though. At very high concentrations the values of E_{HCl} are probably too high.

For H_2SO_4 (Table 10) the mean value of $\Delta E_{H_2O} \cdot \frac{(m+2)^2}{2-\alpha}$ is 33.69. This value is probably too high owing to the great influence of the values of ΔE_{H_2O} at 25 and 70 % H_2SO_4 by weight. A weighted average seems to be closer to 31.6, the value given in eq. (2).

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Investigations on the Phosphorus Metabolism in *Rhodotorula gracilis*. II. Phosphate and Phosphatide Amounts in Protein Yeast and Fat Yeast

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If *Rhodotorula gracilis* is cultivated with a sufficient amount of nitrogen, a yeast containing 40—50 % protein and about 10 % fat is obtained. If it is cultivated in a nutrient solution with a low nitrogen content, a yeast containing 10—12 % protein and 50—60 % fat can be obtained. The considerable difference between the protein and fat content of these two yeast forms involves essential variations in their chemical composition [1,2]. In the following, the results of investigations concerning the presence of phosphorus and phosphatide in fat yeast and protein yeast from *Rh. gracilis* will be presented.

EXPERIMENTAL

The yeast was cultivated in a nutrient solution with varying nitrogen content. In order to be able to make comparisons with previous experiments, the amount of phosphate and sugar in the solution was also varied. This latter variation, however, is not of any greater importance as the phosphate content in all experiments was superoptimal.

In producing the protein yeast, the following nutrient solution was used: 15 g asparagin — 4.7 g KH_2PO_4 — 3 g $MgSO_4$, $7H_2O$ — 1.5 g NaCl — 1.5 g $CaCl_2$, $6H_2O$ — 0.015 g $FeCl_3$,$6H_2O$ — 60 g glucose per litre. The pH of this solution was 4.8.

In producing the fat yeast, the following solution was used: 1 g asparagin — 1 g KH_2PO_4 — 3 g $MgSO_4$, $7H_2O$ — 1.5 g NaCl — 1.5 g $CaCl_2$, $6H_2O$ — 0.015 g $FeCl_3$ — 40 g glucose per litre. pH 4.8.

The yeast was cultivated in 750 ml Erlenmeyer flasks with 300 ml of solution. The flasks were shaken at 25° C.

After 72 hours the yeast was analyzed. In the analyses the dry matter, nitrogen content, fat content and phosphate content were determined. Phosphate was determined according to Fiske-Subbarow [3], and the other analyses as indicated in Ref.[4].

From the phosphorus content of the yeast the phosphatide content was calculated. In this calculation it was assumed that the phosphatides of the yeast contain 3.98 % phosphorus which corresponds to the phosphorus content of lecithine. Even if compounds containing other percentages of phosphorus may occur in the fat, they exist in such small quantities that it is not necessary to take into consideration the slight error thereby resulting in the calculations. This method of calculation was used when the phosphatide

content was so low that a direct determination was difficult to make, *i. e.* especially in the case of the yeast containing the very high percentage of fat. In a few cases the amount of phosphatide was also determined directly, however, and the values obtained in these determinations correspond well with the results of the calculations.

RESULTS

In all experiments the yeast was cultivated (a) in solutions with a high nitrogen content, where only small amounts of fat were formed, and (b) in solutions with a low nitrogen content, where a large amount of fat was formed. Three experiments were made. The yeast was cultivated for 72 hours after which analyses were made.

In Table 1, the chemical analyses of the yeast from all the experiments are shown.

Table 1. Analyses of yeast after 72 hours.

	Experiment	1	2	3
Fat in yeast, %	Protein yeast	11.6	12.1	12.2
	Fat yeast	50.8	42.1	53.6
Protein in yeast, %	Protein yeast	46.6	52.9	55.2
	Fat yeast	12.1	19.8	14.3
P in yeast, %	Protein yeast	1.35	1.20	1.20
	Fat yeast	0.92	0.82	0.88
P in fat, %	Protein yeast	1.12	1.34	1.57
	Fat yeast	0.16	0.18	0.11
Phosphatide in yeast, %	Protein yeast	3.26	4.67	4.81
	Fat yeast	2.04	1.90	1.48
Phosphatide in fat, %	Protein yeast	28.11	33.63	39.41
	Fat yeast	4.02	4.52	2.76
Phosphatide N in yeast	Protein yeast	0.44	0.55	0.54
	Fat yeast	1.05	0.60	0.65
Protein + fat in yeast, %	Protein yeast	58.2	65.0	67.4
	Fat yeast	62.9	61.9	67.9

As indicated by Table 1, 3 typical protein yeasts and 3 typical fat yeasts have been analyzed. In the 3 samples of protein yeast, the fat content varied between 11.6 and 12.2 % and the protein content between 46.6 and 55.2 %. In the 3 samples of fat yeast, the fat content varied between 42.1 and 53.6 % and the protein content between 12.1 and 19.8 %.

In the lowest section of Table 1 the sum of the fat and protein percentages in the yeast is shown. This sum is rather constant, varying only between 58.2 and 67.9 % of the dry substance of the yeast. Other components thus constitute a relatively constant percentage of the yeast, *i. e.* about 35 % irrespective of whether the yeast has a high fat percentage or not.

The phosphorus content is somewhat lower in fat yeast than in protein yeast. Fat yeast contains from 0.82 to 0.92 % phosphorus and protein yeast 1.20 to 1.35 %. There is consequently only a slight difference between fat yeast and protein yeast as far as phosphorus content is concerned.

The percentage of phosphatide (as mentioned above, calculated on the basis of the amount of phosphorus contained in the fat) is considerably lower in fat from fat yeast than in fat from protein yeast. In Table 2 the values found in the experiments to represent the phosphatide content in the fat are indicated and the yeast is listed according to fat content. In addition, the phosphatide content in percentage of the fat-free yeast substance has been calculated.

Table 2. Phosphatide content of yeast with varying fat content.

Fat in yeast %	Phosphatide in fat %	Phosphatide in fat-free yeast %
11.6	28.11	3.69
12.1	33.63	4.51
12.2	39.41	5.48
42.1	4.52	3.28
50.8	4.02	4.15
53.6	2.76	3.19

As the figures indicate, the phosphatide content in the fat decreases considerably as the amount of fat increases. In protein yeast, the phosphatide content varies between 28.11 and 39.41 %, but in the case of fat yeast, it decreases in proportion to the increase of fat and is only 2.76 % in the yeast with the highest fat content. In spite of this considerable variation, none of these values lie outside the limits previously established for microorganisms. For *Rh. gracilis*, Holmberg [5] found that in one sample where the yeast contained 50 % fat phosphatides constituted 3 % of the fat, which is in accordance with our findings.

The phosphatide content calculated in per cent of fat-free yeast varies much less, even though the protein yeast may have a somewhat higher phosphatide content. The reason for the very slight difference in these figures is the fact that the high fat content in the fat yeast in part compensates the low percentage of phosphatide. According to Belin [6] a certain amount of phosphatide is necessary for the microorganisms, and consequently it should be expected that the phosphatide content in protein yeast and fat yeast should be about the same.

The phosphatide/nitrogen relation has previously been investigated by Davidson and Leslie [7], who found it to be relatively constant. In Table 1 this relation has been indicated for our experiments. The figures vary somewhat and tend to be slightly lower for protein yeast than for fat yeast. The variations in question, however, are rather small.

SUMMARY

The phosphatide content in the fat is dependent to a considerable extent on the fat content of the yeast. The higher the percentage of fat in the yeast, the lower the amount of phosphatide. While fat from protein yeast contains between 30 and 40 % phosphatide, fat from fat yeast only contains between 3 and 5. The lowest percentage of phosphatide is found in the yeast with the highest percentage of fat and there is a regular inverse proportion between the fat content of the yeast and the phosphatide content of the fat.

If the phosphatide content of fat-free yeast is calculated, the variations are quite insignificant, which seems to indicate that there must exist a certain amount of phosphatide in the yeast irrespective of the amount of fat in the yeast.

The phosphatide/nitrogen relation does not vary to any greater extent between protein yeast and fat yeast.

The sum of protein and fat per g of yeast is relatively constant, protein and fat constituting about 65 % of the yeast. Thus the quantities of other substances hardly vary although the fat content may increase.

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A Study of Phosphopeptones from Casein

I. Methods of fractionation

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Recently, phosphoproteins have been the object of several chemical investigations and a considerable amount of speculation. Several authors have attempted to isolate and study phosphoproteins from liver and other organs [1–4], and the physiological significance of these is much discussed at present [5]. However, most of the studies have been made on hydrolytic products of the phosphorus-containing proteins of milk and egg [6,7]. These are, of course, convenient to study because of their ready availability.

After enzymatic hydrolysis of casein, Shinn and Nicolet [7,8] isolated a phosphorus-containing fraction with a total of 8 amino acids. Mellander [9], who has made an extensive review of the literature, prepared barium and calcium salts of casein phosphopeptones, using a modification of the method described by Damodaran and Ramachandran. His product contained 13 different amino acids [10,11]. Later, Mellander and Isaksson [12,13] demonstrated an interesting physiological property of their phosphopeptones: calcium phosphopeptones are resorbed and can be used to promote bone calcification in rachitic children.

In this laboratory we have also been interested in phosphoproteins, but it has become evident that before their physiological significance and metabolism can be unravelled much further, more work will have to be done toward the isolation of these substances in a purified form. We have therefore concentrated our efforts on the development of methods of isolation and analysis, using phosphopeptones from casein as suitable models. These have been obtained after hydrolysis with either hydrochloric acid or proteolytic enzymes. Phosphoserine and phosphothreonine have been crystallized from acid hydrolysates [14–16]. Moreover, some low molecular peptides from such hydrolysates have also been isolated [17]. The present paper deals mainly with methods used in attempts to fractionate phosphopeptones of higher molecular weight, obtained by enzymatic hydrolysis.

METHODS AND RESULTS

Material. The calcium phosphopeptones used in the following experiments were obtained through the courtesy of Professor Mellander, Institute of Physiological Chemistry, Gothenburg, and were prepared according to the method used by him [9]. In this method large amounts of casein are digested for 12 days at pH 2 and 37° C with three successive additions of pepsin. A constant amino nitrogen value is then reached. Subsequently, the pH of the digest is adjusted to 7.5 and it is exposed to the action of repeatedly added amounts of trypsin at 37° C for 8 days, at which time a constant amino nitrogen value is obtained. The phosphopeptones are then precipitated as lead salts, decomposed by means of hydrogen sulfide, and finally obtained as calcium salts. Our preparation had the following composition: N 8.8; P 4.0; Ca 10.7 %; and also contained small amounts of Pb and Fe. Standard micro-analytical methods have been employed for the analysis of the elements shown in our Tables. For nitrogen the common micro-modification of Kjeldahl's method (*cf.* Pregl-Roth [18]) was used. Phosphorus was determined according to Teorell [19]. For amino nitrogen the manometric method of van Slyke [20] was used. Calcium was determined by the method of Widmark and Vahlquist [21] and iron as ferro-*o*-phenanthrolin according to the modification described by Blix [22]. For analysis the phosphopeptones were dried to constant weight *in vacuo* over phosphorus pentoxide at 70° C.

Preliminary experiments showed that the phosphopeptone preparation not only contained several metals and some basic nitrogen impurities, but also that it was a mixture of many peptides. It therefore seemed advisable to desalt the material and to carry out a rough fractionation of the peptides before attempting a more sensitive chromatographic resolution.

Ionophoresis. The first step of this procedure utilized an ionophoresis apparatus composed of four large plexiglass compartments, and built on the same principle as that described by Sanger and Tuppy [23]. Compartment 1, the anode cell, was separated from compartment 2 by a membrane consisting of two cellophan sheets; compartments 2 and 3 were separated by a goat skin membrane; and compartment 3 was separated from compartment 4, the cathode cell, by a double paper parchment membrane. The electrodes were 4.5 × 4.5 cm platina sheets. Compartment 1 was filled with 0.3 *N* H_2SO_4, compartment 3 with a solution containing 2 g of calcium phosphopeptone, and compartments 2 and 4 were filled with distilled water. When thus filled and ready for use, the apparatus contained about 800 ml solution.

At the beginning of an experiment the voltage was adjusted so that the current was about 300 mA, and then, as the current dropped, was gradually increased to 500 V. The content of the cathode compartment was replaced with distilled water several times during the experiment. After the current strength had dropped to about 50 mA, the content of the anode compartment was replaced with 0.3 *N* H_2SO_4. Finally, after a few hours, the content of compartment 2 was replaced with distilled water. The experiment was discontinued after about 30 hours, when the current had reached a limiting value of about 20 mA.

With this method most of the metal and basic nitrogen impurities (peptides) migrated to the cathode, and a crude separation of the phosphopeptides into 4 fractions was obtained. About 150 mg of the original 2 g remained in compartment 3. Approximately half of this had precipitated out of solution during the experiment. This was taken as fraction 1. The phosphopeptone remaining in solution was taken as fraction 2. About 1 g phosphopeptone had migrated to cell 2, and about 150 mg could be recovered from the combined and neutralized solutions from compartment 1. To this end the solubility of several heavy metal salts of phosphopeptone was studied. Water solutions of phosphopeptones were not precipitated by the acetates of cadmium, cobalt, iron, or mercury, while insoluble salts were formed by those of bismuth, copper, lanthanum, silver, and uranium. The copper phosphopeptone was the salt most easily and quantitatively decomposed by electrodialysis, a procedure which we considered preferable to decomposition by hydrogen sulfide. Moreover, it was just as insoluble as the other salts, and had been used in the preparation of phosphopeptones by Rimington as early as 1927 [24]. Thus, to obtain fraction 4 in the form of a free acid, it was first precipitated from Na_2SO_4 solution at pH 3 as its copper salt, and then the latter was decomposed by electrodialysis in an apparatus similar to that described by Theorell and Åkesson [25].

The composition of the four fractions obtained by ionophoresis is listed in Tables 1 and 2. As can be seen, a considerable amount of iron has remained in compartment 3 and traces even appear om cell 2. This is of special interest, since the natural occurrence of iron phosphoproteins has already been established by several authors [26–28].

Table 1. Composition of fractions from ionophoresis of phosphopeptone.

Cell	N	P	Ca	Fe	Amino N in % of total N
1	10.8	6.9	0	0	7
2	10.0	4.9	0	trace	6
3 (soluble)	11.9	4.4	1.8	3.2	9
3 (insoluble)	11.3	2.1	1.6	1.9	7

Methods of Indication. Before attempting a further separation of the ionophoresis fractions, it was necessary to find suitable methods for the identification of phosphopeptones. Three different methods were chosen, the ninhydrin and biuret methods for the detection of peptides, and the ammonium molybdate-benzidine reaction for the specific detection of the phosphate ion. Each of these can be used as a spot test for the indication of peptides on paper chromatograms.

The usual ninhydrin reagent (0.25 % ninhydrin in butanol containing 1 % acetic acid) gave no color reaction with the phosphopeptones, even when spots containing 1 000 μg were dried onto a filter paper and sprayed with the reagent. However, a color reaction was obtained with the modification of the ninhydrin reagent recently described by Lewis [29]. With this reagent 5 μg phosphopeptone could be detected.

The biuret reaction used was a modification of that used by Killander [30] on paper chromatograms. 0.1 mg of the phosphopeptone, dried onto an area of 1 cm^2 of filter paper gave a violet spot with the combined reagents. It has not been possible to increase the sensitivity of the reaction. A reagent, stable for longer periods, is the following. A mixture of 10 ml of ethanol and 1 ml of glycerine is saturated with copper acetate. To this solution is added 40 ml of ethanol and 50 ml of a 25 per cent solution of tetra-methyl-ammonium hydroxide. With this reagent 100 μg of phosphopeptone could be detected.

The phosphate reaction used was the ammonium molybdate-benzidine spot test [31], modified for use with organic phosphate. The procedure is as follows: The papers are sprayed with 96 % ethanol saturated with potassium hydroxide, and are subsequently heated at 100° C for 10 minutes. This procedure is repeated once. This causes the papers to assume an intense yellow color, and causes hydrolysis of the phosphopeptone without destroying the paper (the paper used throughout in these experiments has been Munktell No. OB, washed with acetic acid and water according to Hanes and Isherwood [32]). Before applying the reagents the papers are suspended in a glass jar containing an atmosphere concentrated with hydrochloric acid for 30 to 40 minutes. This causes the papers to turn white, and the molybdate reagent can then be applied. The papers are lightly and evenly sprayed with the ammonium molybdate solution and allowed to dry for 15 minutes. Subsequently, they are sprayed with the benzidine solution and immediately afterwards suspended in a second glass jar, containing an atmosphere of concentrated ammonia. Within a very short time blue spots appear corresponding to the positions of phosphopeptides. The limit of identification on a chromatogram is about 25 μg of phosphopeptone with a phosphorus content of 5 per cent.

Of the three methods mentioned above the ninhydrin and ammonium molybdate-benzidine reactions have been of the greatest value. Usually these two reactions have been carried out in succession on the same paper, allowing the detection of phosphorus-free peptides as well as phosphorus-containing peptides.

Paper Chromatography

Paper chromatography was found to be of value not only as a resolution technique in its own right, but also as a means of indicating fractionation attained in experiments with ion exchange resins and carbon.

Before suitable solvent systems were found, a large number were tried which were miscible with water in both small and large proportions such as: ethanol, propanol, acetone, phenol, pyridine/amyl alcohol, pyridine, *n*-butanol, *n*-butanol/acetic acid, and benzyl alcohol. In many cases there was a tendency to form multiple-banded spots, but when the bands were separately eluted with water, electro-dialysed and again analysed by paper chromatography, it was shown that no real separation had occurred. The apparent separation seemed to have been caused by the formation of addition products of solvent and solute. Some of the solvent systems which did give a useful separation were the following: *iso*butyric acid/water, propionic acid/*iso*valeric acid

(1 : 1)/water, and propionic acid/water mixtures. At present the most generaliy used mixture is the following: 25 ml benzene / 25 ml water / 62 ml propionic acid.

For the development of one-dimensional chromatograms, solutions of phosphopeptone containing about 100 μg were applied to a filter paper strip as spots of 1 cm diameter at 3 cm intervals along the starting line. Ascending chromatography was generally used with the benzene/water/propionic acid mixture; the front reached the upper part of the paper in 9—10 hours. The papers were dried at room temperature.

Amino Acid Determinations

Fractionation experiments on ion exchange and carbon columns were controlled by analyses for total nitrogen, amino nitrogen, and phosphorus in order to determine differences in elementary composition. Moreover, it was felt that valuable complementary information would be disclosed by amino acid analyses. For that purpose a rapid method, capable of yielding a scanning estimation was necessary, and this seemed to be afforded by a modification of the colorimetric method, recently described by Lewis [29] for estimation of amino acids on twodimensional paper chromatograms.

An extensive comparison of washed [33] and unwashed Munktell No. OB, Whatman No. 1, and Whatman No. 4 papers was carried out. In all cases the washed papers gave a better and more rapid separation of the amino acids. The Munktell papers were more easily washed free from impurities and yielded as good a separation as the Whatman papers; they were therefore used in the following. The dimensions of the papers were 38 $\times$ 28 cm[33]. Ascending chromatography with phenol-ammonia was used in the long direction, and it required about 15 hours for the front to reach the upper edge of the paper. Freshly destilled phenol was always used. The sheets were then dried at room temperature for 24 hours in a cabinet with good ventilation, and the yellowish brown material deposited by the phenol front was cut away. Subsequently, the papers were developed in the second direction using descending chromatography with pyridine/amyl alcohol for 6 hours. This solution could be used 2—3 times without change. The papers were then dried, sprayed with the ninhydrin reagent described by Lewis, and allowed to dry again for 24 hours. For separation of leucine and isoleucine, one-dimensional chromatograms were developed with lutidine/tertiary amyl alcohol on washed papers for 35—40 hours. Glass containers of suitable sizes were employed for all runs, and control papers with amino acid mixtures resembling those of the samples both in quality and quantity were always run parallel in the same jar.

The spots were cut out and eluted from the dried papers as described by Lewis, and the extracts were transferred quantitatively to a colorimeter cell, and measured in the Beckman B spectrophotometer at 570 mμ (440 mμ for the proline extracts). The value read was corrected for the blank obtained by extracting a circle of the same size as the colored spot from the developed paper. The corresponding amino acid values were read from standard curves constructed for each amino acid by running large series of different amounts

of all the amino acids on two dimensional chromatograms. When necessary, a correction factor was used, calculated from the control, run in parallel with the sample. In most of the experiments the standard error of a single estimation was ± 10—15 per cent. Some values obtained from ionophoresis and Dowex 2 (see below) fractions are listed in Table 2. The recoveries are comparatively good, but in general about 20 % too low. The same result is obtained if the amino nitrogen is calculated in per cent of the total nitrogen.

Table 2. Amino acid composition of fractions from ionophoresis and Dowex 2. Values expressed as percentage for moisture-free material.

Preparation	Asp	Glu	Ser	Gly	Thr	Ala	Lys	Arg	His	Pro	Val	Ileu	Leu	α-amino butyr. ac.	Total	Total + PO_4
Cell 1	8.4	20.5	14.0	2.5	4.2	4.7	0.6	—	—	3.4	5.8	5.0	1.0	—	70.1	90.7
Cell 2	10.4	24.3	13.6	3.2	4.6	2.4	1.9	—	0.3	3.8	5.3	5.5	1.8	—	77.1	91.8
Cell 3 (soluble)	10.0	23.6	12.0	3.1	4.7	2.9	4.4	0.9	3.2	5.7	4.3	5.8	2.7	—	83.3	96.5
Cell 3 (insoluble)	9.9	27.5	12.3	1.7	1.6	1.8	3.1	0.9	3.2	6.3	4.2	1.9	1.7	—	76.1	82.4
Fraction 2 Dowex	10.7	26.5	13.0	1.8	7.8	2.6	5.1	—	1.9	7.5	3.5	2.0	0.5	—	82.9	96.7
Fraction 3 Dowex	20.2	33.6	11.0	2.6	4.4	1.9	8.9	—	0.8	0.9	2.3	3.6	1.4	7.2	98.8	107.2
Fraction 4 Dowex	13.9	28.4	15.7	3.1	5.2	2.4	3.6	—	0.7	3.3	3.3	6.5	2.4	4.7	93.2	108.5
Fraction 5 Dowex	13.1	24.2	12.8	2.6	2.8	5.5	4.4	—	—	4.4	5.0	5.6	1.5	4.9	86.2	102.1
Fraction 6 Dowex	8.9	24.3	15.2	3.5	3.2	3.8	2.7	—	—	6.2	5.3	7.4	3.0	—	82.9	99.7
Fraction 7 Dowex	10.0	29.9	19.3	2.8	7.8	4.1	2.7	—	—	5.2	5.7	8.4	3.1	3.9	102.9	118.2

A comparison between the four ionophoretic fractions shows very clearly that the two fractions from cell 3 contain considerable amounts of all of the three basic amino acids, while the fraction from cell 2 only contains lysine and histidine and the material from cell 1 only inconsiderable amounts of lysine.

Ion Exchange Columns

Some preliminary experiments were carried out with the ion exchange resin, Dowex 50 (approx. 10 % cross linking, and 250—500 mesh) using 0.01—2 *N* HCl as displacer. However, this resin did not yield a satisfactory separation and about half of the phosphopeptone put onto the column could not be recovered from the effluent solution. This also diminished the value of Dowex 50 as a desalting material.

Experiments with Dowex 2 columns (approx. 10 % cross linking, and 200—400 mesh) carried out according to a procedure similar to that described by Cohn [34,35] gave better results. In these experiments a large 6.5 × 50 cm glass column fitted with a glass filter was used for preparative work. 2 g of material from compartment 2 of the ionophoresis apparatus were dissolved by adding dilute NaOH to a volume of 20—30 ml and a pH of 5. The solution was transferred to the column, which was in the chloride form, and had been washed until the effluent was of pH 5. Fraction 1 was washed from the column with distilled water, fractions 2 and 3 were removed with 0.01 *N* HCl,

Table 3. Composition of fractions from separation on Dowex 2 columns.

Fraction	N	P	Amino N in % of total N	Yield in g from 4 g
1 (water)	11.7	3.7	—	0.20
2 (0.01 *N* HCl)	13.2	4.6	4	1.02
3 (0.01 *N* HCl)	13.2	2.8	7	0.17
4 (0.02 *N* HCl)	12.9	5.1	5	0.15
5 (0.05 *N* HCl)	12.4	5.1	7	0.28
6 (0.2 *N* HCl)	11.3	5.4	4	0.07
7 (2 *N* HCl)	11.3	5.4	—	0.08

and fractions 4, 5, 6, and 7 with 0.02 *N* HCl, 0.05 *N* HCl, 0.2 *N* HCl, and *2 N* HCl, respectively. In each case the volume of displacer used was such that control papers sprayed with the phosphate reagent showed a nearly complete removal of the fraction. The fractions displaced with 0.02—2 *N* HCl were immediately brought to pH 3, precipitated with copper acetate, and electrodialysed. Total nitrogen, phosphorus, and yield are summarized in Table 3. For amino acid composition of the fractions, see Table 2.

As can be seen from the Tables, Dowex 2 suffers from one of the same defects as Dowex 50, for about half of the material put onto the column cannot be displaced with the concentrations of HCl used. Experiments are now in progress with modified forms of Dowex 2 in order to improve the yield. Nevertheless, use of unmodified Dowex 2 does permit a fractionation of the mixture of peptides from compartment 2. There is a clear tendency toward the displacement of more highly acidic peptides with increasing concentrations of HCl. And there is also a tendency for the later fractions to contain higher concentrations of leucine and isoleucine. Moreover, the occurance of α-aminobutyric acid in some of the later fractions is to be noted. So far, however, no single pure peptide has been obtained directly by fractionation on Dowex 2.

Carbon Columns

At present the routine procedure used for the further separation of the ionophoresis fractions has employed chromatography on carbon columns as the second step. The carbon used has been Norit FNX Special. Before use, the carbon has been washed with several liters of 20 per cent acetic acid, 96 per cent alcohol, and finally with distilled water. In addition, to improve the yields of the fractions displaced from the column, the carbon has been pretreated with stearic acid in a manner closely similar to that described by Hagdahl, Williams, and Tiselius [36] and Hagdahl (personal communication). 100 g of the charcoal is suspended in an ether solution of stearic acid and stirred mechanically for 24 hours, adding more ether at intervals. The ether is then filtered off. Under these conditions the Norit takes up no more than 20—25 per cent of stearic acid even though an excess amount of the latter is present.

The carbon obtained by this procedure is suspended in distilled water and poured into a shallow glass column. Several liters of water are again run through the column before the start of the experiment. A water solution of 200 mg phosphopeptone is then transferred to the column, and the column is washed with 2 liters of distilled water, causing the removal of about 20 per cent by weight of the phosphopeptone. This fraction, as tested by paper chromatography, has been found to be quite different from the next fraction, displaced from the column by a saturated solution of *iso*valeric acid in water (neither 20 per cent acetic acid nor 20 per cent propionic acid cause a displacement of any of the phosphopeptone). A third fraction, rather similar to the second, can be displaced by a 10 per cent solution of *iso*valeric acid in 20 per cent acetic acid. Together, the two *iso*valeric acid fractions amount to about 40—45 per cent of the phosphopeptone put onto the column. A fourth fraction can be displaced with lauric acid and a fifth with stearic acid (both acids in the form of sodium salts in a saturated solution of 50 per cent ethanol). The phosphopeptones displaced with these solutions bring the total up to about 85—95 per cent of that originally put on the column. Chromatograms of the fractions have shown that a definite separation of the ionophoresis fractions is attained. Nevertheless, all of the fractions have been shown to contain several peptides.

Paper Powder Columns

In order to further separate the fractions obtained from the carbon columns, paper chromatography on columns has been used. Paper columns have been used in this laboratory for several years for the separation of amino acids. These columns have been largely of two sizes, 1.8 × 70 cm and 6.5 × 40 cm. The powder has been of the same quality as that used to manufacture Munktell No. OB paper, and has been ground several times in a Wiley mill and thoroughly washed with water, ethanol, and ethyl ether on large Buchner funnels.

The smaller sized columns have usually been filled with 0.5—1 g portions of dry powder and packed with flat stainless steel stamps, of a diameter a few mm less than that of the glass chromatographic tube. The large columns have been packed with 2—3 g portions of paper using alternately a flat and a rounded stamp. This procedure was shown to be necessary in order to obtain a uniform flow rate of solvent through the column, as controlled by observation of the movement of a band of methyl orange through the tube with *n*-butanol/acetic acid/water [37] as solvent.

The solvent to be used in a given experiment is run through the dry column until the effluent is colorless, and a mixture of the dry amino acids is packed evenly onto the upper surface of the column with a glass stamp. The amino acid mixtures are usually mixed with a small amount of paper powder to insure a more even distribution over the top of the column. After cautiously driving the amino acids into the column with small additions of solvent, the reservoir is connected, and the solution allowed to drop into the tubes of a fraction collector. Usually atmospheric pressure is all that is needed to obtain a satisfactory flow of solvent. Using this method and the *n*-butanol/acetic acid/water mixture, a satisfactory separation of up to 200 mg of leucine, valine,

α-amino-butyric acid, and alanine can easily be obtained on the larger column. It is our impression that the resolution is nearly as good as that obtained on paper strip chromatograms.

The paper column methods worked out for amino acids have worked equally well for phosphopeptones, and several of the Norit fractions have been further separated into fractions giving a ninhydrin reaction but no phosphate reaction, fractions giving a phosphate reaction but no ninhydrin reaction, and into fractions giving both a ninhydrin and a phosphate reaction. Up to now, only the smaller columns have been used, 150 mg samples of each fraction being put onto the column and eluted with the same benzene/water/propionic acid mixture used in the paper chromatograms mentioned earlier.

Using the methods outlined above it has been possible to obtain larger quantities of phosphopeptides, at least partially separated and purified from non-phosphorus containg peptides. Some chemical properties of these peptides will be described in a following paper.

SUMMARY

Some methods have been outlined for the separation and purification of phosphopeptones obtained from bovine casein. Those methods routinely used have been ionophoresis, and chromatography on carbon, ion exchange, and paper powder columns.

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Beef Liver Glucose Dehydrogenase

I. Purification and Properties

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Liver glucose dehydrogenase (GDH) was discovered by Harrison [1] and partially purified by him and by a number of other workers [2–5]. It is a diphosphopyridine nucleotide (DPN)-linked enzyme [6], but it has been reported by several investigators that triphosphopyridine nucleotide (TPN) can replace DPN as the coenzyme [5,7–9]. Further purification of the glucose dehydrogenase from beef liver is reported here, together with some other information concerning its properties and possible physiological role.

EXPERIMENTAL

Enzyme Assay. The GDH was assayed spectrophotometrically by measuring the initial rate of reduction of DPN by the enzyme in solution at pH 7.6 in the presence of excess quantities of glucose and the coenzyme, as described by Strecker and Korkes [5], and employing the enzyme unit defined by them. The protein content was determined spectrophotometrically by measurement of the absorption at 340 mμ; an assumed absorption coefficient of $E^{1\%}_{1cm} = 10$ was used.

Purification Procedure

Beef liver which had been stored in a frozen state was allowed to thaw partially overnight at 3°, cut into *ca.* 1 cm. cubes, and homogenized in a blendor with about 2 volumes of acetone. The mixture was poured with stirring into 10 volumes of acetone, filtered, and the filter cake again homogenized with acetone and poured into 10 volumes of fresh acetone. After filtration, the second filter cake was broken up into 3 volumes of ether, the mixture stirred, and filtered. All of these operations were carried out in a cold-room at −18°; the acetone and ether used were previously chilled to this temperature. The filter cake was rapidly dried to constant weight at room temperature. One kg of beef liver generally yielded 250 to 300 g of acetone powder. The dry powder could be stored for several weeks at −18° with no detectable loss of activity. The acetone powder was extracted by stirring with 8 volumes of distilled water for 45 minutes at room temperature. The resulting extract was centrifuged for 45 minutes at room temperature and 2 300

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r.p.m. and the precipitate discarded. The GDH activity of the supernatant solution was generally 20—25 units/mg, with a yield of about 14 000 units per g of acetone powder. The solution could be stored for a few days at 3° if desired.

First Ammonium Sulfate Fractionation. To each 1-liter volume of the aqueous extract was added 174 g of ammonium sulfate, and the solution brought to pH 6.8—7.2 by dropwise addition of concentrated ammonium hydroxide. The solution was allowed to stand for 15 minutes, and then centrifuged for 45 minutes at 2 300 r.p.m. The precipitate was discarded. Ammonium sulfate, 137 g per liter, was added to the supernatant, and it was again adjusted to pH 6.8—7.2. After the mixture had stood for 15 minutes, it was centrifuged for 1 hour and the supernatant discarded. The precipitate was dissolved in 0.05 *M* pH 7.6. phosphate buffer, using about 100 ml for each liter of the original water extract. These operations were done at room temperature. The recovery of activity was 80—85 %, at a level of 60—70 u./mg. The solution was stored at 3° overnight, but preferably not longer.

Second Ammonium Sulfate Fractionation. The cold (3—5°) solution from the above step was diluted with water to a final protein concentration of about 80 mg/ml, and treated with 61.5 g of ammonium sulfate/liter. After 15 minutes, it was centrifuged for 1 hour at 5° and 6 000 r.p.m., and the precipitate discarded. The same quantity of ammonium sulfate used before was added to the supernatant, and after standing at 5° for a few minutes the mixture was again centrifuged. The supernatant was discarded, and the precipitate dissolved in 0.05 *M* pH 7.6 buffer to give a final volume of not more than 40 % of that at the beginning of this fractionation step. The solution was dialyzed overnight at 3° against about 8 volumes of 0.02 *M* pH 7.6 buffer containing 50 mg/l of cysteine hydrochloride. This step generally gave GDH concentrates of *ca.* 100—120 u./mg in yields of about 80—90 %.

Third Ammonium Sulfate Fractionation. The dialyzed solution from the last step was diluted with 0.02 *M* pH 7.6 buffer to a protein concentration of not over 50 mg/ml. Ammonium sulfate was added to 30 % saturation, and the resulting precipitate removed by centrifugation and discarded. Amounts of saturated ammonium sulfate solution, pH 7, were added dropwise to the supernatant to give first 36 % and then 43 % saturation, and the two resulting precipitates collected by centrifugation and dissolved in 0.05 *M* pH 7.6 buffer, all of the operations being done at 3—5°. The two fractions were dialyzed as indicated above, with two changes of the dialysis solution. Most of the activity was usually found in the second fraction, precipitated between 36 % and 43 % saturated ammonium sulfate. This fraction ordinarily contained 40—50 % of the activity, at a level of about 350 u./mg.

Ethanol Fractionation. The dialyzed solution from the above step was made 0.1 *M* with respect to sodium chloride and the pH brought to 5.9—6.1 with 10 % acetic acid. It was then chilled to 0°, and fractionated with 50 % (v/v) ethanol which had previously been cooled to —18°. The ethanol solution was added slowly, so that the temperature of the mixture did not rise above —2° for the first two fractions, or above —3° to —4° for subsequent fractions. Precipitates were collected by centrifugation, dissolved in 0.05 *M* pH 7.6 buffer, and dialyzed as usual. In a typical experiment, a first fraction, precipitated by 0.20 volume of 50 % ethanol, was discarded. Subsequent fractions were obtained by additions of 0.10, 0.10, 0.15, and 0.20 volumes of the 50 % ethanol. Fractions 3 and 4 (usually) contained about 40 % of the activity, at about 550—600 u./mg.

The purification operations up to this point were sufficiently reproducible that they could be carried out by a technician following a standardized procedure. The subsequent steps were carefully controlled, with each fraction being assayed.

Fractionation with 4.5 M Phosphate. Material from the ethanol fractionation was further purified by fractional precipitation with 4.5 *M* phosphate buffer, pH *ca.* 7. The concentrated buffer was added dropwise to the enzyme solution until the first appearance of a precipitate, generally at a phosphate concentration of about 1.0—1.2 *M*. This was removed by centrifugation, and the addition of phosphate continued in increments of not more than 0.1 *M*; the resulting precipitates were collected and dissolved in 0.05 *M* buffer for assay and dialysis. In a typical fractionation, the total recovery of GDH activity was about 90 %, with about 60 % of the activity precipitated at phosphate concentrations between 1.15 and 1.30 *M* and at a specific activity level of *ca.* 850 u./mg.

Final Purification. GDH concentrates of specific activity 800—900 u./mg were next fractionated with saturated ammonium sulfate solution, this procedure generally affording

a product of 1 300—1 400 u./mg activity in some 40 % yield, as well as other fractions of lower activity. The best material was dialyzed overnight and then fractionated again with 4.5 *M* phosphate. This yielded concentrates of activity 1 900—2 200 u./mg with a recovery of about 40 %. Immediate refractionation of such products with 4.5 *M* phosphate gave GDH of specific activity 3 200—3 400 u./mg in 40 % yield, as well as some less highly active fractions.

Yields. The over-all yield from the original aqueous extract (20—25 u./mg) of the acetone powder through eight steps to a product of activity 3 400 u./mg, a roughly 150-fold purification, was about 0.5 %, when only the best fractions from each step were used for the subsequent step. However, the yield could be increased several times by reworking fractions of intermediate activity.

Stability. Solutions of the enzyme in dilute pH 7.6 buffer could be stored in the refrigerator for one or two weeks without significant loss of activity, and could be kept many weeks in the cold as a precipitate under 55 %-saturated ammonium sulfate solution. However, solutions containing small amounts of ammonium sulfate frequently lost considerable activity on standing overnight.

Michaelis Constants

Coenzymes. The determinations of the Michaelis constants for the coenzymes (DPN, TPN, deaminated DPN, see below) were carried out by measuring the initial rates of reduction of the coenzyme as indicated by the increase in absorption at 340 mμ, using a Beckman Model B spectrophotometer. Solutions were prepared so that they contained 0.37 *M* glucose, 0.05 *M* phosphate, pH 7.00, and varied concentrations of the coenzymes. For each coenzyme concentration, three or four 3.00 ml aliquot portions were pipetted into 1 cm cuvettes. The reaction was initiated by adding at the Beckman 200 units of GDH in a volume of 0.02 ml; the values of Δd_{340} for 15 and 30 seconds so obtained in the replicate determinations were averaged. The enzyme used generally had a specific activity of 2 000 u./mg or greater; in a few cases when 1 000 u./mg material was used, no differences could be noted. All determinations were made at 21—22°, Higher temperatures increased the initial reaction rates considerably, but had less effect on the values of the Michaelis constants so obtained.

Glucose. In this case, the solutions were 2.1×10^{-4} *M* with respect to DPN, and again 0.05 *M* in phosphate. The glucose concentration was varied between 0.03 *M* and 0.12 *M*. Otherwise, the determinations were done as described above, except that the increase in absorption of the solution at 340 mμ was followed for one minute.

Equilibrium Constant

Solutions were prepared to contain glucose, 0.13 *M* phosphate buffer, and 11 000 units of GDH (sp. activity 2 500 u./mg) in 2.98 ml volume in a cuvette, and their absorption spectrum determined in the range 320—360 mμ. The reaction was initiated by the addition of 0.02 ml of DPN solution containing sufficient DPN to give a concentration of about 4×10^{-5} *M* in cuvette; the absorption readings were corrected for the addition of such a quantity of oxidized DPN. When the absorption at 340 mμ had become constant (*ca.* 45 minutes), 0.50 ml of 20 % aqueous glucose solution was added, and the absorption again determined, with appropriate corrections for dilution and the absorption of the added glucose. Finally, the pH at equilibrium was checked. The DPN concentration of the stock solution was assayed with liver alcohol dehydrogenase according to Bonnichsen[10].

RESULTS AND DISCUSSION

Purity. Strecker and Korkes [5], using aqueous extraction of acetone powder, ammonium sulfate and pH fractionations, and calcium phosphate gel treatments, obtained GDH concentrates with a specific activity of 900—1 100 u./mg. The purification procedure described in the present paper has given material

of some three times this potency, but there is no assurance that it is homogeneous. A sample of partially purified GDH of activity 1 400 u./mg was examined by electrophoresis in 0.046 *M* phosphate buffer at pH 6.96. This revealed two major components of nearly equal size and difficultly separable from each other; and a third, quite small component, which moved much faster. It was possible to isolate the small, mobile component. This proved to contain a negligible quantity of GDH, the mixture of the two large components accounting for nearly all of the enzymic activity. On this basis, it is possible that the material further fractionated to a level of 3 400 u./mg potency was nearly pure. Attempts to crystallize the small amounts of such preparations which were obtained were unsuccessful.

It is of some interest to calculate an approximate turnover number for liver glucose dehydrogenase. By assuming an activity of 3 400 u./mg and a molecular weight of 100 000, one finds that at pH 7.6, a mole of GDH reduces about 55 moles of DPN per minute. Although the absolute value of such an estimated turnover number is open to question, it is at least evident that the enzyme, if it is nearly pure, is indeed a sluggish one. In this respect it may be compared to horse liver alcohol dehydrogenase, for which a maximal turnover number of 140 for the oxidation of ethanol has been reported [11]. The turnover numbers for most other dehydrogenase systems are several orders of magnitude higher.

Substrate Specificity. The best preparations of Strecker and Korkes [5] oxidized xylose at about one-fourth the rate of the oxidation of glucose, while galactose and arabinose were oxidized at about 3 to 4 percent of the glucose rate. Our preparations, tested under the usual assay conditions, oxidized xylose at about 30 percent of the rate with glucose, at several levels of purification of the enzyme. A crude (71 u./mg) GDH preparation showed an activity of 10.5 u./mg with D-arabinose; but the ability to oxidize arabinose decreased with increasing purification of the enzyme until, at levels of 2 000 u./mg and above, no oxidation of arabinose could be detected. Even the most potent preparations still retained some ability to oxidize galactose, but the results varied with different galactose samples, and may possibly be ascribable to contamination of the carbohydrate.

Coenzyme Specificity. It has been recently reported that GDH preparations of specific activity 1 000 u./mg oxidized glucose in the presence of either DPN or TPN, the relative rates being dependent on pH [5]. With the usual assay conditions of pH 7.6 and both glucose and coenzyme in excess, we could detect no difference in the rate of glucose oxidation with DPN or TPN, using a GDH preparation of 2 100 u./mg. A sample of deaminated DPN was prepared according to Kaplan, Colowick and Ciotti [12]. This material was not significantly less active than DPN or TPN under the assay conditions.

The Michaelis constants for DPN, TPN, and deaminated DPN were determined at pH 7.00 and 21.5° as described in the experimental section. The results are shown in Fig. 1. It may be noted that the Michaelis constants are 4.3×10^{-6} *M* for DPN, 6.2×10^{-6} *M* for TPN, and 9.0×10^{-6} *M* for deaminated DPN. The relative activities of DPN and deaminated DPN in a number of other enzyme systems have been studied by Pullman, Colowick, and Kaplan [13].

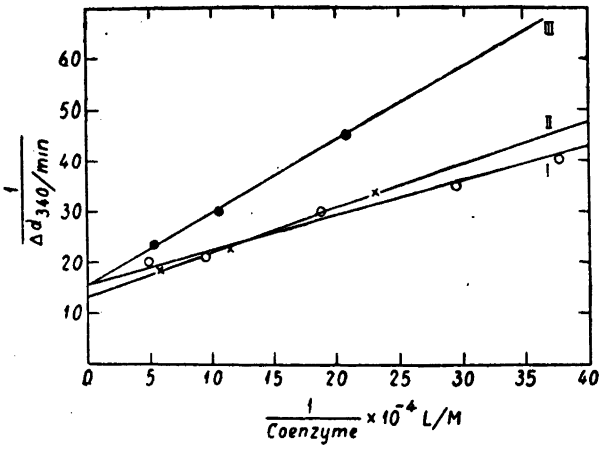

Fig. 1. Michaelis constants for the three coenzymes. Glucose, 0.37 M, 0.05 M phosphate buffer, pH 7.00. GDH, 67 u./ml. Temperature, 21.5°. Curve I: DPN; curve II, TPN; curve III, deaminated DPN.

pH and Initial Reaction Rates. Fig. 2 summarizes the effect of the pH on the initial reaction rates in the presence of excess DPN and two concentrations of glucose. It may be observed that with both glucose concentrations, maximum rates were observed near pH 8. The data from Fig. 2, plus additional measurements, were used to calculate Michaelis constants for glucose at the different pH's. As is shown in Fig. 3, the Michaelis constant for glucose has a minimum near pH 7, where its value is 3.1×10^{-2} *M*, and increases markedly below pH 6.7 and above pH 7.8. Strecker and Korkes observed values of 0.15 *M* at pH 7.0 and of 0.07 *M* at pH 7.6 and 8.2.

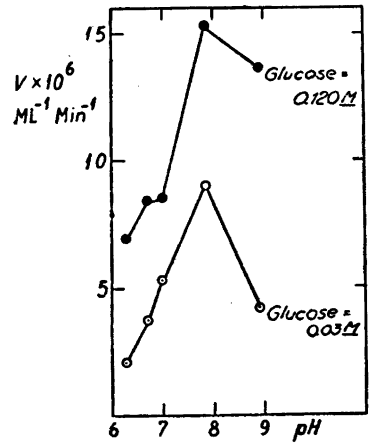

Fig. 2. Initial reaction velocity at different pH values. DPN, 2.1 × 10⁻⁴ M. Phosphate or pyrophosphate buffer, 0.05 M. GDH, 67 u./ml. Temperature, 21.5°.

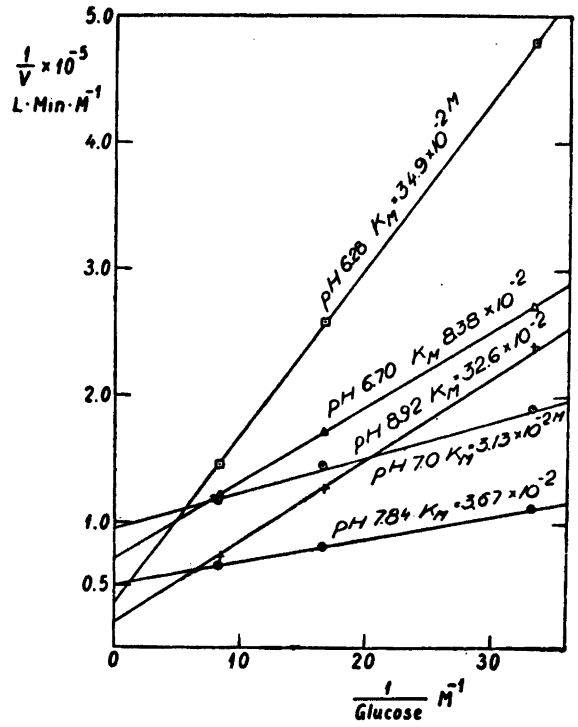

Fig. 3. Michaelis constants for glucose at different pH values.

Equilibrium Constant. The equilibrium constant for the reaction

$$\text{Glucose} + \text{DPN} \overset{\text{GDH}}{\rightleftharpoons} \text{Gluconic acid lactone} + \text{DPNH} + \text{H}^+$$

was determined at pH 7.0 and 21°, using 3 670 units of purified GDH per milliliter. In one experiment, the initial glucose concentration was 1.33×10^{-4} *M* and that of DPN, 4.04×10^{-5} *M*; at equilibrium, 88.5 percent of the DPN was reduced. In a second experiment, starting with 6.67×10^{-5} *M* glucose and 3.94×10^{-5} *M* DPN, 78.2 percent of the DPN was reduced at equilibrium. Lower glucose concentrations were not used, because of the greatly lengthened time necessary to achieve equilibrium. From these experiments, values for the equilibrium constant of 2.9 and 3.3×10^{-7} were calculated. Strecker and Korkes [5] observed an average value of 15 for the equilibrium constant at pH 6.7, using an expression which did not include hydrogen ion concentration. Their value may be compared with those reported here by multiplying by 2×10^{-7}. This gives a value of 30×10^{-7}, approximately ten times as great as what we have observed.

The detection of an apoenzyme-reduced DPN complex by observation of a shift in the absorption maximum of reduced DPN from 340 mμ to 325 mμ in the presence of large (approximately equimolecular) amounts of enzyme was observed by Theorell and Bonnichsen [11,14] in the case of liver alcohol

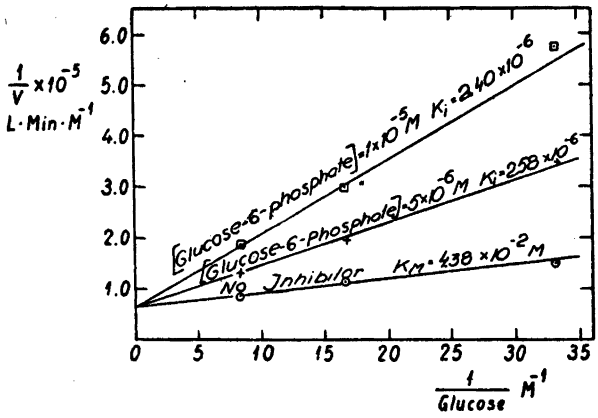

Fig. 4. Competitive inhibition of GDH system by glucose-6-phosphate. DPN, 2.1 × 10^{-4} M, 0.05 M phosphate buffer, pH 7.00. GDH, 67 u./ml. Temperature, 22°.

dehydrogenase; and later by Chance and Neilands [15] with lactic dehydrogenase, using especially sensitive spectrophotometric apparatus. Neilands [16], studying the same system with conventional equipment, failed to detect the existence of such a complex. In the equilibrium experiments described above, the molar concentration of enzyme could be estimated to have been perhaps of the order of one-tenth that of the reduced DPN. No shift in the absorption maximum of the reduced DPN could be detected.

Inhibition by Hexose Phosphates. Liver glucose dehydrogenase, according to Strecker and Korkes [5], failed to oxidize hexose diphosphate, glucose-6-phosphate, fructose-6-phosphate and ribose-5-phosphate, and these esters inhibited the oxidation of glucose by the enzyme. No quantitative data were given. We have examined the inhibition of the GDH system by fructose-1,6-diphosphate and by glucose-6-phosphate in experiments in which DPN was present in excess and the glucose concentration was varied. As can be seen from Fig. 4, glucose-6-phosphate strongly inhibited the reaction in competition with glucose; the common intercept for the lines representing the inhibited and non-inhibited reactions demonstrates the competitive nature of the inhibition [17]. A similar result was obtained with fructose-1,6-diphosphate. From the values of the inhibition constants, 6.2×10^{-5} *M* for the fructose ester and *ca.* 2.5×10^{-6} *M* for glucose-6-phosphate, it can be seen that although the enzyme is unable to oxidize these two esters, they are bound to the same reactive centers on the enzyme surface as is glucose. Moreover, the binding is some 500 times as tight in the case of fructose diphosphate, and over 10 000 times as tight with glucose-6-phosphate, as compared with the binding to the enzyme of glucose itself.

It is of interest that in the presence of excess glucose, 10^{-3} *M* glucose-6-phosphate strongly inhibited the reaction at three concentrations of DPN but, as expected, the inhibition was noncompetitive in nature. The initial reaction rates were essentially the same for each DPN concentration employed.

The Physiological Function of GDH. It is evident from consideration of the magnitude of the estimated turnover number of the enzyme and of its large Michaelis constants for glucose, that GDH oxidizes glucose only slowly at high concentrations, and below glucose concentrations of about 10^{-2} *M*, hardly at all. Furthermore, the reaction is almost completely blocked for concentrations of glucose below about 0.05 *M* by glucose-6-phosphate at levels of approximately 10^{-5} *M*. Normal liver contains about 6 per cent of glycogen, and tissues containing glycogen always have a high equilibrium concentration of glucose-6-phosphate [18]. Hence it seems quite likely that the GDH in liver is incapable of oxidizing glucose to any large extent, particularly as compared with the normal pathways for glucose metabolism. We have observed that aqueous extracts of freshly ground liver were devoid of GDH activity in the usual assay, although some activity appeared after precipitation with 50 %-saturated ammonium sulfate; this may perhaps be explained in terms of the discussion above. Stetten and Stetten [19], on the basis of isotopic studies, concluded that the direct oxidation of glucose by liver GDH contributes little if any gluconic acid to the body fluids.

Rather than assume that liver GDH is merely a useless artifact, it would seem preferable to suppose that the enzyme does play some significant role in the body's metabolism, possibly as a DPN-linked dehydrogenase, but with some substance other than glucose as its normal substrate.

SUMMARY

1. Beef liver glucose dehydrogenase has been purified by fractionations with ammonium sulfate, ethanol and phosphate to a specific activity of about 3 400 u./mg. There is no direct evidence that such preparations are homogeneous.

2. The best preparations are capable of oxidizing only glucose, xylose, and perhaps galactose. DPN, TPN and deaminated DPN can all serve as coenzymes.

3. The Michaelis constants for DPN, TPN and deaminated DPN have been determined at pH 7.00; and for glucose at a number of pH values. An equilibrium constant has been calculated for the reaction at pH 7.00 and a temperature of 21°.

4. Strong inhibition of the enzyme system has been observed with glucose-6-phosphate, which competes with glucose, but not with DPN, for the protein surface. Fructose-1,6-diphosphate also inhibits in competition with glucose.

5. It is suggested that the oxidation of glucose to gluconic acid lactone is not the normal metabolic role of liver glucose dehydrogenase.

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Beef Liver Glucose Dehydrogenase

II. Inhibition Studies with Compounds Related to the Coenzymes

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In 1942, v. Euler [1] observed inhibition of liver glucose dehydrogenase and lactic dehydrogenase by compounds related to nicotinic acid, and in some cases it was shown that the inhibitor competed with diphosphopyridine nucleotide (DPN). Recently, a number of workers have reported the competitive inhibition of various dehydrogenases by nicotinamide [2,3] and by adenine, adenosine and adenosine triphosphate (ATP) [4]. With the availability of highly purified beef liver glucose dehydrogenase (GDH) [5], it seemed desirable to make an investigation of the types of compounds related to DPN (and deaminated DPN, which also functions as a coenzyme in this system [5]) which might cause inhibition, and of the qualitative and quantitative nature of the inhibitions. Such findings might be expected to contribute to an understanding of the nature of apoenzyme-coenzyme interactions.

EXPERIMENTAL

The inhibitor to be tested was dissolved in 0.2 *M* phosphate buffer, pH 7.0, and the pH of the resulting solution adjusted to 7.00 by the addition of dilute alkali or mineral acid if necessary; then the solution was diluted to a phosphate concentration of 0.076 *M*. A 6.86 ml volume of the inhibitorbuffer solution was mixed with 3.50 ml of 20 % aqueous glucose solution and 0.14 ml of coenzyme solution of known concentration. Three 3.00 ml aliquots of the reaction mixture were pipetted into 1 cm Beckman cuvettes for triplicate determinations. The reaction was initiated at the Beckman by the addition of 200 units of purified (1 000—2 500 u./mg) glucose dehydrogenase in a volume of 0.02 ml. The absorption of the solution at 340 mμ was read within 5 to 10 seconds after mixing and the increase in absorption at 340 mμ noted after 15 and 30 seconds. All determinations were carried out at temperatures between 21 and 22°.

Calculations [6]. The initial reaction rate, v, was calculated from the observed increase in optical density at 340 mμ per 30 seconds, $\triangle d_{30}$, using the expression $v = \frac{2 \triangle d_{30}}{6.23 \times 10^3}$ moles liter^{-1} minute^{-1}. This was in turn derived from the absorption coefficient of reduced DPN, $E^{1\%}_{1\,cm} = 93.8$ at 340 mμ [7]. When deaminated DPN was the coenzyme, the rate

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Table 1. Inhibition by substituted pyridines.

Inhibitor	Conc. of Inhib., M/L	Coenzyme	Slope	Intercept	K_i, M/L
None	— — —	DPN	0.420	0.98×10^5	$K_M = 4.28 \times 10^{-6}$
None	— — —	Deam.-DPN	0.875	»	$K_M = 9.0 \times 10^{-6}$
Pyridine	1.31×10^{-1}	DPN	0.60	»	3×10^{-1}
Nicotinamide	1.00×10^{-1}	DPN	1.985	»	2.7×10^{-2}
»	2.00×10^{-2}	DPN	0.794	»	1.9×10^{-2}
»	5.00×10^{-2}	Deam.-DPN	5.50	»	1.0×10^{-2}
Nicotinic acid	1.31×10^{-1}	DPN	2.83	»	2.3×10^{-2}
» »	2.20×10^{-2}	DPN	0.885	»	2.0×10^{-2}
*Iso*nicotinic acid	5.00×10^{-2}	DPN	0.42	»	No inhib.
*Iso*nicotinic acid hydrazide	7.00×10^{-2}	DPN	0.545	»	2×10^{-1}
2-Methyl-nicotinic acid	3.37×10^{-2}	DPN	0.994	»	2.5×10^{-2}
Quinolinic acid	4.00×10^{-2}	DPN	0.42	»	No inhib.
Trigonelline	6.12×10^{-2}	DPN	0.42	»	» »
β-Hydroxy-pyridine	1.00×10^{-2}	DPN	0.875	»	0.92×10^{-2}
Pyridoxal*	5.00×10^{-3}	DPN	4.03	»	6×10^{-4}
»	1.00×10^{-3}	DPN	0.830	»	1×10^{-3}
4-Pyridoxic acid *	1.00×10^{-3}	DPN	1.84	»	3×10^{-4}

was calculated on the assumption that reduced deaminated DPN has the same absorption coefficient at 340 mμ as reduced DPN. A plot of the reciprocal of the velocity against the reciprocal of the molar concentration of coenzyme gave a straight line, for which the slope and the intercept on the $(1/v)$-axis were noted. In cases of competitive inhibition, the lines representing the reaction in the presence and absence of inhibitor have a common intercept, but different slopes. The inhibition constant, K_i, was calculated from the relationship:

$$\text{Slope} = (K_M/V_{\text{Max.}}) \left(1 + \frac{[I]}{K_i} \right)$$

where K_M is the Michaelis constant for the coenzyme, $1/V_{\text{Max}}$ is the intercept on the $(1/v)$-axis, and [I] is the molar concentration of the inhibitor.

The results are presented in Tables 1 and 2. The compounds marked with an asterisk had such high absorption at 340 mμ that it was difficult to obtain accurate readings, and the values recorded should be regarded as approximate.

One compound, *nebularine* ** (9-D-ribosylpurine) [8], was available only in limited amounts as a 3 mg/ml solution in physiological saline. In this case the inhibitor-buffer solution was prepared by addition of 4.5 *M* phosphate buffer, pH 7.00, to the proper phosphate concentration before mixing with glucose and DPN. Only a single determination was made at each DPN concentration. A special determination of the Michaelis constant for DPN under the same conditions was made. From the observed intercept of 0.98×10^5 and slope of 0.635, a K_M of 6.48×10^{-6} was calculated. For the reaction in the presence of 1.0×10^{-3} *M* inhibitor, a slope of 0.885 and an intercept of 0.98×10^5 gave a value for K_i of 2.5×10^{-2} *M*. This value must of course be regarded as approximate.

** Obtained through the courtesy of Dr. N. Löfgren, Stockholm, Sweden.

Table 2. Inhibition by substances related to the adenyl moiety of the coenzymes.

Inhibitor	Conc. of Inhib., M/L	Coenzyme	Slope	Intercept	K_i. M/L
Phosphate	1.5	DPN	1.385	0.98×10^5	7.6×10^{-1}
»	1.0	DPN	0.910	»	8.6×10^{-1}
Adenine	1.75×10^{-2}	DPN	1.035	»	1.2×10^{-2}
»	8.75×10^{-3}	DPN	0.720	»	1.2×10^{-2}
Adenosine	4.58×10^{-2}	DPN	2.42	»	9.7×10^{-3}
»	9.2×10^{-3}	DPN	0.857	»	8.8×10^{-3}
3-Adenylic acid	1.0×10^{-2}	DPN	1.646	»	3.3×10^{-3}
» »	3.48×10^{-3}	DPN	0.815	»	3.7×10^{-3}
ATP	4.8×10^{-3}	DPN	1.55	»	1.8×10^{-3}
»	2.4×10^{-3}	DPN	1.01	»	1.7×10^{-3}
»	4.0×10^{-3}	Deam.-DPN	1.95	2.49×10^3	Non-competitive
Inosinetriphosphate	1.2×10^{-3}	DPN	0.23	1.65×10^5	Non-competitive
Inosinetriphosphate	9.0×10^{-4}	Deam.-DPN	1.28	0.98×10^5	2.0×10^{-3}

DISCUSSION OF RESULTS

Competition of Substituted Pyridines and DPN. Pyridine-3-sulfonic acid inhibited GDH in competition with DPN, according to v. Euler [1], and it was noted that at a fixed DPN concentration the reaction was also inhibited by nicotinic acid and amide, but not by trigonelline. In the present study, pyridine and ten substituted pyridines have been examined with respect to their ability to inhibit the GDH-DPN system. Of these eleven compounds, six caused fairly strongly inhibition, two inhibited weakly, and no inhibition could be detected with three others. All of the inhibitors operated competitively with DPN. A typical example is represented graphically in Fig. 1. From the competitive nature of the inhibition, it is evident that the inhibitors complex with the enzyme on the same point or points on the protein surface as does DPN. The ratio K_i/K_{DPN}; a measure of the relative tightness of the binding to the protein of DPN and inhibitor, is large for all the compounds tested. Even with the strongest inhibitor of this type tested, 4-pyridoxic acid, the coenzyme was bound seventy times more tightly than the inhibitor.

The results are summarized in Table 3, where the inhibitory abilities of the compounds relative to that of nicotinamide are presented. It would appear that for such a compound to be a reasonably good inhibitor in this system, substitution of the pyridine ring at the 3-position is required. At this position, substitution of a carboxyl group for an amide group did not significantly alter the inhibitory activity, and replacement of the carboxylic acid amide by a hydroxyl group markedly increased it. The largest inhibitions were produced by pyridoxal and 4-pyridoxic acid, compounds containing negative groups in both β-positions (3 and 5), as well as at position 4. It is of interest that when the carboxyl group was moved from position 3 to position 4 of the pyridine ring the ability of the substance to inhibit this enzyme system vanished. The

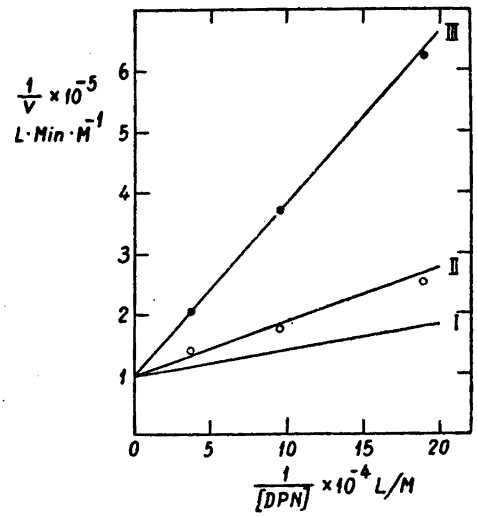

Fig. 1. Inhibition by nicotinic acid. Curve I: No inhibitor. Curve II: 2.2 × 10^{-2} M inhibitor. Curve III: 1.31 × 10^{-1} M inhibitor.

addition of a methyl group to the nicotinic acid structure had no effect on the inhibiting ability, but upon the addition of a second carboxyl group in the 2-position, the inhibitory activity was abolished. In view of the structural similarity between DPN and trigonelline, it is of particular interest that no inhibition was caused by the betaine, a finding reported earlier by v. Euler [1].

Competition of Inorganic Phosphate and Compounds Related to Adenine with DPN. Williams [4] has described the competitive inhibition of malic dehydrogenase by adenine, adenosine and adenosinetriphosphate (ATP). In this system it appeared that both adenine and adenosine possessed the same K_i, whereas ATP was probably but not certainly a stronger inhibitor. It was concluded that one point of attachment of DPN to the enzyme surface is at the adenine moiety, and perhaps another through the phosphate groups,

Table 3. Relative Inhibitory Activity of Pyridine Derivatives.

Compound	Relative Inhibitory Activity
Pyridine	0.08
Nicotinamide	1.0
Nicotinic acid	1.1
*Iso*nicotinic acid	0
*Iso*nicotinic acid hydrazide	0.1
2-Methylnicotinic acid	0.9
Quinolinic acid	0
Trigonelline	0
β-Hydroxypyridine	2.5
Pyridoxal	29
4-Pyridoxic acid	77

Table 4. Relative Inhibitory Activity of Compounds Relative to Adenine

Compound	Relative Inhibitory Activity
Adenine	1.0
Adenosine	1.3
3-Adenylic Acid	3.4
ATP	6.9
9-D-Ribosylpurine	0.5

but with the ribose portion of DPN not involved. We have examined the same three metabolites in the glucose dehydrogenase system (Tables 2 and 4). It can be seen that here ATP was clearly a much stronger inhibitor than adenine, and it is probable that adenosine was somewhat more tightly bound to the protein than was the simple purine. 3-Adenylic acid was intermediate in inhibitory activity, falling in this respect between adenosine and ATP. The inhibitions by adenine and ATP are shown in Fig. 2.

The recent isolation and structure determination of *nebularine*, 9-D-ribosylpurine [8], made available an ideal compound for investigating the role of the 6-amino group of the adenine nucleus in the protein-coenzyme binding, since *nebularine* differs from adenosine only in the absence of this amino group. It was found that GDH was competitively inhibited by nebularine, but less than half as strongly as it was by adenosine.

Glucose dehydrogenase was also inhibited in competition with DPN by relatively high concentrations of inorganic phosphate; at a phosphate concentration of 1.0 *M*, a K_i of 0.86 was observed, and one of 0.76 at 1.0 *M* phosphate. This appears to be much weaker inhibition by phosphate than was observed by Theorell in 1935 [9] in the case of the Zwischenferment of Warburg and Christian.

Inhibitors Tested Against Two Coenzymes. It was of interest to examine the behavior of inosinetriphosphate (ITP) in the GDH system, since in this com-

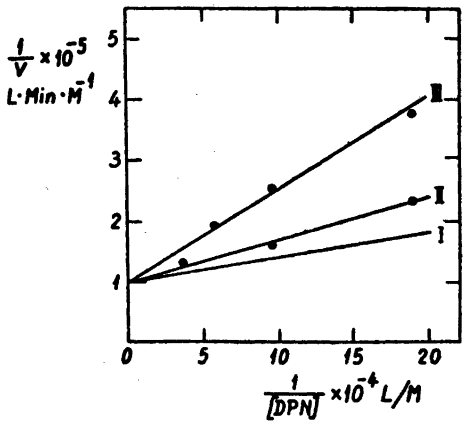

Fig. 2. Inhibition by adenine and ATP. Curve I: No inhibitor. Curve II: Adenine, 8.75 × 10⁻³ M. Curve III: ATP, 4.8 × 10⁻³ M.

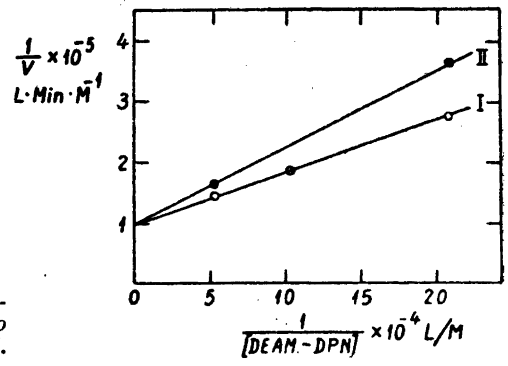

Fig. 3. Inhibition by ITP in the GDH-deaminated DPN system. Curve I: No inhibitor. Curve II: ITP, 9.0 × 10⁻⁴ M.

pound the 6-amino group of the purine nucleus has been replaced by a hydroxyl group. Thus, ITP bears the same structural relationship to ATP that deaminated DPN does to DPN. As can be seen from Table 2 and Figs. 3 and 4, the GDH system was inhibited by ITP; the substance competed with deaminated DPN, but the inhibition was non-competitive when DPN was the coenzyme. In view of this result, ATP was examined in the GDH-deaminated DPN system. The resulting inhibition was non-competitive in nature (Table 2). Finally, nicotinamide was tested, and found to inhibit the action of the enzyme in competition with deaminated DPN as well as with DPN itself.

Conclusion. From the degree and nature of the interactions between the apoenzyme and inhibitors structurally related to the coenzyme, one can draw certain conclusions regarding the structural features of the coenzyme molecule which are involved in the linkages in the enzyme-coenzyme complex.

It appears that DPN is attached to the GDH surface through linkages involving several parts of the coenzyme molecule. It is quite evident that the nicotinamide moiety is involved, with a key structural feature being the carboxylic acid amide group at the 3-position of the pyridine ring. While there is no direct evidence for participation by the ribosyl group attached to the nicotinamide ring, the failure of trigonelline to inhibit perhaps suggests some importance for this carbohydrate portion of the molecule. The adenine

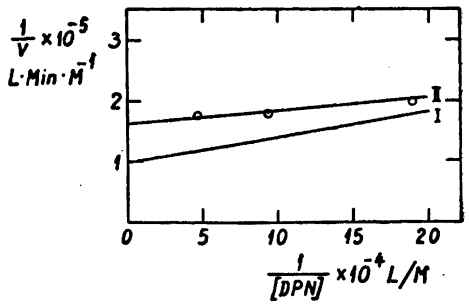

Fig. 4. Inhibition by ITP in the GDH-DPN system. Curve I: No inhibitor. Curve II: ITP, 1.2 × 10⁻³ M.

moiety clearly contributes to the GDH-DPN binding and the probable increased inhibition by adenosine makes it likely that the ribose structure of this portion of the molecule is also involved. 9-D-Ribosylpurine inhibited the GDH-DPN system about as strongly as did nicotinamide, but less than half as strongly as did adenosine; and from these results it may be concluded that both the purine ring structure and the 6-amino group of the adenyl moiety of DPN are important in the protein-coenzyme complex. The participation of the phosphate portion of the structure is clearly demonstrated by the magnitude of the inhibition produced by ATP, as well as by the weak inhibition caused by inorganic phosphate.

The Michaelis constants for DPN and deaminated DPN (Table 1) indicate that DPN is bound to the apoenzyme more tightly than is deaminated DPN, and it would hence appear that the 6-hydroxyl group contributes less to the binding than does the corresponding amino group. However, the hydroxyl group is involved, and a qualitatively different type, or site, of binding is suggested by the results obtained when ATP and ITP were each studied against both coenzymes.

Deamination causes no change in the pyridine portion of DPN and, as expected, nicotinamide inhibition was competitive with respect to both coenzymes. The smaller value observed for the K_i against deaminated DPN is in accordance with the larger Michaelis constant for the deaminated coenzyme.

There is reason to believe that the oxidation of glucose is not the true physiological role of liver glucose dehydrogenase [5,10]. In this connection, it is of interest that of the various substituted pyridines studied as inhibitors, pyridoxal and the closely related 4-pyridoxic acid were bound to GDH many times more tightly than were the simpler compounds of this class (Table 3). It seems at least conceivable that GDH might be able to function as a pyridoxal phosphate-linked transaminase. However, this has not been subjected to experimental verification.

It is evident that the conclusions presented here could be strengthened and broadened by the examination of additional compounds for inhibitory activity. Further, it should be emphasized that these ideas may be valid only for the particular enzyme system studied here; differences are to be expected with other enzymes.

SUMMARY

1. Eighteen compounds structurally related to DPN have been examined for their ability to inhibit beef liver glucose dehydrogenase activity.

2. From the nature and intensity of the inhibitions observed, some conclusions have been drawn concerning the portions of the coenzyme molecules which are involved in the binding of the apoenzyme-coenzyme complexes.

3. It appears that most moieties of the coenzyme molecules contribute to the linkage with the protein surface. In the DPN molecule, the pyridine ring and its 3-amide group, the phosphoric acid residues, the ribose moiety attached to adenine, and the adenine nucleus and its 6-amino group all participate. The substitution of a hydroxyl group for the amino group in the deamination of DPN results in a weaker and qualitatively different type of binding in the altered coenzyme.

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Studies on the Interaction of Paraffin Chain Alcohols and Association Colloids. I. The Solubility of Decanol-1 in Sodium Oleate and Sodium Myristyl Sulphate Solutions above the Critical Micelle Concentration

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In 1947 Harkins [1,2] suggested the term "penetration" to define the process by which polar-nonpolar compounds such as paraffin chain alcohols and amines are brought into aqueous solution by association colloids. The term was introduced to differentiate this process from the process of hydrocarbon solubilization. From X-ray studies Harkins concluded that the molecules of polar-nonpolar compounds are built into the micelles in a different way than nonpolar hydrocarbon molecules; whereas the latter enter into the lipophilic inner region of the micelles between the palisade layers, the former penetrate between the ions in the palisade layer and with their polar groups in direction of the surface of the micelle. The studies later conducted by Klevens [3,4] have confirmed that different regions of the micelle are involved in the hydrocarbon solubilization and in the penetration of polar-nonpolar compounds and hence these are two different processes. Ekwall and coworkers [5–7] have further pointed out that the solubilization of hydrocarbons and polar-nonpolar substances differ from each other in many respects.

The different behaviour of polar-nonpolar compounds is due to the operation of a new factor not involved in the solubilization of hydrocarbons, namely the interaction between the hydrophilic dipole end groups of the polar-nonpolar molecules and the water dipoles on one hand and between former and the ionic groups of the colloid on the other. As Ekwall and Danielsson have shown [6,7] this interaction occurs at very low association colloid concentrations, at the so-called limiting association concentration of the colloid, L.A.C., which is much below the critical micelle concentration, C.M.C. Owing to this interaction the properties of the colloid solutions are altered in many respects. Frequently it happens that the interaction leads to the precipitation of a complex substance composed of polar-nonpolar compound, association colloid and water.

The penetration process has been the subject of numerous studies during the last six years, but despite this it is still incompletely understood in several respects.

We have therefore considered it desirable to undertake a detailed study of the penetration process in its most typical form, as it appears when long paraffin chain alcohols are dissolved in aqueous solutions of ionic association colloids of the paraffin chain type. The experiments were conducted mainly with decanol-1 and the association colloids used were fatty acid soaps and monoalkyl sulphates. In the following we will describe the results of quantitative determinations of the solubility of decanol-1 in sodium oleate and sodium myristyl sulphate solutions above their critical micelle concentrations.

EXPERIMENTAL

The decanol-1 (Light & Co) used was purified by repeated recrystallization.

Sodium oleate was prepared in the usual manner by neutralizing purest oleic acid (British Drug Houses Ltd.) with sodium ethylate in absolute ethanol.

Sodium myristyl sulphate was prepared from Duponol M E (E. J. du Pont de Nemours & Co, Inc.) by repeated extraction with and recrystallization from absolute ethanol. Analyses and molecular weight determinations indicated that the substance contained small amounts of homologous compounds.

The aqueous colloid solutions were prepared by weighing both the colloid and the water (specific conductance $\varkappa = 1.0 \cdot 10^{-6}$ mho).

The solubility of decanol in the soap solutions was determined by the turbidity method. Equal amounts of the same soap solution were transferred to ampoules, and different amounts of decanol were introduced into the ampoules with an Agla micrometer syringe. The ampoules were then sealed and shaken 2—4 days at 40° C. During this period the solutions either dissolved the decanol or became saturated with the alcohol or its complex with soap. When the alcohol added was in excess of the amount required for saturation, the solutions remained turbid. The turbidities were measured with a Pulfrich Step Photometer.

Comparative experiments were conducted to determine whether the shaking time and the method of preparation of the solutions affected the solubility values. Oleate solutions of different concentrations containing decanol were prepared in three different ways: method 1, as described above; method 2, by warming several minutes at 90° C on a water bath and then shaking at 40° C; method 3, by warming to 90° C, allowing to stand two days at 20° C, and then shaking at 40° C. In some cases the period of shaking at 40° C was prolonged to 30 days during which the turbidity was measured several times. The length of shaking period was not found to be of any significance with method 1. In the case of the other two methods, somewhat greater deviations were observed in some cases and the character of the turbidity underwent slight changes with time; however the turbidity values agreed fairly well with those obtained using method 1. It was established that the solubility values determined by the turbidity method used in this study did not deviate more than $\pm$ 0.2 ml decanol per liter.

RESULTS

There is a limit to the amount of decanol-1 that is dissolved by association colloid solutions. When the alcohol is added in excess of this amount, the solution becomes turbid. The substance causing the turbidity is composed of decanol, association colloid and water [8]. The amount of decanol dissolving in the solution before turbidity appears is dependent on the soap concentration of the solution. Tables 1 and 2 give the maximum amounts of decanol that

Table 1. The maximum amounts of decanol dissolved by sodium oleate before the solutions become turbid.

Moles oleate per 1 000 g solution	Millilitres decanol per 1 000 g solution
0.00500	0.1
0.00997	0.2
0.0200	0.5
0.0300	1.2
0.0395	1.5
0.0500	1.9
0.0638	2.4
0.0782	3.3
0.101	4.2
0.111	4.5
0.125	5.2
0.144	6.3
0.171	7.8
0.198	9.0
0.215	9.9
0.224	10.3
0.233	10.9
0.241	11.2
0.250	11.6
0.267	12.2
0.275	12.4
0.279	13.0
0.300	13.5
0.304	13.7
0.308	13.7
0.329	14.9
0.357	15.7
0.387	16.8
0.412	17.5
0.434	17.9

Table 2. The maximum amounts of decanol dissolved by sodium myristyl sulphate before the solutions become turbid.

Moles myristyl sulphate per 1 000 g solution	Millilitres decanol per 1 000 g solution
0.00999	0 65
0.0300	2.5
0.0700	5.7
0.0902	7.1
0.109	8.2
0.130	10.4
0.150	11.8
0.170	13.6
0.190	15.0
0.210	17.2
0.230	18.6
0.260	21.3
0.288	23.7
0.310	25.3
0.347	28.1
0.401	31.8
0.495	37.7
0.593	43.5

are dissolved by sodium oleate and sodium myristyl sulphate solutions before the solutions become turbid (referred to as the solubility in the following).

Fig. 1 illustrates the concentration dependence of the solubility of decanol in the oleate and myristyl sulphate solutions. Up to oleate concentrations of about 0.12 *M*, the solubility of decanol increases linearly with the oleate concentration. Above this concentration the increase appears to be somewhat more rapid but above 0.25 *M* it diminishes perceptibly. The points marked with a cross refer to experiments in which the solutions were prepared in a different manner than usual (warmed, cooled, longer shaking period). It is seen that these values have not been greatly affected by the difference in treatment. In the myristyl sulphate solutions the solubility increases linearly up to a myristyl sulphate concentration of about 0.18 *M* after which the slope of the curves increases at first and then clearly begins to decrease when the concentration exceeds 0.3 *M*.

The dotted lines for both substances indicate, how the solubility should increase with the association colloid concentration if the relation continued to

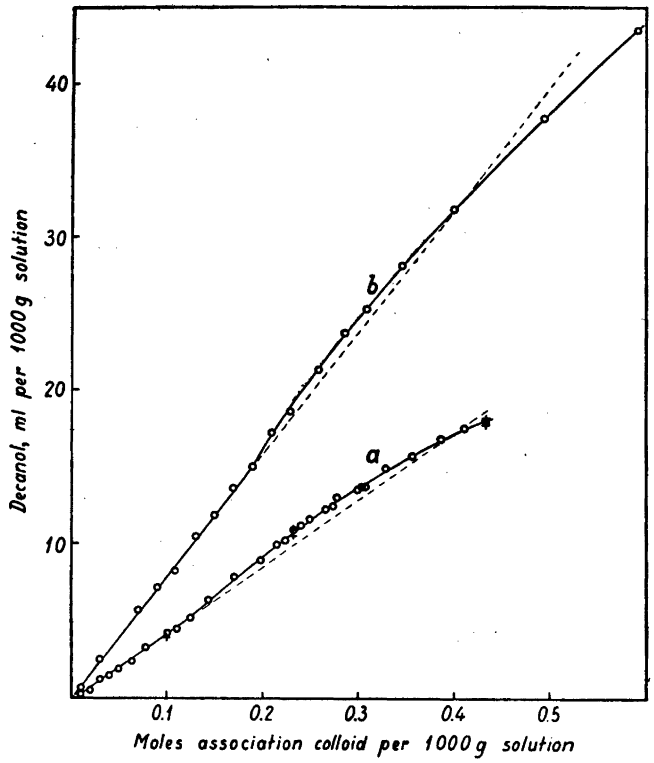

Fig. 1. The maximum amounts of decanol dissolved before the solutions become turbid.
a) Sodium oleate solutions
b) Sodium myristyl sulphate solutions

be linear as at the low concentrations. The deviations from linearity are greater than the relatively wide error limits of the method, ± 0.2 ml decanol per liter, and must hence be considered significant.

DISCUSSION

According to the measurements of Harkins and his co-workers [2] the curves representing the solubility of heptanol-1 in potassium dodecanoate and potassium tetradecanoate solutions are concave toward the colloid concentration axis, whereas the corresponding solubility curves for decanol-1 and dodecanol-1 are slightly convex towards the same axis. Klevens [4] is also of the opinion that the solubility curve for heptanol-1 is concave, but he states that the curves for alcohols with ten or more carbon atoms appear to be linear.

As was shown above, our measurements have given a somewhat different result; the solubility curves for decanol are linear above the C.M.C. up to relatively high concentrations of the association colloid, after which it tends to rise slightly and then curves downwards at still higher concentrations.

Table 3. Composition of the mixed micelles when the solutions become turbid; the proportion of decanol in moles per mole micellar soap.

Association Colloid.	Concentration range	Maximum proportion of decanol in moles decanol per mole micellar soap
Sodium oleate	0.005—0.12 *M*	0.23
	0.15 *M*	0.30
	0.4 *M*	0.14
Sodium myristyl sulphate	0.005—0.18 *M*	0.41
	0.22 *M*	0.50
	0.5 *M*	0.30

It may be mentioned in this connection that the solubility of decanol in solutions of several other paraffin chain soaps also increases linearly with the soap concentration above their C.M.C., *i.e.* within the small micelle range.[8]

When hydrocarbons and many other substances are solubilized, the excess separates in the pure state. From the slopes of the solubility curves it is in such cases possible to calculate the saturation capacity of the micellar substance for the solubilized substance. The turbidity which is observed in oleate and myristyl sulphate solutions when decanol is added in excess is not, however, due to pure decanol, but is due to a mixture of decanol, association colloid and water [5,7,8]; with increasing excess of decanol, a part of the colloid is precipitated.

The solubility curves shown in Fig. 1 are hence not directly comparable with those obtained in the study of hydrocarbon solubilization. Owing to the penetration there are formed mixed micelles; the surfaces of the micelles become less hydrophilic when more decanol molecules are built into the micelles, and the solubility of the micelles decreases. It seems most probable that the solutions become turbid, when these mixed micelles begin to separate. Our solubility determinations thus give the maximum amount of decanol which the micelles are able to take up before this separation begins. From the slopes of the curves the composition of this mixed micelle substance can be estimated. In Fig. 2 it is shown how the decanol content of the mixed micelles, expressed in moles of decanol per mole of micellar oleate and micellar myristyl sulphate, varies with the association colloid concentration. Their decanol content remains constant up to a association colloid concentration of 0.12—0.18 *M*, after which it tends to increase to a slightly higher value, but falls off rapidly with further increase in the colloid concentration. Data taken from these curves are given in Table 3.

It is known that the solubilization power for hydrocarbons increases with the chain length of the association colloid [4,5]. One of us has previously shown that the saturation capacity of sodium oleate for *p*-xylene is 40 per cent higher than the saturation capacity of myristyl sulphate for the same hydrocarbon [5].

The opinion prevailing in the literature seems to be that the ability to dissolve alcohols increases with the chain length of the colloid [4,9]. This is not

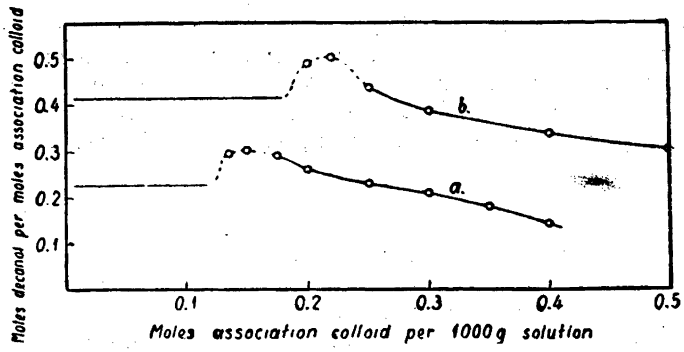

Fig. 2. The decanol content per mole association colloid of the mixed micelles.
a) Sodium oleate solutions
b) Sodium myristyl sulphate solutions

correct, at least not in all cases, as shown by the results of the present study. The ability of myristyl sulphate to dissolve decanol is about 80 % higher than that of oleate although the latter has the longer chain length. This must be attributed primarily to the different ionic end groups in the two colloids. The sulphate group is more hydrophilic than a carboxyl group, and the incorporation of decanol in the palisade layer has evidently a greater influence on the hydrophilic properties of the micelle surface in the case of the latter end group. This is confirmed by the fact that the solubilization power of sodium myristate for decanol is less than that of sodium myristyl sulphate [8].

In this connection it may be mentioned that unpublished measurements carried out in this laboratory [8] show that the "solubilization" of decanol by sodium myristate, sodium laurate and sodium caprinate solutions above their C.M.C. (in the small micelle range) increases in the order mentioned and is greater for all three soaps than the "solubilization" by oleate. This proves that the solubility of decanol increases with decreasing chain length when the ionic group is the same and indicates that the solubility of the alcohol-containing mixed micelles increases when the ratio of the total number of hydrophobic CH_2-groups to the number of hydrophilic groups in the micelle decreases.

The fact that the upper limit for the composition of the soluble decanol-association colloid micelles expressed in moles of decanol per mole of soap is constant over an extended concentration range above the C.M.C. shows that the micellar substance has constant properties in this concentration range. This latter range is the region where the small micelles occur. It is interesting to note that deviations first become evident in oleate solutions above 0.12 —0.25 *M* and in myristyl sulphate solutions above 0.18—0.3 *M*. (See Figs. 1 and 2.) At these points the ability of the micelles to incorporate decanol without precipitions begins to decrease. It may be noted that also other properties of the micellar substance undergo change at the same concentrations; the saturation capacity for hydrocarbons (*p*-xylene), which remains constant over the small micelle range, begins to increase when the corresponding oleate and myristyl sulphate concentrations, about 0.2 *M*, are exceeded [6,10,11].

Our studies on the solubilization of decanol indicate that the properties of oleate and myristyl sulphate solutions undergo pronounced alterations in the concentration ranges referred to. Whether these concentration limits, which undoubtely are real, indicate the occurrence of a new step in micelle formation or requires another explanation, will be considered in a later paper.

SUMMARY

The maximum amounts of decanol-1 that are solubilized by sodium oleate and sodium myristyl sulphate solutions of various concentrations before turbidity occurs have been determined. In the small micelle range of both association colloids the decanol solubility increases linearly with the soap concentration, but in the concentration range 0.12—0.18 *M* the solubility curves reveal a tendency to rise more rapidly. At still higher concentrations the curves become concave toward the soap concentration axis. The deviations from linearity occur at approximately the same concentration regions at which earlier studies have shown the properties of oleate and myristyl sulphate solutions to undergo marked changes.

The solubilities of decanol and other paraffin chain alcohols in aqueous solutions of association colloids of the paraffin chain type can not be directly compared with the solubilities of hydrocarbons in these solutions. In the latter case the excess substance separates from the solution in the pure state, whereas when the alcohol is solubilized, a substance composed of alcohol, association colloid and water separates. It seems probable that the latter substance is a mixed micelle substance which is formed by the incorporation of alcohol in the palisade layer of the micelle as a result of which the surface of the micelle becomes less hydrophilic. The measurements show that the solubility of decanol is greater when the ionic end group is a sulphate group than when it is a carboxyl group and increases with decreasing length of the paraffin chain of the association colloid.

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The Evaluation of Complexity Constants from Polarographic Data

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The possibility of studying the equilibria of complex compounds polarographically was recognized by Heyrovsky and Ilkovic [1] as early as 1935. Since then several polarographic determinations of equilibrium constants of various systems have been reported in the literature.

Hitherto essentially the last complexity product of a system has been measured, whereas little attention has been paid to the consecutive constants illustrating the stepwise formation of complex compounds. Some authors even seem to deny the possibility of measuring these constants polarographically.

In 1950 the present authors [2] suggested the use of the polarographic method for the investigation of the stepwise formation of complexes, and recently de Ford and Hume [3] have presented a method of calculating the consecutive constants from polarographic data. Furthermore Hume, de Ford and Cave [4] have reported their results on the equilibrium constants of the cadmium thiocyanate system.

De Ford and Hume [3] assume that the ligand concentration at the drop surface is the same as the concentration in the body of the solution, and their calculations refer to the case where the central metal ion is reversibly reducible at the electrode. In this article we intend to show that the polarographic method is capable of still wider application. It can be used when the ligand concentrations at the drop surface and in the body of the solution are not the same and even when the central metal ion is not reversibly reducible. In the last case, however, it is necessary to use an indirect method based on the behaviour of an „indicator ion".

DIRECT POLAROGRAPHIC METHOD

The method generally used for the determination of the last complexity product is based on the following principle:

Let M be the central atom and A the ligand of a complex compound MA_n. If E_1 is the half-wave potential of a solution containing no ligand — *e.g.*

a metal perchlorate solution — and E_2 is the half-wave potential in the presence of a large excess of ligand, the following equation is valid:

$$\Delta E_{\frac{1}{2}} = E_2 - E_1 = -\frac{RT}{mF} \cdot \ln(\beta_n \cdot A^n) \qquad (1)$$

It is assumed that the m-valent metal is reduced to the metallic state. β_n is the last complexity product of the following system of equilibria:

$$\frac{MA}{M \cdot A} = \beta_1 ; \frac{MA_2}{M \cdot A^2} = \beta_2 ; \cdots\cdots ; \frac{MA_n}{M \cdot A^n} = \beta_n \qquad (2)$$

The letters denote concentrations of different species. For the sake of convenience, activity coefficients have not been taken into consideration. The relationship between the complexity products and the individual complexity constants is

$$b_1 \cdot b_2 \cdot \cdots\cdots b_n = \beta_n \qquad (3)$$

where

$$b_n = \frac{MA_n}{MA_{n-1} \cdot A} \qquad (4)$$

Equation (1) is based on the assumption that the concentration of the ligand at the electrode is the same as in the bulk of the solution and that only the last of the consecutive complex compounds needs to be taken in account. In other words, the equation cannot be used unless the final complex is strong and there is a large excess of ligand in the solution.

If a more generally valid equation is required all the complexity equilibria must be taken in consideration, and it is further necessary to pay attention to the fact that the concentrations at the electrode surface and in the solution are not the same. Polarographic equilibrium calculations must always be made on the basis of the concentrations at the surface. According to general principles of polarography we can write:

	Solution	Electrode surface
Total concentration of ligand	c_A	c_A
Total concentration of metal	c_M	$\frac{i_d - i}{i_d} \cdot c_M$
Concentration of free ligand	A	A_o
Concentration of free metal ion	M	M_o

The concentrations at the electrode surface are designated by the subscript o, i is the measured current, i_d is the diffusion current. The total concentration of the ligand at the electrode surface is known, and the total concentration of the metal can be calculated if i and i_d are also known. A_o will be discussed later. M_o can be calculated from the equation

$$\Delta E = -\frac{RT}{mF} \cdot \ln \frac{\frac{i_d - i}{i_d} \cdot c_M}{M_o} \qquad (5)$$

ΔE is the difference between the potential of a metal perchlorate solution with ligand present and without any ligand at a defined current value. If $i = \frac{1}{2} i_d$, then $\Delta E = \Delta E_{1/2}$, the difference between half-wave potentials. Also other current values can be employed if the small corrections for the change in the residual current and the diffusion current (due to the changes in the diffusion coefficients) are taken into account.

If weak complexes are formed, the slope of the curve will usually be the theoretical one, but if the complex formed is strong and if only small amounts of the ligand are present, the slope, especially in the upper part of the curve, will be smaller. This is because A_o increases when metal is removed from the solution. Consequently M_o decreases as the current increases, and the curve shifts towards more negative values. Of course, the change in the slope is no indication of any irreversible reaction.

If consecutive complexity products are to be determined, ΔE must be measured at different ligand concentrations c_A. For the calculations, ΔE and c_A must be expressed as functions of the equilibrium constants. This is easily done, since the total concentration equals the sum of the concentrations of all the components. In other words:

$$\frac{i_d - i}{i_d} \cdot c_M = M_o \cdot (1 + \beta_1 \cdot A_o + \beta_2 \cdot A_o^2 + \cdots + \beta_n \cdot A_o^n) \qquad (6)$$

From (5) and (6) we obtain

$$\Delta E = -\frac{RT}{mF} \cdot \ln(1 + \beta_1 \cdot A_o + \beta_2 \cdot A_o^2 + \cdots + \beta_n \cdot A_o^n) \qquad (7)$$

Furthermore

$$c_A = A_o + M_o \cdot (\beta_1 \cdot A_o + 2 \cdot \beta_2 \cdot A_o^2 + \cdots + n \cdot \beta_n \cdot A_o^n) \qquad (8)$$

The problem of computing individual complexity constants is hereby reduced to a purely mathematical problem. One simply conducts a series of c_A and ΔE measurements and calculates A_o and the β constants from equations (5), (7) and (8). If n = 1 or 2, the calculations are very simple. If n is larger than 2, equations of higher order are obtained, and their solution is not quite simple. An approximate value for β_1 can be obtained from measurements at a low ligand concentration by assuming that only the first complex is formed. In an analogous way an approximate value for the last complexity product β_n can be obtained — if the complex is strong enough — from measurements at high ligand concentrations. Equation (7) will then be transformed into

$$\Delta E = -\frac{RT}{mF} \cdot \ln(\beta_n \cdot A_o^n) \qquad (9)$$

This equation is identical with the previous equation (1), which neglects all the complexes with less than n ligands.

Correct values for the other constants can be obtained by successive approximations. Leden [5], in a potentiometric study of the cadmium complexes,

was the first to determine consecutive complexity constants by measuring the concentration of the central metal ion. He used a partly graphical method. Fronaeus [6] has later modified this method. De Ford and Hume [3] used a method similar to Leden's. For details one must refer to the original papers and to a following paper by Eriksson [7].

De Ford and Hume assume that the ligand concentration at the drop surface is the same as in the body of the solution. It may be remarked that this assumption is not essential. According to equations (5)—(8) it is possible to determine all concentrations in question at the drop surface.

It may some time be of advantage to measure the potential shift at a smaller current than $\frac{1}{2}\, i_d$.

In the following paper [7], the polarographic determinations of the complexity constants of cadmium chloride and bromide will be described.

If the central metal ion is not reversibly reducible or oxidizable but the ligand is, the consecutive complexity constants can be determined polarographically in an analogous way. The calculation will then be simpler and can be made according to the method of J. Bjerrum [8].

INDIRECT POLAROGRAPHIC METHOD

Even if polarography offers a possibility of determining individual complexity constants, the method seems at first sight to be of only limited value as the application of polarographic methods to equilibrium problems presupposes a reversible reaction at the electrode surface. Unfortunately, only a few metals — *e.g.* Cd, Pb, Tl — are reversibly reducible at the mercury electrode.

It may be noted, however, that a non-reducible metal can be titrated amperometrically after adding a suitable indicator ion, as shown by Ringbom and Wilkman [9], and in an analogous way it is possible to determine the concentration of a non-reducible metal ion by using another ion as an indicator.

As an example we may take the case in which the equilibrium constants of the compounds of a metal M and the ligand A are to be determined. If cadmium, for example, forms complexes of known strength with the same ligand, the cadmium ion can be used as an indicator, provided that the cadmium wave appears before the M-wave.

Applying the equation (7) to cadmium we get

$$\Delta E = -\frac{RT}{2F} \cdot \ln(1 + \beta_1 \cdot A_o + \beta_2 \cdot A_o^2 + \cdots + \beta_n \cdot A_o^n) \qquad (10)$$

If all the β constants are known, the equation (10) represents a quite general method for the determination of anion (or molecule) concentrations. One merely adds a little indicator, in this case cadmium perchlorate, to the metal solution and measures the half-wave potential with reference to the half-wave potential of a cadmium perchlorate polarogram. From a graph showing the relation between ΔE and A_o the ligand concentration can be immediately obtained.

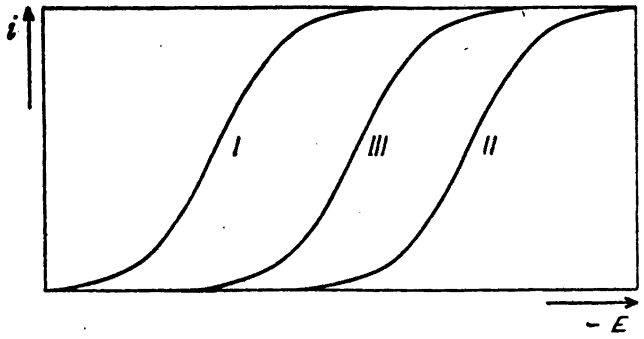

Fig. 1. Polarograms illustrating the indicator method of measuring ligand concentrations.

I. Indicator ion.
II. Indicator ion + ligand.
III. Indicator ion + metal ion + ligand.

It is clear that when M ions are added to a solution containing Cd ions and A ligands, they will react with part of the ligands; in other words, A_o will decrease. In Fig. 1 three polarograms are plotted: curve I has been recorded in a cadmium perchlorate solution, curve II has been taken in a solution containing cadmium and the ligand, and curve III in the same solution as II after adding M ions in a known concentration.

It may be emphasized that the mathematical calculation of the complexity constants by the indirect method is simpler than in the direct determination of the central ion. It is suitable to use the average ligand number $\bar{n}$ introduced by J. Bjerrum [8] *i.e.*

$$\bar{n} = \frac{\text{number of bound ligands}}{\text{total number of metal atoms}} \tag{11}$$

The average ligand number of cadmium at the electrode surface can be calculated from the equation

$$\bar{n}_{oCd} = \frac{\beta_1 \cdot A_o + 2 \cdot \beta_2 \cdot A_o^2 + \cdots + n \cdot \beta_n \cdot A_o^n}{1 + \beta_1 \cdot A_o + \beta_2 \cdot A_o^2 + \cdots + \beta_n \cdot A_o^n} \tag{12}$$

It is convenient to plot $\bar{n}_{oCd}$ as a function of log A_o. If only mononuclear complexes are formed, $\bar{n}_{oCd}$ does not depend on c_{Cd} and $\bar{n}_{oM}$ can then be computed from the equation

$$\bar{n}_{oM} = \frac{c_A - A_o - \bar{n}_{oCd} \cdot \frac{i_d - i}{i_d} \cdot c_{Cd}}{c_M} \tag{13}$$

For the complexity constants of the M—A-complexes the following method suggested by J. Bjerrum [8] can be used:

Equation (12) is written as follows

$$\bar{n}_{oM} + (\bar{n}_o - 1) \cdot A_o \cdot \beta_1 + (\bar{n}_o - 2) \cdot A_o^2 \cdot \beta_2 + \cdots + (\bar{n}_o - n) \cdot A_o^n \cdot \beta_n = 0 \quad (14)$$

From the log A_o versus $\bar{n}_{oM}$ curve, A_o values corresponding to $\bar{n}_{oM} = 0.5$, 1.5, 2.5 etc. are taken. The constants are then calculated from these values by means of equation (14). Some approximations can usually be made.

When cadmium is used as an indicator, it seems probable that the concentrations of the following ligands can be determined: chloride, bromide, iodide, nitrite, thiocyanate, azide, acetate, several organic anions, ammonia. Cyanide ions form a strong complex with cadmium, but according to Kolthoff and Lingane [10], the wave is not perfectly reversible. The complexes with nitrate, chlorate, bromate and fluoride seem to be too weak for the purpose in question.

In the indirect method suggested above there is one weak point. If the metal M is not reversibly reducible, the reaction between M and the ligand does not occur instantaneously. Consequently, even if it is possible to calculate all the concentrations at the electrode surface, it is not certain that the M ions are in equilibrium with the ligands liberated at the surface.

If A and A_o do not differ very much, *i.e.* if the slope of the curve is the theoretical one, the error caused by the unattained equilibrium can be neglected. All the cadmium complexes mentioned above, except the cyanide complex, seem to be so weak that no considerable differences between A and A_o exist if $i = \frac{1}{2} i_d$ or less. But if there is a marked decrease in the slope, then it is better to measure potential differences using a lower current strength than the half-wave current, or to measure ΔE at various current strengths and to extrapolate the values to $i = 0$. In this way the concentration in the bulk of the solution is obtained, and the polarographic method is similar to a potentiometric method, the indicator system representing an electrode of the second order.

The accuracy of the method described above is of course dependent on the strength of the indicator complex. If the indicator complex is a very weak one, the accuracy is low, but if the complex is strong, the attainable accuracy can be surprisingly high. A simple calculation illustrates this.

In direct measurements of cadmium potentials, the error at 25° is

$$\frac{dE}{\frac{dCd}{Cd}} = \frac{RT}{2F} = 12.3 \text{ mV} \quad (15)$$

This means that ± 1 mV corresponds to an error about ± 8 %. If on the other hand, the concentration of ligand A is measured by means of cadmium potentials, the error is given by the equation

$$\frac{dE}{\frac{dA}{A}} = -\bar{n}_{Cd} \cdot \frac{RT}{2F} \quad (16)$$

This means that if the average ligand number $\bar{n}_{Cd}$ is larger than 1, the theoretical accuracy is greater than in the direct measurement of cadmium concentrations. If cadmium forms a strong complex, so that $\bar{n}_{Cd}$ approaches the value 4, then $\pm$ 1 mV will correspond to an error of only $\pm$ 2 %. Of course, other factors can affect the results, which largely depend on the accuracy of the determination of the indicator complexity constants. It may also be mentioned that if the M-complexes are weak, the accuracy in determining $\bar{n}_M$ and hence the equilibrium constants according to equation (13) is essentially dependent on the accuracy of the determination of the difference $c_A - A_o$. Consequently, it is necessary to have a rather high concentration of M in order to decrease A_o sufficiently.

The influence of activity coefficients has not been considered above. If possible, it is advantageous to make all the measurements in a concentrated sodium perchlorate solution, as is usual in potentiometric measurements.

The polarographic method is probably in some instances superior to the potentiometric method, since the dropping mercury electrode is more convenient than amalgam electrodes, which demand great care. Moreover, when low concentrations are measured, the polarographic method gives no polarization troubles. Which of the two methods can be used depends on the case in question; this aspect will not be discussed here. It seems, however, worth while to emphasize that polarography as well as potentiometry offer possibilities for the measurement of the concentrations of anions or molecule groups that form complexes with a reversibly reducible metal. It also appears to be theoretically possible to determine consecutive complexity constants of other metal complex compounds, even when neither the central ion nor the ligand can be determined polarographically.

SUMMARY

The possibility of determining consecutive complexity constants from polarographic data are discussed.

An indicator method is proposed for the determination of ligand concentrations. Complexity constants can be determined polarographically by this method even if neither the metal nor the ligand of the complex is reduced at the electrode.

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Thermochemical Investigations on Organic Sulfur Compounds

III. On the possible use of thianthrene as a secondary standard in bomb calorimetry for sulfur compounds *

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In the determination of heats of combustion of organic compounds in a bomb calorimeter, the presence of sulfur in the molecule may cause several systematic errors. In 1934 the IUPAC Standing Commission for Thermochemistry pointed out the importance of calibrating the calorimetric system with a standard substance containing the same element as the substance being studied.[1] Thus, many errors of a systematic character will tend to be minimized. Two years later the Standing Commission proposed sym-diphenylthiourea or thioglycolic acid as sulfur-containing test-substances [2].

From the following discussion concerning the requirements which a suitable secondary standard substance should fulfil, it is concluded that neither sym-diphenylthiourea nor thioglycolic acid can be accepted as a satisfactory secondary standard.

REQUIREMENTS FOR A SECONDARY STANDARD

Huffman has summarized the requirements which a standard substance for bomb calorimetry should fulfil [3]:

1. *It should be readily obtainable in a pure state.*
2. *It should be easy to dry and should remain so without special precautions.*
3. *It should be completely stable at elevated temperatures.*
4. *It should not be volatile.*
5. *It should pellet readily.*
6. *It should be readily inflammable.*
7. *It must burn completely.*

However, further requirements are necessary for a standard substance containing sulfur. It is obvious that the common practice of burning sulfur

* Paper I and II in this series: References 4 and 5.

compounds together with an auxiliary substance such as paraffin oil should be avoided in the case of the secondary standard.

8. *It should be burned without use of an auxiliary substance.*

The last requirement follows from the attempt to minimize the influence from possible (unknown) systematic errors due to the presence of sulfur. Some of these errors may vary significantly if widely varying amounts of sulfur are burned either as elemental sulfur or as an organo-sulfur compound *. It is impossible in practice to maintain constant the amount of sulfur present in combustions of various substances.

The energy equivalent of the calorimeter restricts the quantity burned for substances with low sulfur content. For example, a reasonable amount of octadecylmercaptane for a combustion in an ordinary bomb calorimeter would be 0.5 g, a quantity which contains less than 2 milliatoms of sulfur.

The upper limit for the amount of sulfur in a single combustion is set partly by the increasing risk of incomplete oxidation to sulfuric acid with increasing amounts of sulfur and partly by the necessity of avoiding sulfuric acid in high concentrations in the final state. It was found in this laboratory that 8 milliatoms of sulfur in each combustion experiment were oxidized completely to hexavalent sulfur (*cf.* below). Waddington and co-workers burned by "The Huffman-Ellis Method" [6] an amount of thiophene corresponding to 10 milliatoms of sulfur without observing any sulfur dioxide [7]. In this case, if 10 ml of water is used in the bomb, the sulfuric acid obtained will be 2 *N* which, at the present, is a reasonable upper limit for the concentration of the acid, considering the approximations made in correcting the actual to the idealized bomb process [4]. It is not advisable to increase the amount of water in the bomb as the errors in the calculation of the heat of solution of carbon dioxide then no longer may become significant [4].

Therefore, when burning a substance with a high percentage of sulfur, the quantity used is limited and thus the heat evolved from the combustion of the compound is rather small. In the use of the method of comparative measurements the total heat evolved should be kept constant, and the use of an auxiliary substance is required. This decreases the accuracy with which the heat of combustion of the sulfur compound can be determined. In such cases it is desirable to burn as large a quantity of the compound as possible.

From what has been said it is evident that the amount of sulfur used in a combustion must be varied from one compound to another — in practice between approximately 2—3 and 8—10 milliatoms of sulfur. Thus, it is not possible to standardize the calorimeter by burning a standard substance "under conditions identical with those which will characterize later on the combustion of the substance investigated"[1]. The choice of a standard substance must therefore be the result of a compromise, the amount of substance burned should correspond to 5—7 milliatoms of sulfur. This is the basis for the statement of the next requirement.

9. *The quantity of standard substance burned should contain a sufficient amount of the element in question (sulfur) to give a final state comparable to that*

* For small variations in the amount of sulfur present in a combustion, such errors are negligible; *cf.* communication IV (to be published).

found after combustions of compounds containing an average amount of the same element.

Neither of the two substances proposed by the Standing Commission for Thermochemistry fulfils the last two requirements. The heat of combustion of the sym-diphenylthiourea is calculated to be approximately 1 680 kcal per mole. In a calorimeter of conventional design in which the total heat evolved is between 5 000 and 8 000 calories only about 3 to 5 millimoles of this substance may be burned. On the other hand, it is necessary to use about 15 to 20 millimoles of thioglycolic acid to obtain the same amount of heat. Thus the sulfur content of diphenylthiourea is too low and that of thioglycolic acid too high to make the substances desirable as standards.

In searching for a suitable standard substance for bomb calorimetry of sulfur compounds, the behaviour of thianthrene was investigated.

SYNTHESIS AND PURIFICATION OF THIANTHRENE

Thianthrene was synthesized from pure benzene, freshly distilled sulfur monochloride and aluminum chloride (Fries and Vogt [8]). The crude thianthrene was recrystallized from benzene, distilled at 0.2 mm Hg in an all-glass apparatus without standard joints, twice recrystallized from benzene and subjected to sublimation at 10^{-3} mm Hg and 100° C. The products from three different syntheses, all showing the same m.p. 155.9° C, were combined (sample A, 750 g). Two sulfur analyses both showed 29.8 % S (calculated value 29.64 %). Sample A was subjected to a slow freezing-out process whereby 75 % of the substance crystallized (sample B) and the rest was discarded. The purity of samples A and B was checked by observation of melting point curves * in an apparatus described by Smit [9]. From the result it was evident that one freezing-out operation greatly improved the purity. The melting interval decreased from 0.25° C to 0.18° C (± 0.02°). At the same time both the initial and the final melting temperatures increased, from 155.70 to 155.90° and from 155.95 to 156.08° C. For sample A premelting began at 155.25 and for sample B at 155.70° C. On the basis of certain assumptions the amount of impurity may be roughly estimated from the melting point curve [9,10]. By this method the depression of the melting point of sample B due to the presence of impurities was calculated to be 0.02° C. Determination of the cryoscopic constant of thianthrene gave a mean value of 24.5° C per mole of impurity per 100 g of solvent. Thus a depression of 0.02° corresponds to 0.8 millimole of impurity per 100 g of thianthrene (0.18 mole-%). The purity of sample B was hence estimated to be 99.8 mole-%.

SUITABILITY OF THIANTHRENE AS A SECONDARY STANDARD

A preliminary investigation concerning the fulfilment of requirements 1 to 9 gave the following results:

1. Thianthrene is readily synthesized in large quantities. It has the advantage that it can be purified according to a variety of methods: recrystallization, distillation, sublimation and freezing-out. It is expected that by a carefully controlled freezing-out operation the purity could be increased considerably beyond the purity of sample B.

2. The high temperature conditions encountered in the purification process warrant a completely dry product. The hygroscopicity of thianthrene was investigated as follows. A finely pulverized sample (2 g) of thianthrene was kept

* These curves were obtained during a visit with Prof. J. Coops, Vrije Universiteit, Amsterdam; we wish to express our thanks to Dr. Coops and his co-workers for their experienced advice and helpfulness.

at 21.3° C and 98 % relative humidity in a small dish. Table 1 gives the weight of the dish plus substance at different time intervals. No absorption of moisture could be detected within the accuracy of the balance.

Table 1. Hygroscopicity of thianthrene.

Day	1	2	3	6	8	10	13	15	23
Weight (grams)	15.11869	69	66	66	69	68	64	69	69

3. The thianthrene was distilled at 200° C and then frozen out at 156° C over a period of several hours, yet the purity increased. However, the thermal stability should be investigated further.

4. The volatility of a 2 g sample of powdered thianthrene in an even layer of 10 cm^2 surface was examined at 21.3° C in the open air (Table 2). On the average the volatility was found to be 0.01 mg per 24 hours.

Table 2. Volatility of thianthrene.

Hours	3	23	47	119	167	216	288	360
Weight (grams)	19.71775	75	73	70	69	69	60	53

5. Thianthrene is easy to pellet.

6—7. No difficulties have been encountered in igniting thianthrene and under the conditions employed it burns completely.

8—9. In the calorimetric system used, the amount of thianthrene burned corresponded to 6 milliatoms of sulfur which is a suitable amount according to the preceding discussion. However, the amount of thianthrene which should be used for different calorimetric systems is variable and depends ultimately on the volume of the bomb used. The generally accepted procedure for the determination of the energy equivalent of a bomb calorimeter is to burn a standard sample of benzoic acid under standardized conditions. According to the recommendations of the Standing Commission for Thermochemistry [2] three grams of benzoic acid should be used per litre of bomb volume. Thus, the bomb volume determines the amount of heat that should be evolved in the standardizing experiments and also in all subsequent combustions of other substances carried out under equal conditions. In practice, the amount of thianthrene which should be burned in different bomb calorimeters can be expected to correspond to amounts of sulfur varying between 5 and 10 milliatoms.

CALORIMETRIC APPARATUS AND METHOD

The calorimeter and accessory apparatus, experimental procedure and methods of calculation have been described in detail [4]. However, a new method has been adopted for the determination of nitric acid. A systematic study of different methods showed that more reproducible results could be obtained in the estimation of the nitric and nitrous acids by using a direct method rather

than the earlier indirect one [11]. The nitrogen acids were reduced to ammonia with Devarda's alloy and the ammonia formed was distilled and titrated with 0.1 *N* hydrochloric acid [7]. As previously, the amount of nitrous acid was determined colorimetrically using the method of Griess.

Energy equivalent of the calorimeter. The energy equivalent of the calorimeter was determined by burning National Bureau of Standards benzoic acid lots 39f and 39g with identical certified heats of combustion of 26.4294 ± 0.0026 int. kj or 6317.8 ± 0.6 cal per gram mass when burned under specified conditions at 25° C. This corresponds to $-\Delta Ec(p = 1; 293.16)$ * = 6314.2 ± 0.6 cal per gram mass for the reaction $C_7H_6O_2$ (s, 1 atm) + 7.5 O_2 (g, 1 atm) = 7 CO_2 (g, 1 atm) + 3 H_2O (liq, 1 atm). Table 3 shows a series of determinations of the net energy equivalent, S_B, of the calorimeter, which includes the 10 ml of water in the bomb and also the 30 atm of oxygen, but excludes the charge of substance(s) burned and the combustion products. These are included in S_F [4]. S_B and S_F are given in cal per temperature unit, which is, as in previous publications the temperature difference between two fixed points on the Beckmann thermometer, the lower corresponds to 20.00° C and the upper to 21.35° C (the difference between these two temperatures is the Interval Unit, I.U.). The first column of Table 3 gives the mass of benzoic acid, the second column the increase in temperature in interval units and the third column the sum of the heats evolved from the formation of the nitrogen acids (13.8 cal per millimole nitric and −6.9 cal per millimole nitrous acid formed from nitrogen, oxygen and water). The fourth column gives the heat of combustion of the cotton thread and the last column the net energy equivalent of the calorimeter.

Table 3. Benzoic acid. S_F = 0.6 cal per I. U. Correction for dissolved carbon dioxide (CO_2-corr) = 6.9 cal. Washburn corrections (W_c) = 3.3 cal.

Bencoic acid mg mass	ΔT_{corr} I. U.	HNO_3+HNO_2 cal	Cotton thread cal	S_B cal/I. U.
820.54	0.99263	8.4	13.9	5 251.5
821.42	0.99384	8.4	14.4	5 251.3
822.08	0.99453	8.4	14.3	5 251.8
821.08	0.99367	8.4	14.8	5 250.5
822.04	0.99468	8.4	15.4	5 251.8
822.06	0.99435	8.4	13.1	5 251.4
822.76	0.99547	8.4	13.9	5 250.6

Mean: S_B = 5 251.3 ± 0.4 (average deviation) cal per I. U. at 20.00° C.

* Unfortunately, a list of symbols approved by the Standing Commission for Thermochemistry is lacking. The notations used are in conformity with those used by Rossini in his "Chemical Thermodynamics" [12]. In order to simplify the type-setting the standard reference state is indicated within brackets together with the temperature in degrees Kelvin. Thus ΔEc ($p = 1$; 293.16) signifies the increase in internal energy of the combustion reaction at 293.16°K with both reactants and reaction products in given states (g = gaseous; liq = liquid; s = solid) and at unit pressure (1 atm). ΔHf ($f = 1$; 298.16) signifies the standard heat of formation at 298.16° K with gaseous reactants and, if stated gaseous, the reaction product at unit fugacity; liquid and solid components are at unit pressure (1 atm). Thus, it has the same meaning as $\Delta Hf°_{298.16}$. In each case it is specified if the energy quantities refer to one gram mass or to one mole.

HEAT OF COMBUSTION OF THIANTHRENE

Two series of determinations of the heat of combustion of thianthrene are summarized in Table 4.

Table 4. Thianthrene.

Mol.wt 216.316 Density $d_4^{20} = 1.440$ *

$S_F = 0.2$ cal per I. U. CO_2-corr = 6.2 cal. $W_c = 1.2$ cal.

$S_B = 5\,251.3$ cal per I. U. (next to last combustion 5 255.1). $-\Delta Ec$ ($p = 1$; 293.16) given for formation of CO_2 and H_2SO_4, 115 H_2O.

Thianthrene mg mass	ΔT_{corr} I. U.	HNO_3+HNO_2 cal	Cotton thread cal	$-\Delta Ec$ ($p=1$; 293.16) cal per g mass
644.76	0.98810	9.8	14.3	7 995.9
654.32	1.00222	9.8	14.0	7 992.5
653.49	1.00091	9.8	14.2	7 992.1
652.94	1.00100	9.1	17.8	7 995.1
652.48	0.99968	9.2	14.6	7 994.8
655.64	1.00408	9.4	15.6	7 992.4
651.61	0.99783	9.4	13.8	7 994.2
654.25	1.00196	9.4	13.7	7 995.3
653.34	0.99994	9.4	14.4	7 994.9
651.40	0.99758	9.4	13.3	7 995.6

Mean for the first series: 7 994.1 ± 1.4 cal per g mass
Mean for the second series: 7 994.5 ± 1.0 cal per g mass
Mean for both series: 7 994.3 ± 1.2 cal (average deviation) per g mass
± 0.4_5 cal (standard deviation of the mean)

Thus, for the reaction

$C_{12}H_8S_2(s) + 17\ O_2(g) + 228\ H_2O\ (liq) = 12\ CO_2(g) + 2\ H_2SO_4,\ 115\ H_2O\ (liq)$

$-\Delta Ec$ ($p = 1$; 293.16) = 1729.3 ± 0.16 ** kcal per mole.

SUMMARY

The requirements for a substance considered suitable as a standard in bomb calorimetry for sulfur compounds are discussed. The amount of sulfur present in a combustion (as an organo-sulfur compound) cannot be kept constant and, in practice, it will vary between 2 and 10 milliatoms. It is therefore not possible to follow rigorously the recommendation of the IUPAC Standing Commission for Thermochemistry of the International Union of Pure and Applied Chemistry: to standardize the calorimeter by burning a standard substance "under conditions identical with those which will characterize later on the combustion of the substance investigated". The choice of a standard substance must be the result of a compromise, the amount of substance burned should correspond to an average amount of sulfur in the range between 5 and 7 milliatoms.

* The density was determined on single crystals by a buoyancy method (Thomson's method). On two samples of crystals the values obtained were 1.440_0 and 1.439_5.

** The "overall" standard deviation [13,4].

Thianthrene was investigated as to its suitability as a standard substance and was found to be promising. However, further investigations, especially concerning the purification of thianthrene, are necessary before a definite proposal can be made for the use of thianthrene as a standard substance.

The heat of combustion of thianthrene to carbon dioxide and a diluted sulfuric acid (H_2SO_4, 115 H_2O) was determined to be $-\Delta Ec\ (p = 1; 293.16) = 1\ 729.3_0 \pm 0.32$ * kcal per mole.

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* Twice the "overall" standard deviation [13].

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Low-molecular Carbohydrates in Algae

I. Investigation of *Fucus vesiculosus*

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Studies on the carbohydrate constituents of various lichens have been reported in previous communications [1] from this institute. Lichens are composed of algae and fungi and similar studies on algae have now been commenced. The present paper records an investigation of the brown alga *Fucus vesiculosus*.

The only low-molecular carbohydrate previously observed in brown algae is D-mannitol, which is a major constituent. Three additional substances of carbohydrate nature have now been isolated by fractionation of the carbohydrate fraction from *Fucus vesiculosus* on carbon and hydrocellulose columns.

The first substance, m.p. 124—125°, $[\alpha]_D^{20}$ + 4°, had a R_F-value rather close to that of glycerol, but on the carbon column it moved slower than sucrose. It gave the hydroxamate test for esters and on hydrolysis yielded mannitol and acetic acid. Analyses were consistent with the formula of a mannitol monoacetate. The substance consumed 4 moles of periodate with formation of 3 moles of formic acid and is consequently 1-D-mannitol monoacetate. The carbohydrate fraction, after successive treatments with lead acetate and hydrogen sulfide, contained acetic acid which was removed when the solution was concentrated to dryness. The acetate, consequently, might possibly be an artefact, but when, however, a solution of mannitol in 50 % acetic acid was concentrated to dryness, the material was recovered unchanged and not even traces of mannitol acetates could be detected.

The second substance, m.p. 140—141°, $[\alpha]_D^{20}$—18°, moved as a disaccharide on the paper chromatogram and on the carbon column. It was nonreducing and on acid hydrolysis yielded equimolecular amounts of mannitol and glucose. It consumed 6 moles of periodic acid with formation of 4 moles of formic acid, which together with the optical rotation indicates that the substance is 1-D-mannitol β-D-glucoside.

The third substance was amorphous, $[\alpha]_D^{20}$-14°, and moved as a trisaccharide on the paper chromatogram and on the carbon column. It was nonreducing and on acid hydrolysis yielded 1 mole of mannitol and 2 moles of glucose. It consumed 6.5 moles of periodate with formation of 3.4 moles of formic acid. No formaldehyde was formed. The expected values for 1,6-D-mannitol di(β-D-glucopyranoside) are 7 moles of periodate and 4 moles of formic acid,

and bearing in mind that the substance was amorphous and certainly not perfectly pure, the agreement is rather satisfactory.

In addition to these substances the presence of several other minor constituents was demonstrated by paper chromatography. Sucrose and its products of hydrolysis, glucose and fructose were recognized, but the other spots were due to unidentified substances. One of them, however, might be due to α,α-trehalose.

Acetates of glycitols have not previously been observed in Nature. During the investigation of carbohydrates in lichens, however, spots with R_F-values corresponding to that of mannitol monoacetate were frequently observed, the spots were not identified and the amounts of substance were too small to permit isolation. It now appears not impossible that these spots were due to glycitol acetates.

Some glycitol glycosides have been isolated from natural sources. A glycerol α-D-galactoside, floridoside [2], and a D-glyceric acid α-D-mannoside [3] have been isolated from red algae, and a D-arabitol β-D-galactoside, umbilicin[1], from several lichens. Clavicepsin [4], isolated from ergot *(Claviceps purpurea)* is of special interest in this connection. It is a diglucoside of mannitol, and judging from its high specific rotation and α-glucoside, and is thus isomeric with the substance described above.

When the alga was collected no special precautions were taken immediately to inactivate the enzymes. Therefore the possibility that the substances isolated are products of an enzymatic transformation is not excluded.

EXPERIMENTAL

The alga (kindly supplied by Dr. E. Vasseur) was collected on the Swedish west coast and immediately dried in the sun. The crushed alga (975 g) was extracted in a continuous extractor with ether for 3 days and then with methanol for 9 days. The methanol extract was concentrated to dryness under reduced pressure, the residue was 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 sulfide. The solution was then concentrated to dryness. Another 9 days extraction with methanol yielded more material, and the two fractions (30 g and 6 g respectively) were combined.

In a preliminary experiment part of the carbohydrate fraction (7 g) was dissolved in 1 % ethanol and absorbed on a carbon-Celite column [5] (35 × 4.5 cm). The column was eluted with aqueous ethanol, the concentration of the ethanol being continuously increased from 1 % to 15 %. The eluate was divided into fractions, which were investigated by paper chromatography. (Solvents: butanol-ethanol-water, 4 : 1 : 5 and ethyl acetate-acetic acid-water, 3 : 1 : 1) Similar fractions were combined and concentrated to dryness under reduced pressure. The results of the separation are summarized below.

0— 750 ml	1.6 g	Salts.
750—1 200 ml	3.5 g	Mannitol, identical with an authentic specimen.
1 200—1 220 ml	0.08 g	Traces of mannitol, glucose, fructose, and several unidentified substances.
1 220—2 870 ml	0.13 g	Traces of unidentified substances.
2 870—3 080 ml	0.06 g	Mannitol monoacetate, traces of sucrose, of a non-reducing substance with the same R_F-value as α,α-trehalose and of unidentified substances.
3 080—3 210 ml	0.19 g	Mannitol monoacetate and monoglucoside.
3 210—3 590 ml	0.23 g	Mannitol monoglucoside, small amounts of mannitol monoacetate.
3 590—4 000 ml	0.03 g	Mannitol monoglucoside.

When the column was eluted with 50 % ethanol, a further amount of material (0.42 g) consisting of the mannitol diglucoside and unidentified substances with lower R_F-values was obtained.

In a new run a larger amount of material (25 g) was absorbed on the same column, and eluted with aqueous ethanol (4 000 ml), the concentration of which was continuously increased from 1 % to 25 %. A fraction (2.5 g) containing a mixture of mannitol monoacetate and mannitol monoglucoside was obtained together with a fraction of chromatographically pure mannitol diglucoside (0.6 g).

The first fraction was combined with the corresponding fractions from the previous run, and the two components separated on a hydrocellulose column (60 × 3 cm), using a mixture of *iso*propanol-butanol-water (7 : 1 : 2) as solvent. The separation was followed by paper chromatography and on concentration of the fractions mannitol monoacetate (0.4 g) and mannitol monoglucoside (2.1 g) were obtained.

Mannitol monoacetate

The mannitol monoacetate, m.p. 100—120, crystallized when the solvent was vaporated. After two recrystallizations the melting point was constant, 124—125°. $[\alpha]_D^{20}$ + 4° (water, C = 2).

A sample (10 mg) was hydrolysed with 0.1 *N* hydrochloric acid (0.5 ml) at 100° overnight, neutralized with sodium hydrogen carbonate and concentrated to dryness. The residue was acetylated with acetic anhydride in pyridine and from the reaction mixture mannitol hexaacetate (11 mg), m.p. 120—121, $[\alpha]_D^{20}$ + 23° (chloroform, C = 2), identical to an authentic specimen, was isolated. Mannitol hexaacetate was also obtained by direct acetylation of the product with acetic anhydride in pyridine.

The volatile acid was identified as acetic acid by Dyer's method [6]. The salt from the acyl group determination was transformed to the free acid and steam distilled at constant volume (15 ml). The distillate was divided into 10 ml-fractions which were titrated with 0.01 *N* sodium hydroxide. A comparable amount of authentic acetic acid (0.05 mmole) was distilled under the same conditions, and the course of distillation for the two samples was found to be identical.

Distillate, ml	Unknown acid %	Acetic acid %
10	27	27
20	44	46
30	58	59
40	70	70
50	77	78
60	84	84
70	88	88
80	91	91

$C_6H_{16}O_7$ (224.2)	Calc.	C 42.9	H 7.19	$COCH_3$ 19.2
	Found	C 42.8	H 7.05	$COCH_3$ 18.9

On periodate oxidation with 0.1 *M* solution at room temperature and pH 3.5 overnight the substance consumed 4.0 ± 0.1 mole periodate. By oxidation with sodium metaperiodate under similar conditions 3.0 ± 0.1 mole of formic acid were formed.

Mannitol monoglucoside

The amorphous product was dissolved in methanol and kept at 0°. After some days the substance (1.55 g) crystallized, m.p. 137—138°. After one further crystallization from methanol the m.p. increased to 140—141°, $[\alpha]_D^{20}$ —18° (water, C = 2). The substance (100 mg) was hydrolyzed with 1 *N* sulphuric acid at 100° overnight. (With 0.1 *N* acid the hydrolysis was not complete.) The sulphuric acid was neutralized with barium carbonate and the barium sulphate removed by filtration. The presence of two substances

in the hydrolysate was demonstrated by paper chromatography. These had the same R_F-values and colour reactions with different developing agents as glucose and mannitol, respectively. As the reducing component was readily fermented with baker's yeast, its identity with glucose is demonstrated. After the fermentation of glucose, the mixture was purified by successive treatments with lead acetate, hydrogen sulfide and the Amberlite resins IR 120 and IR 4B. Mannitol (45 mg) was obtained when the solution was concentrated, and after one recrystallization from methanol melted at 161—163°, undepressed when mixed with an authentic sample. A quantitative analysis of the hydrolysate (0.07 ml about 2 % solution) was made by paper chromatography (the method of Hirst and Jones [7]). Solvent: Ethyl acetate-acetic acid-water, 3 : 1 : 1. The amounts of glucose and mannitol found were 0.782 mg and 0.758 mg, respectively, the proportion 1.04 : 1 being very close the theoretical value.

By periodate oxidation, under the same conditions as the mannitol monoacetate, the substance consumed 6.3 ± 0.1 mole of periodic acid and yielded 4.0 ± 0.1 moles of formic acid.]

$C_{12}H_{24}O_{11}$ (344.3)	Calc.	C	41.9	H	7.03
	Found	»	41.7	»	7.31

Mannitol diglucoside

The material eluted from the carbon column was chromatographically pure but did not crystallize. $[\alpha]_D^{20} -14°$ (water, C = 2). The substance was subjected to the same investigations as the monoglucoside. Mannitol (30 mg) was isolated from the hydrolyzed product (100 mg) after fermentation of the glucose. The proportion of glucose to mannitol was found to be 1.95 : 1 by quantitative paper chromatography. A partially hydrolysed product gave a spot on the chromatogram, identical to that of the monoglucoside. The substance consumed 6.5 moles of periodic acid and yielded 3.4 moles of formic acid.

SUMMARY

Three new carbohydrate components, 1-D-mannitol monoacetate, 1-D-mannitol β-D-glucopyranoside and 1.6-D-mannitol di(β-D-glucopyranoside) have been isolated from the brown alga *Fucus vesiculosus*.

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Low-molecular Carbohydrates in Algae

II *. Synthesis of l-D-Mannitol Monoacetate and 1,6-D-Mannitol Diacetate

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The isolation of l-D-mannitol monoacetate from the brown alga *Fucus vesiculosus* was reported in a previous communication *, and the synthesis of this substance was therefore a matter of some interest. It is known that primary hydroxyl groups are more readily acylated than secondary ones. Thus by benzoylation of mannitol with 2 moles of benzoyl chloride in pyridine, 1,6-mannitol dibenzoate could be obtained in a yield of 80 % [1]. Mannitol was consequently acetylated with acetic anhydride (1.5 moles) in pyridine and the reaction products separated on a carbon-Celite column, affording mannitol, monoacetates, diacetates and triacetates in yields of 15, 32, 28 and 16 % respectively, calculated on the mannitol. The total recovery, 91 %, is exactly the same when calculated on acetic anhydride. As a rather large yield of triacetates was obtained, it is evident that secondary hydroxyl groups have been acetylated to a considerable extent, and it was also evident that the fractions contained different isomerides. Thus the yields of crystalline l-mannitol monoacetate and 1,6-mannitol diacetate were 26 and 15 %, respectively, while only traces of crystalline material could be obtained from the triacetate fraction. The monoacetate was identical with that isolated from *Fucus vesiculosus*.

The method seems to be suitable for the preparation of glycitols, where all primary hydroxyl groups are acetylated. For the preparation of derivatives, in which one of two primary hydroxyl groups is acetylated, however, it is probably restricted to the favourable cases such as mannitol, where these two groups are equivalent.

EXPERIMENTAL

Mannitol (12.1 g) was dissolved in boiling pyridine (250 ml) and acetic anhydride (10.2 g, 1.5 moles) freshly distilled over phosphorus pentoxide, was added. The solution was refluxed for 4 hours and then concentrated to a syrup under reduced pressure. The residue was dissolved in water and concentrated again. The last traces of pyridine, which

* Part I. Preceding paper.

would be harmful to the carbon column, were removed by filtration of an aqueous solution through a column of Amberlite IR 120. The solution was concentrated to 200 ml and put on a column of animal charcoal — Celite, 1 : 1, (35 × 4.5 cm) which was eluted with ethanol (4 000 ml), the concentration of which was increased continuously from 1 to 30 %. The eluate was divided into fractions which were investigated by paper chromatography. Solvent: butanol, ethanol, water, 4 : 1 : 5, reagent: silver nitrate and sodium ethoxide [2]. Appropriate fractions were combined and concentrated.

400—1 060 ml	Mannitol 1.8 g
1 230—1 990 ml	Mannitol monoacetates 4.7 g
2 190—3 500 ml	Mannitol diacetates 4.9 g

When the column was eluted with 50 % ethanol (2 000 ml), mannitol triacetates (3.3 g) were obtained.

No separation within the different fractions could be observed either in the carbon column or on the paper chromatogram.

1-D-Mannitol monoacetate

The mannitol monoacetate fraction crystallized when concentrated to dryness and by recrystallization from anhydrous ethanol a rather pure substance (4.0 g) m.p. 121—122°, was obtained. Further recrystallizations from the same solvent raised the m.p. to 124—125°, undepressed on admixture with 1-D-mannitol monoacetate from *Fucus vesiculosus*. $[\alpha]_D^{20}$ + 5° (water, C = 2).

1,6-D-Mannitol diacetate

The mannitol diacetate fraction partly crystallized when concentrated to dryness, and by recrystallization from anhydrous ethanol rather pure material (2.6 g) of m.p. 120—122° was obtained. Further recrystallizations raised the m.p. to 125—126°. $[\alpha]_D^{20}$ + 3 (water, C = 2). On periodate oxidation the substance consumed 3.3 moles of periodic acid with formation of 2.2 moles of formic acid. The calculated values are 3 and 2 moles, respectively. No formaldehyde was formed. (Found: C 45.1; H 6.91; Calc. for $C_{10}H_{18}O_8$ (266.2): C 45.1; H 6.81.)

D-Mannitol triacetate

A small quantity of crystalline material (30 mg), melting at 115—121° after several recrystallizations from ethanol, could be obtained from the triacetate fraction. It was not further investigated.

SUMMARY

1-D-Mannitol monoacetate and 1.6-D-mannitol diacetate have been prepared. The former substance is identical to the mannitol acetate isolated from *Fucus vesiculosus*.

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Short Communications

A Colour-Reaction for Detection of Methylenedioxy Groups

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All methods for detection of methylenedioxy groups are based on splitting off formaldehyde with hot sulphuric acid and detection of this by some specific reaction.

Labat [1], Pictet and Cramers [2], and Leonhart and Fay [3] made use of the green colour produced by gallic acid, whereas Weber and Tollens [4] carried out the reaction with phloroglucinol dissolved in concentrated sulphuric acid. The formaldehyde split off reacts with phloroglucinol forming an insoluble amorphous precipitate while the solution turns red. Clowes and Tollens [5] used the same procedure in a semiquantitative analytical method for determination of methylenedioxy groups.

Eegriwe [6] showed that chromotropic acid (1,8-dihydroxy-3,6-naphthalenedisulphonic acid) produces a purple colour with formaldehyde in strong sulphuric acid, this being a very sensitive and specific reaction. Also compounds yielding formaldehyde under the conditions used in the reaction produce the colour. Consequently the reaction can be used for detection of methylenedioxy groups as well.

Unfortunately many compounds give red colours with sulphuric acid alone. By diluting with water the dark by-products are precipitated while the colour produced by formaldehyde and chromotropic acid remains.

Piperonal, safrole, narcotine, piperine, berberine, methysticin, *o,o'*-di-methoxymethoxy-benzilic acid, *o*-methoxymethoxybenzaldehyde, and methylene-di-*p*-chlorphenylether have been used as test substances. The reaction has been checked on a great variety of compounds not giving off formaldehyde with hot strong sulphuric acid, and no implications have been noted from any of them. Eegriwe [6] found that a red colour was produced with furfural. With this modification in procedure, however, the test with furfural is found to be negative.

Procedure.

1) About 4—5 mg of the compound are dissolved in 4—5 ml of 90 % sulphuric acid. The solution is divided into two equal parts in two test tubes, and 4—5 mg of chromotropic acid are added to one of them.

The test tubes are kept at 70—80° for 20 minutes. After cooling 10 ml of water are added to each test tube. If necessary, the solutions are filtered. If methylenedioxy groups are present, the solution containing chromotropic acid is purple to bluish-purple while the other solution is colourless or faintly yellow.

2) About 0.2 mg of the compound is dissolved in 4—5 drops of 90 % sulphuric acid. The solution is divided into two equal parts in two small centrifuge cones (1 ml), and about 1 mg of chromotropic acid is added to one of them. The cones are kept at 70—80° for 20 minutes. Two drops of water are added to each cone, and the contents are stirred with a glass thread and centrifuged. As to the colours of the supernatant liquids, see procedure 1.

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Synthesis of Conjugated Bile Acids. Bile Acids and Steroids 5

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Bile acids conjugated with glycine or taurine were first prepared with Curtius' azide method by Bondi and Müller [1,2]. Cortese and Bauman [3-5] have used the formylated chloride of the bile acid in a Schotten-Baumann procedure but Cortese's modification [6] of the azide method seems the best method published.

For work with labelled bile acids we needed a method more suitable for synthesis of conjugates on a small scale. Of the numerous peptide syntheses that have been published in recent years we have found the method using carbonic-carboxylic acid anhydrides introduced independently by Wieland and Bernhard [7], Boissonnas [8], and Vaughan *et al.*[9,10] very suitable for this purpose. We have used the conditions worked out by Boissonnas with some minor modifications described below in the experimental section.

One of the advantages is that bile acids with free hydroxyl groups can be treated directly with ethyl chlorocarbonate for the preparation of the anhydride. The anhydride is then directly mixed with an aqueous solution of the sodium salt of glycine or taurine. Starting from cholic acid and glycine the crystalline glycocholic acid can be prepared in less than an hour. When the synthesis is made on a micro scale the product is conveniently purified by partition chromatography [11,12].

A report on the preparation and properties of the taurine and glycine conjugates of all the common bile acids will be published shortly.

EXPERIMENTAL

Glycocholic acid: 1.021 g of cholic acid (2.5 mmoles) was dissolved in 5 ml of dioxane containg 0.59 ml of tri-*n*-butylamine. The solution was cooled to + 10° and 0.238 ml of ethylchlorocarbonate was added. After 15 minutes at this temperature a solution of 187 mg of glycine in 2.5 ml of *N* sodium hydroxide was added at once and the mixture rapidly mixed. After 15 minutes when the gas evolution had subsided and the solution had set to a gel, enough water was added to give a clear solution which was evaporated *in vacuo* to a syrup; the latter was redissolved in about 20 ml water. Acidification with hydrochloric acid gave a sticky precipitate that was washed by decantation with water until free from HCl. When triturated with a little ethyl acetate it rapidly set to a mass of crystals that were filtered and dried. Yield 880 mg of practically pure glycocholic acid. m.p. 162° *cf.* [3,13]. For analysis a sample was recrystallized twice from ethanol-ethyl acetate and dried at 110° *in vacuo*. (Found: C 66.9; H 9.4; N 2.9. Calc. for $C_{26}H_{43}O_6N$: C 67.0; H 9.3; N 3.0.)

Taurolithocholic acid (Lithocholyltaurine): 0.983 g of lithocholic acid and 0.59 ml of tri-*n*-butylamine was dissolved in 5 ml of dioxane, 0.238 ml of ethyl chlorocarbonate was added as described above. After 15 minutes at + 10°, 312 mg of taurine in 2.5 ml *N* sodium hydroxide was added. After 1 hour 10 ml of water was added and the solution evaporated to a syrup *in vacuo*. After addition of about 15 ml of water and acidification to pH 1 with hydrochloric acid a precipitate formed that was filtered off. The dried precipitate was dissolved in 10 ml hot ethanol and then diluted with 15 ml ethyl acetate. After one more recrystallization from ethanol-ethyl acetate the m.p. was 212—213° (hot stage, corr.) and remained unchanged on further recrystallizations. Yield 45 %. Dried at 110° *in vacuo* for analysis. (Found: C 64.3; H 9.5; N 2.8; S 5.9. Calc. for $C_{26}H_{45}NO_5S$: C 64.6; H 9.4; N 2.9; S 6.6.)

Microanalyses by K. Pääbo at this institute and B. Rolander at the Medical Nobelinstitute, Stockholm.

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The Stoichiometry of the Reaction between Hydrazoic Acid and Phenanthrenequinone

STIG VEIBEL and THOR BAK

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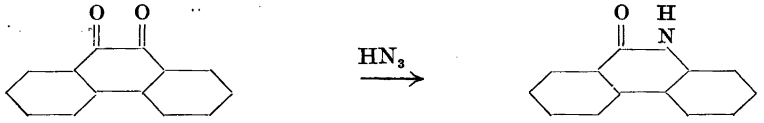
Caronna [1] states that phenanthrenequinone and retenequinone dissolved in concentrated sulphuric acid reacts with hydrazoic acid to give phenanthridone and 1-methyl-7-*iso*-propyl-phenanthridone respectively.



The initial stage of this reaction is possibly the same as in the Schmidt reaction in which compounds containing a carbonyl group react with hydrazoic acid in the presence of strong Lewis acids to give amides. (For a review of the Schmidt reaction see H. Wolff [2].) The sequence of reactions following the initial stage of the reaction considered here must, however, be different from that of the Schmidt reaction as the overall reaction in the former case is the substitution of a NH group for a CO group, whereas in the latter an amide is formed by interposing a NH group between the CO group and a group attached to it. Accordingly the stoichiometry of the two reactions appears to be entirely different.

The Schmidt reaction has the following stoichiometry

$$R^1-CO-R^2 + HN_3 \rightarrow R^1-CO-NHR^2 + N_2$$

as side reactions are probably responsible for the small production of ammonia observed. In the reaction studied by us we observed formation of ammonia to a considerably greater extent, and we interpret this as a property in the stoichiometry.

The stoichiometrical proportions were determined in the following way: The reaction was carried out with dilute solutions (0.1 *M*) of phenanthrenequinone and hydrazoic acid in concentrated sulphuric acid. After some hours the reaction mixture was diluted with water, the residual hydrazoic acid distilled off in a Kjeldahl-apparatus and titrated with 0.1 *N* ceric sulphate [3]. The liquid was made alkaline and the ammonia was distilled off and titrated in the usual way.

The molar ratio between converted hydrazoic acid and the ammonia formed is calculated, and, when hydrazoic acid is present in excess, the molar radio between converted hydrazoic acid and converted phenanthrenequinone can be calculated as well.

We found	Mean value	Standard deviation
Hydrazoic acid : Ammonia	1.95	0.06
Hydrazoic acid : Phenanthrenequinone	2.18	0.17

These ratios are independent of the ratio between the initial concentrations of hydrazoic acid and phenanthrenequinone within the range examined (0.5—6.0 for $HN_3 : NH_3$; 2.0—6.0 for $HN_3 : C_{14}H_8O_2$).

The gas evolved from the sulphuric acid solution was analysed and found to be pure nitrogen. Only after the addition of water an evolution of carbon dioxide took place. This suggests the following stoichiometry for the overall reaction:

$$C_{14}H_8O_2 + 2\ HN_3 + H_2O \rightarrow C_{13}H_9ON + 2\ N_2 + CO_2 + NH_3$$

When ice is added together with water to dilute the reaction mixture a black tarry intermediate product is often observed, which within a certain time decomposes, forming the white phenanthridone. The same stoichiometry is found when the reaction is carried out with retenequinone instead of phenanthrenequinone. We have prepared 1-methyl-7-*iso*-propylphenanthridone as follows:

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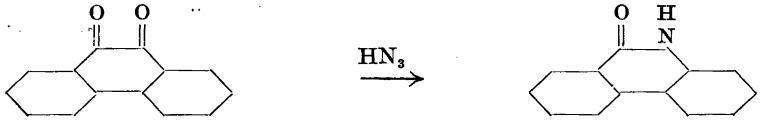

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20 g of retenequinone is dissolved in 200 ml of concentrated sulphuric acid. To the dark green solution is added 15 g of sodium azide in small portions over a period of 10 minutes during which the temperature rises to 70—80°. The reaction mixture is diluted with 800 ml of water, and the solution is kept at 60—70° for one hour. The phenanthridone is filtered off by suction and is dried at 100°. The product formed has a m.p. of 200—205°, and the yield is almost quantitative. After one recristallisation with charcoal from 500 ml of benzene a product melting at 220° (corrected) is obtained in a yield of about 60 %.

The investigation of the reaction is being continued.

1. Caronna, G. *Gazz. chim. ital.* **71** (1941) 481.
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Liquefaction of Endolymph from Sharks by Hyaluronidase

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On account of the great similarity between vitreous humor from the eye and the endolymph of the inner ear we have investigated whether the latter like the former might contain hyaluronic acid. We have made the following experiments on the endolymph from about twenty sharks *(Ascanthias vulgaris)*.

One ml of the endolymph was incubated with a trace of testicular hyaluronidase (Schering) at room temperature for 72 hours. After some hours the jelly began to liquify. After 72 hours the viscosity of the fluid which prior to the addition of enzyme was very high, was close to that of water. This experiment was repeated with the same result. In a further trial endolymph liquified within 4 hours. A sample without enzyme did not change its consistence within the same time.

As the enzym is known to be highly specific [1,2,3] this result proves that the endolymph contains hyaluronic acid or at least a substance closely related to that acid.

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Crystalline Rhodanese

I. Purification and Physicochemical Examination

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The purification of rhodanese, an enzyme catalyzing the reaction between thiosulfate and cyanide to form sulfite and thiocyanate, was attempted by Lang [1], who obtained a 20-fold purification from liver and by Cosby and Sumner [2], who reported a 100-fold purification from beef liver. The present author [3] later obtained a 150-fold purification from the same source.

In this paper the isolation of rhodanese in the crystalline state from beef liver and some physico-chemical measurements on the enzyme are described. A preliminary report of a part of this work has already been published [4].

EXPERIMENTAL

Determination of Rhodanese Activity and Purity

The successful isolation of the enzyme was made possible only after a suitable assay system had been worked out. None of the earlier methods for determination of rhodanese [1,2,5,6] was found to be applicable to the purified enzyme. Rhodanese is to a large extent inactivated, if diluted with water or buffer before assay, but this inactivation is prevented in the presence of thiosulfate [5-7]. The purified rhodanese is, however, also partly inactivated if incubated with thiosulfate of too high concentration, and it was not found permissible first to mix the diluted enzyme with thiosulfate of the high concentration used in the test and then start the reaction by adding cyanide as in earlier tests [5,6]. Thiosulfate and cyanide had thus to be mixed before the enzyme was added. The enzyme was diluted with dilute thiosulfate containing serum albumin, as the latter was found to increase the activity of the purified enzyme. The effect of albumin may be attributable to a removal of trace metals or a protection against surface denaturation.

The enzyme was assayed with the following test system. After dilution in the presence of 0.0125 *M* thiosulfate and 0.025 % bovine albumin (Armour) 0.5 ml enzyme was added to a mixture of 1.0 ml 0.125 *M* $Na_2S_2O_3$, 0.5 ml 0.20 *M* KH_2PO_4 and 0.5 ml 0.25 *M* KCN. The conditions in the test system were then: thiosulfate 0.052 *M*, cyanide 0.05 *M*, phosphate 0.04 *M* and pH 8.6 and were optimal for the beef liver enzyme. The reaction vessels were 50 ml Erlenmeyer flasks kept in a thermostate at 20° and the enzyme was added from a 0.5 ml transfer pipette, treated with silicone in order to minimize emptying time. After 5 minutes at 20° C the reaction was stopped by the addition of 0.5 ml 38 % formaldehyde, whereupon 2.5 ml of a ferric nitrate reagent (containing 100 g $Fe(NO_3)_3 \cdot 9\ H_2O$ and 200 ml HNO_3, sp.g. 1.40, and distilled water to 1 000 ml) was added. After dilution with 25 ml distilled water, the optical density at 460 mμ in a 1 cm cuvette was determined in the

Beckman DU spectrophotometer. The use of formaldehyde in the colorimetric determination of thiocyanate was found to be a great improvement. When ferric ions are added to a solution containing thiocyanate in an excess of thiosulfate, a blue colour due to a complex of ferric ions and thiosulfate first develops, but rapidly fades, whereupon the red colour of the iron-thiocyanate complex becomes visible. As also the latter colour is unstable, previous investigators have usually determined the red colour after a fixed time interval after the addition of ferric nitrate. But in the presence of formaldehyde no blue colour develops and the colour of the iron-thiocyanate complex is completely stable for at least one hour even in bright daylight.

The test system was calibrated with an argentometrically standardized thiocyanate solution. A plot of thiocyanate formed against amount of enzyme in the test gave an approximately straight line. The relative error of the method is less than 3 %. One rhodanese unit (RU) was taken as the amount of enzyme, which in the test system formed 10 μ-equivalents of thiocyanate, and was equivalent to the density reading 1.08. The purity of the different enzyme preparations was expressed in RU per mg of dry weight. The latter was sometimes determined by drying a sample to constant weight at 105—110° C after removal of salt impurities by dialysis against distilled water. As rhodanese is inactivated by dialysis against distilled water [1], protein determinations were mostly carried out instead of dry weight determinations, in order to avoid losses of valuable material. Protein was determined with the turbidimetrical method given by Bücher [6]. This method was found not to be influenced by the presence of moderate amounts of salts or organic solvents in the sample. The determination was carried out as follows: To the sample, containing about 1 mg of protein, was added 1.0 ml 0.67 % aqueous ammonium sulfate solution and the sample made up to 9.5 ml with distilled water. After the addition of 0.5 ml 1.5 *M* trichloroacetic acid the optical density at 350 mμ in a 1 cm cuvette was determined in the Beckman spectrophotometer. The test was calibrated with a solution of crystalline rhodanese, the dry weight of which was determined after dialysis against 0.01 *M* sodium sulfate and correction of the obtained dry weight for the ash content. The optical density was found to be proportional to the protein content in the test up to 1.6 mg protein and 1 mg of crystalline rhodanese was equivalent to the density reading 0.235 at 1 mm slit width.

Purification

Extraction. Beef liver was reported to be the best source of the enzyme [3], but only 50 % of the total activity in the liver could be extracted, as the rest was bound to sedimentable particles, presumably mitochondria fragments. Different extraction procedures have now been tried in order to improve the yield in the extraction step. The butanol treatment of Morton [9], reported to release many mitochondria linked enzymes into true solution, was found to inactivate a large part of the rhodanese activity. Extraction of minced liver with acetate buffer at pH 5 and 37° C, as described by Kerr and Levy [10] for β-glucuronidase, was found to release about 80 % of the rhodanese, and similar results were obtained by using acetone dried liver as the starting material. However, no improvement in purity was reached after the following step, and none of these time-consuming procedures were consequently used in the final fractionation scheme. Treatment of liver homogenate with Teepol (a surface active agent), reported by Walker and Levvy [11] to release β-glucuronidase, was found to destroy the rhodanese. Autolysis of liver brei at an alkaline pH, which was found by Baumann *et al.*[12] to solubilize liver esterase, had no effect on rhodanese. The extraction procedure used in the previous work [3] was consequently retained. It consists of homogenizing beef liver (usually first frozen and then thawed, as this treatment was found to increase the yield, *cf.* Walker and Levvy [11]) with water and then precipitation of some impurities by the addition of a small amount of basic lead acetate. After centrifugation a deep red, usually clear extract is obtained. Any turbidity, sometimes encountered when the liver was stored a long time before use, was removed during the following steps, and did not affect these.

Ammonium sulfate fractionation. The fractionation was carried out as described before [3] first at pH 3.8 in the cold room, and collecting the enzyme between 1.36 and 1.91 *M* ammonium sulfate. The precipitates obtained during fractionation were separated from the solutions either by centrifugation in a refrigerated centrifuge or by suction filtration with the aid of Hyflo Super Cel. The yields obtained with the latter procedure were

usually 10—20 % lower than with the first, presumably due to surface-denaturation of the enzyme during the filtration. The precipitate, containing the enzyme, was dissolved in a solution containing secondary phosphate and thiosulfate, the latter present in order to stabilize the enzyme during the subsequent fractionation with ammonium sulfate, carried out at pH 7.7. The enzyme was here collected between 1.95 and 2.55 *M* concentration of ammonium sulfate.

Dialysis. Before the obtained preparation could be fractionated with organic solvents, the remaining ammonium sulfate had to be removed by dialysis. Rhodanese is partly inactivated by dialysis against distilled water, but can be dialyzed against buffers in a certain pH-range without inactivation. After the appropriate conditions for the following acetone fractionation step had been established, it was found convenient to remove the remaining ammonium sulfate by dialysis against 0.01 *M* sodium acetate. No appreciable inactivation was encountered in this step.

Organic solvent fractionation. The conditions for fractionation with organic solvents at low temperature according to the principles given by Cohn *et al.*[13] were now investigated. In preliminary experiments the solubility of the salt fractionated enzyme, freed from ammonium sulfate by dialysis against distilled water, was studied in 20 % ethanol by volume at varying pH. The desired pH and ionic strength was obtained with acetate buffers; the pH of the system measured in the absence of ethanol at room temperature. At an ionic strength of 0.0015 a pronounced solubility minimum at pH 6.0 was observed, at the ionic strength 0.01 the solubility curve was more flat. Better separation of rhodanese and impurities was obtained on the acid side of the solubility minimum and at the higher ionic strength. The enzyme could be freed from some impurities by fractionation with ethanol at pH 5, ionic strength 0.01, but attemps to precipitate out the enzyme from the supernatant by raising the ethanol concentration resulted in an appreciable inactivation of rhodanese, even when all the precautions given by Deutsch [14] were followed. Other organic solvents were then tried, and it was found that rhodanese could be precipitated at low temperature with acetone or methanol without any appreciable inactivation. It was then decided to use acetone as precipitant, and the conditions finally adopted were as follows: Impurities were removed at pH 4.9, ionic strength 0.006, temperature —5° C and acetone concentration 35 % by volume, and the rhodanese then precipitated by raising the acetone concentration to 50 %. Rhodanese has thus a considerable solubility in solutions containing organic solvents, which may be connected with its high optical density at 280 mμ, corresponding to a high content of aromatic amino acids. The solubility of rhodanese in organic solvents could be depressed by the addition of Zn-ions, and preliminary experiments were carried out according to the directions given by Cohn *et al.*[15], but no purification was obtained. The solubility was also depressed in the presence of sulfate ions, but no systematic survey of the purification possibilities in this system was undertaken.

Further fractionation with ammonium sulfate. The acetone remaining in the preparation was removed by dialysis against 0.01 *M* sodium acetate, and the enzyme was further purified by precipitation with 1.75 *M* ammonium sulfate at pH 4.5. The enzyme had now a purity of about 200 RU/mg and was ready for crystallization, if a solution of the enzyme had only a faint yellow colour. Any remaining hemoglobin is difficult to separate from the enzyme by crystallization, but can conveniently be removed by fractionation with ammonium sulfate at pH 7.8, in which case the hemoglobin separates with the precipitate first obtained. When the precipitate from the acid ammonium sulfate was suspended in water, 0.01 *M* sodium acetate or dilute ammonium sulfate pH 7.8, an insoluble fraction sometimes remained, which after centrifugation was obtained as a gelatinous pellet (with a brown precipitate at the bottom). This gelatinous pellet was soluble in 0.1 *M* sodium thiosulfate, giving a solution containing rhodanese of high purity. From this solution the enzyme could be crystallized as usual. The "thiosulfate soluble rhodanese" may represent a polymer in which the enzyme molecules are held together with disulfide bonds, which are broken in the presence of thiosulfate. Its formation could be reduced by carrying out the acid ammonium sulfate precipitation at room temperature and rapidly (in a few hours) collecting the precipitate.

THE CRYSTALLINE ENZYME

Crystallisation. It has thus far been possible to obtain rhodanese as microscopically visible crystals only from ammonium sulfate at pH 7.8. At pH 5.5 a pronounced prethixotropy could be obtained by a gradual addition of ammonium sulfate to the purified enzyme, but no visible crystals were obtained. From ammonium sulfate at pH 7.8 the enzyme crystallized either as rectangular plates (Fig. 1) or as elongated prisms (Fig. 2) or as mixtures of both. They were composed of the same protein as a preparation, which first crystallized as elongated prisms, was recrystallized as plates, and another preparation which first crystallized as plates, was recrystallized as prisms. No conclusions could be drawn concerning the factors governing the appearance of the different crystal forms. Similar results has been obtained with other enzymes [16,17]. The best way to crystallize the enzyme was to precipitate it with ammonium sulfate at pH 7.8 and then dissolve the precipitate in a small volume of about 1 *M* ammonium sulfate of the same pH, when the enzyme immediately crystallized. Crystallization was also accomplished by gradually adding ammonium sulfate to the solution, or even by precipitating the pure enzyme with ammonium sulfate and leave the precipitate in the refrigerator, whereupon the amorphous precipitate in a few days was converted into crystals. Rhodanese could also be crystallized by dialysis against ammonium sulfate of increasing concentration, but no advantage was found in the latter method. The enzyme was sometimes unstable during the crystallisation, as losses up to 25 % were encountered.

Isolation experiment. A typical preparation of crystalline rhodanese is described as follows: 6.70 kg frozen, thawed beef liver was disintegrated in a Turmix blendor together with tap water, 2.5 l water used for each kg liver. The homogenate contained 600 000 RU with a purity of 0.288. 670 ml 20 % basic lead acetate (Sw. P.) was then added to the homogenate and the suspension left over night in the cold room. The suspension was then centrifuged at room temperature, giving 16.0 l turbid extract, containing 396 000 RU of purity 1.07. The extract was brought to + 4° C and to it was added 876 g ammonium sulfate and the pH adjusted to 3.8 with 720 ml 1 *M* HCl. After the precipitate had settled 2 450 g ammonium sulfate was added. (Better yields are, however, obtained if the first precipitate is removed before the ammonium sulfate concentration is increased [3]). After 2 hours the precipitate was removed by suction filtration with the aid of 1 200 g Hyflo Super Cel. 14.4 l turbid filtrate was obtained, to which was added 1 225 g ammonium sulfate and the precipitate was left over night to settle. The precipitate was filtered by suction with the aid of 144 g Hyflo Super Cel and the filtrate discarded. The enzyme was eluted from the filter cake with 700 and 300 ml of a solution 0.05 *M* with respect to Na_2HPO_4 and $Na_2S_2O_3$. 875 ml clear, reddish-brown solution was obtained, containing 177 000 RU of purity 16.8. To this solution was added 490 g ammonium sulfate, dissolved in 875 ml water and containing 4.2 ml ammonia. After 3 hours at room temperature the precipitate was removed by centrifugation and to the 1 960 ml clear supernatant obtained 196 g ammonium sulfate was added. The precipitate was removed by centrifugation in a refrigerated centrifuge after 12 hours at + 4° C and dissolved in 150 ml 0.01 *M*

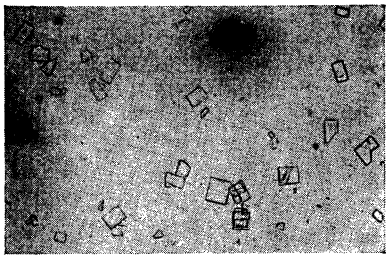

Fig. 1. Crystalline rhodanese as plates, × 150.

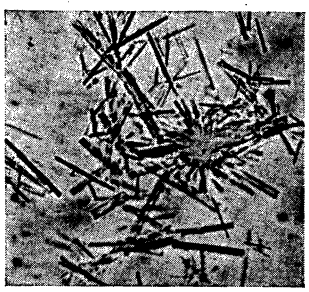

Fig. 2. Crystalline rhodanese as elongated prisms, × 300.

sodium acetate, giving 218 ml deep red solution, containing 130 000 RU of purity 34.2. The remaining ammonium sulfate was removed by dialysis at + 4° C against large volumes of 0.01 *M* sodium acetate, and to the 245 ml solution, still containing 130 000 RU, was added 25 ml 0.1 *M* acetic acid. The solution, which now had a pH of 4.9 was cooled until ice formation was obtained, whereupon 140 ml acetone was gradually added. After 3 hours at −5° C the suspension was centrifuged at the same temperature in a refrigerated centrifuge. From the 370 ml supernatant obtained, the enzyme was precipitated by the addition of 110 ml acetone. After an additional 3 hours at −5° C the precipitate was separated as before and dissolved in 100 ml cooled 0.01 *M* sodium acetate. 110 ml turbid, pink solution, containing 86 700 RU of purity 77.6 was obtained. The remaining aceton was removed by dialysis against 4 l 0.01 *M* sodium acetate at + 4° C. A precipitate appearing in the dialysis bag was centrifuged off, whereupon 122 ml solution, containing 82 700 RU was obtained. The pH was adjusted to 4.5 with 25 ml 0.1 *M* acetic acid and 126 ml 3.78 *M* ammonium sulfate was added. After 3 hours at + 4° C, the precipitate was centrifuged off in a refrigerated centrifuge and suspended in 15 ml 1 *M* ammonium sulfate, pH 7.8. The insoluble portion (containing 13 300 RU "thiosulfate soluble rhodanese") was centrifuged off and 17.3 ml of a solution containing 58 100 RU of purity 201 was obtained. The pH was adjusted to 7.8 with 0.2 ml 1 *M* ammonia, and the enzyme precipitated by the addition of 10.2 ml 3.78 *M* ammonium sulfate pH 7.8. The precipitate was centrifuged off and suspended in 4 ml 0.95 *M* ammonium sulfate pH 7.8. Beautiful crystals in the form of plates were immediately obtained, and the crystallisation was completed by the addition of 0.5 ml 3.78 *M* ammonium sulfate pH 7.8. The crystals were centrifuged down and dissolved in 5 ml distilled water, giving 6.2 ml solution containing 45 800 RU of purity 257. To this solution was added 3 ml 3.78 *M* ammonium sulfate pH 7.8 and an amorphous precipitate was obtained. This was put in the refrigerator and was found next day to have changed into needle-like prisms. 0.5 ml 3.78 *M* ammonium sulfate pH 7.8 was added in order to complete crystallisation. The crystals were separated by centrifugation and dissolved in 5 ml distilled water, giving 6.5 ml solution containing 33 500 RU of purity 267, correspond-

ing to 125 mg of the purified enzyme. The total purification from the starting material was 930 with a total yield of 5.6 %.

Absorption spectrum. The absorption spectrum of crystalline rhodanese is shown in Fig. 3 as determined in the Beckman DU spectrophotometer. The spectrum was not changed in the presence of thiosulfate or cyanide, and no formation of an enzyme-substrate compound could thus be demonstrated spectrophotometrically.

Stability. The crystalline enzyme was more unstable than impure preparations. The enzyme lost about 10 % of its activity in 4 days, when stored as a 1 % solution in phosphate buffer in the refrigerator. It could be frozen without inactivation and is best stored in this condition.

Electrophoresis. Recrystallized rhodanese was investigated in the analytical Tiselius electrophoresis apparatus. The long section cell was used and measurements were carried out in phosphate buffers of ionic strength 0.1 at pH 5.52, 6.53 and 7.44. At the last pH the enzyme appeared homogenous, but at pH 6.53 two components and at pH 5.52 four components were obtained. The enzyme was, however, unstable during the preliminary dialysis in the latter two experiments, thus at pH 6.53 a turbidity and at pH 5.52 a precipitate appeared, which were removed by centrifugation prior to electrophoresis. No definite conclusions concerning the homogenity of the crystalline enzyme could then be drawn from these experiments, as the enzyme was unstable. Nor was it possible to determine the isoelectric point of rhodanese, but even at pH 5.52 all the components migrated anodically, which means that the isoelectric point must be lower, in accordance with observations on impure enzyme [3]. It is of interest that all the patterns obtained in the descending limb with the crystalline enzyme showed a "spike", similar to the β-anomaly of blood serum and also observed with crystalline aldolase [17]. This spike was in the case of aldolase connected with a precipitation of protein, observed in the descending boundary.

Ultracentrifugation. The sedimentation constant for the recrystallized enzyme was determined in the Spinco analytical ultracentrifuge, Model E. Measurements were made at room temperature (20—23° C) in the analytical rotor, type A, at a speed of 59 780 rpm. The sedimentation constants were calculated as described by Shulman [18], and corrected to standard conditions according to Svedberg and Pedersen [19]. The sample contained except rhodanese phosphate buffer pH 7.44, ionic strength 0.1 and 0.17 *M* NaCl. Two determinations were made at 0.32 and 0.20 % protein concentration, and the values obtained for S_{20} were 3.03 and 2.91 · *S* respectively. The value 3.0 · *S* was taken as the sedimentation constant for rhodanese. Only one component was observed at pH 7.4, 6.5 and 5.5 in contrast to the electrophoresis experiments.

Diffusion. The diffusion constant was determined in the long section electrophoresis cell as described by Longsworth [20]. The boundary was, however, observed by the schlieren method and the diffusion followed only in that limb, into which the protein was displaced during the compensation, as the boundary in the other limb was found to be distorted. The diffusion constant was calculated according to the maximum ordinate-area method from the slope of the straight line, obtained by plotting $1/H_m^2$ (H_m:maximum ordinate) against time. The values were reduced to standard conditions (water at 20° C)

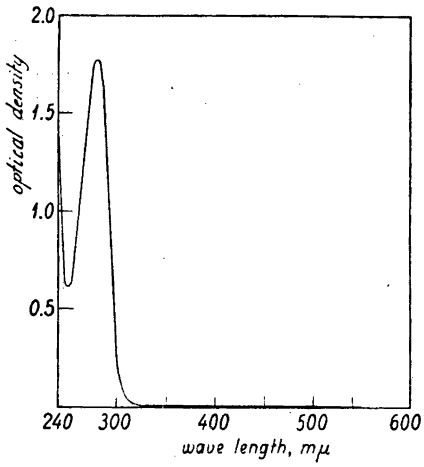

Fig. 3. Absorption spectrum of crystalline rhodanese: D = 1 cm., 0.1 % rhodanese in phosphate buffer pH 7.4.

using the formula given by Sumner *et al.*[21]. Two experiments were carried out in phosphate buffer of ionic strength 0.1 and pH 7.44. In one experiment the protein concentration was 0.176 % and the diffusion was allowed to proceed for 110 000 seconds at + 0.3° C giving $D_{20} = 7.36 \cdot 10^{-7}$ and in the other the diffusion of a 0.338 % rhodanese solution was followed for 140 000 seconds at + 1.1° C giving $D_{20} = 7.66 \cdot 10^{-7}$. The mean value $7.5 \cdot 10^{-7}$ cm^2 sec^{-1} was taken as the diffusion constant for rhodanese.

Molecular weight. The apparent partial specific volume of the recrystallized enzyme was determined pycnometrically at 25° on a solution of the recrystallized enzyme in phosphate buffer pH 7.44, ionic strength 0.1. The obtained value was 0.742. The molecular weight for the enzyme could now be calculated and found to be 37 100. The frictional ratio f/f_0 was 1.28.

Turnover number. The mean value for the specific activity of four different preparations of recrystallized rhodanese was 270 (range 259—286) RU/mg. The corresponding value for the turnover number of the enzyme is of the magnitude 20 000 molecules of thiocyanate per minute per molecule enzyme formed from thiosulfate and cyanide at 20° and pH 8.6.

SUMMARY

The enzyme rhodanese has been purified from beef liver and crystallized. The colorless enzyme was electrophoretically homogenous at pH 7.4, at lower pH:s denaturation occurred. Rhodanese appeared homogenous in the ultracentrifuge with a sedimentation constant of $3.0 \cdot S$.. The diffusion constant was $7.5 \cdot 10^{-7}$ and the partial specific volume 0.74. The molecular weight was 37 000 and the turnover number in the standard test about 20 000 molecules of thiocyanate formed per minute per molecule enzyme.

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Crystalline Rhodanese

II. The Enzyme Catalyzed Reaction

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In the preceeding paper the preparation and physicochemical characterisation of crystalline rhodanese was described [1]. In the present investigation the effect of different factors upon the enzyme catalyzed reaction has been studied. As the question of the reversibility of the reaction has got different answers [2-4] the problem was now reinvestigated. In a study of the substrate specifity of rhodanese we recently [5] found that *p*-toluene thiosulfonate could replace thiosulfate as sulfur donor, and this investigation has now been extended to other thiosulfonates. Inhibitor experiments [6,7] have suggested that the active group in the enzyme is a disulfide bond, and the crystalline enzyme was now consequently tested for these groups with the nitroprusside reaction. The reaction mechanism [7] of the enzyme is also discussed.

EXPERIMENTAL

Materials. Crystallized rhodanese was used throughout this part of the investigation. The sodium thiosulfonates listed in Table 2 were prepared from the corresponding sulfonyl chlorides and sodium sulfide following the directions given by Troeger and Linde [8]. Each was crystallized at least twice from ethanol, after which all but two gave satisfactory analytical values for sulfur. In the case of ethane and *n*-butane thiosulfonate however, even repeated recrystallisations from ethanol did not give more than about 90 % of the theoretical value. Difficulties in obtaining the aliphatic thiosulfonates in a pure form have previously been noted [9].

Methods. In studying the rhodanese catalyzed reaction between cyanide and thiosulfate, the enzyme was always diluted in the presence of 0.0125 *M* thiosulfate and 0.025 % albumin. The other conditions are evident from the figure legends. The obtained thiocyanate was determined as in the standard test [1].

The sensitive pyridine-pyrazolone method as described by Boxer and Rickards [10] was used in an attempt to demonstrate a formation of cyanide from thiocyanate and sulfite in the presence of rhodanese. The concentration of thiocyanate and sulfite was 0.05 *M* and the reaction mixture of total volume 2.1 ml contained also 0.05 *M* phosphate buffer, pH 7.4. After 15 minutes incubation in the "oxidation tube", described by Boxer and Rickards [11], the reaction mixture was acidified to pH 4 with 2 ml 0.05 *M* sulfuric acid, and any hydrogen cyanide formed was removed by aeration and collected in 1 ml 0.1 *M* sodium hydroxide. As sulfur dioxide is also liberated from the acid reaction mixture and interferes with the final color development [10] it had to be removed by oxidation. This

was accomplished by passing the gas stream from the reaction mixture first through an empty trap containing glass wool, and then through a trap containing 2 ml 0.1 *M* potassium permanganate and 2 ml 0.5 *M* sulfuric acid. The purpose of the empty trap was to prevent any spray from the reaction mixture reaching the permanganate, as otherwise cyanide would be formed by oxidation of the thiocyanate present in the reaction mixture. The colorimetric determination of cyanide was carried out as in the original method [10].

The different thiosulfonates were analyzed for sulfur according to Grothe-Krekeler and their content of thiosulfonate was determined by a colorimetric modification of the titrimetric procedure given by Gutman [12]. In this method the thiosulfonate is treated with cyanide in the heat, giving the corresponding sulfinate and thiocyanate. In the original method the sulfinate was titrated iodometrically, but in the present modification the thiocyanate is determined with ferric ions in the presence of formaldehyde. The determination was carried out as follows: To 1 ml of a thiosulfonate solution, containing up to 10 μ-mole thiosulfonate, was added 0.5 ml 0.25 *M* potassium cyanide and the mixture evaporated to dryness on a steam bath. The residue was dissolved in 2.0 ml distilled water and 0.5 ml 38 % formaldehyde, 2.5 ml ferric nitrate reagent [1], and 25 ml water was added. The yield of thiocyanate formed was then obtained from the optical density at 460 mμ.

The ability of the different thiosulfonates to function as sulfur donors in the rhodanese reaction was examined in the following test system, which is an improvement of the earlier method [5]. 1 ml of a 0.125 *M* thiosulfonate solution containing 0.20 *M* phosphate buffer pH 7.4 and 1.0 ml 0.125 *M* potassium cyanide (adjusted to pH 7.4 with hydrochloric acid) were mixed and 0.5 ml of enzyme added to start the reaction. The thiosulfonate was dissolved in the phosphate buffer in order to diminish decomposition of the salt. In the case of the naphtalene salts nevertheless a decomposition of 10 % was obtained in dissolving the salt. The enzyme was always diluted in the presence of the sulfur donor and phosphate buffer pH 7.4 (0.0125 and 0.02 *M* resp.) and 0.025 % albumin. The reaction was stopped after 5 minutes at 20° C by the addition of 0.5 ml formaldehyde and 2.5 ml ferric nitrate reagent. A heavy precipitate now appeared and was left for 30 minutes in order to obtain complete precipitation. 25 ml of distilled water was then added and the precipitate centrifuged off. The thiocyanate formed was then obtained as usual from the light absorption at 460 mμ.

The nitroprusside reaction for sulfhydryl and disulfide groups was carried out on filter paper according to Mirsky and Anson [13].

RESULTS

Effect of pH. The influence of pH on the rate of thiocyanate formation from thiosulfate and cyanide is shown in Fig. 1. A broad pH-optimum between pH 8 and 9 is observed.

Effect of enzyme concentration. As shown in Fig. 2 the thiocyanate formed in 5 minutes was proportional to the amount of enzyme in the range giving not more than 7.5 μ-equivalents of thiocyanate. This value corresponds to 6 % conversion of the substrate.

Rate of thiocyanate formation. The results are shown in Fig. 3. Also here a linear relation was obtained until about 7 μ-equivalents of thiocyanate had been formed.

Effect of substrate concentration. The initial reaction rate was studied at pH 8.5 and varying thiosulfate concentrations, as shown in Fig. 4. The inhibitory effect of sulfite [3,6] is also shown in the same figure. The effect of varying thiosulfate concentrations was also studied at pH 7.4 (Fig. 5) and also the rate dependence on the cyanide concentration. From Fig. 4 it is evident that raising the concentration of cyanide and thiosulfate from 0.05 to 0.10 *M* has only a slight effect on the rate, in disagreement with the results obtained by Saunders and Himwich [14]. When the curves in Figs. 4, 5 and 6 were replotted

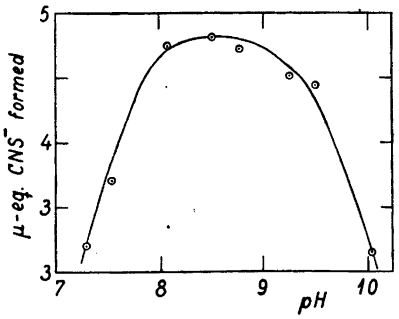

Fig. 1. Effect of pH on rhodanese activity. 0.05 M thiosulfate and cyanide, 0.10 M phosphate, final volume 2.5 ml. 5 minutes incubation at 20° C, pH measured on the complete system.

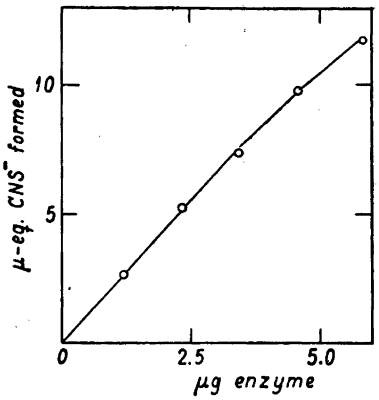

Fig. 2. Relation of enzyme concentration to activity. 0.05 M thiosulfate and cyanide, 0.04 M phosphate, pH 8.5, final volume 2.5 ml. 5 minutes incubation at 20° C.

according to Lineweaver and Burk [15] (the reciprocal of reaction velocity plotted against reciprocal of substrate concentration) approximately straight lines were obtained for the values in Figs. 4 and 6 but not for Fig. 5. The deviations occur at the lower thiosulfate concentrations and are attributed to the inhibitory effect of cyanide [6,14,16]. Consequently no Michaelis constants for thiosulfate and cyanide have been calculated, as the kinetics for the rhodanese reaction are more complex than the simple Michaelis-Menten theory [17] permits. Not only one of the initial reactants (cyanide), but also at least one of the reaction products (sulfite), inhibits the enzyme. The effect of an excess of the other reaction product (thiocyanate) on the velocity could not be investigated, as no satisfactory analytical method for following the initial reaction rate was in this case available.

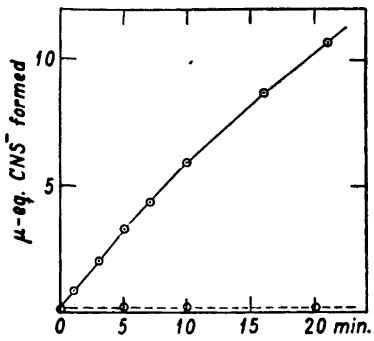

Fig. 3. Reaction velocity and time. 0.05 M thiosulfate and cyanide, 0.04 M phosphate, pH 8.5, final volume 2.5 ml. Temperature 20° C. ⊙ 1.2 μg enzyme. ○ Blank, containing albumin, no enzyme.

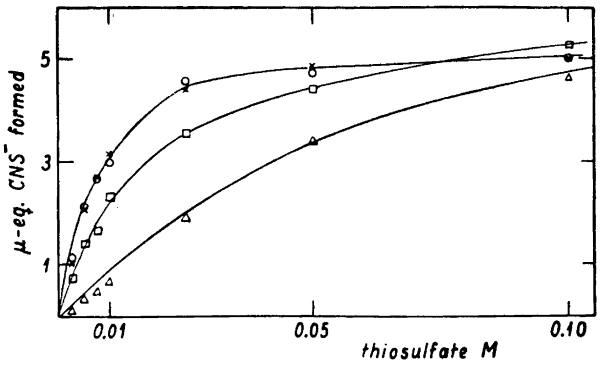

Fig. 4. Relation of thiosulfate concentration to activity at pH 8.5. □ 0.10 M cyanide, 0.08 M phosphate. ⊙ 0.05 M cyanide, 0.04 M phosphate. × 0.025 M cyanide, 0.02 M phosphate. △ 0.05 M cyanide, 0.04 M phosphate and 0.05 M sulfite. Final volume 2.5 ml, 5 minutes incubation at 20° C.

Inhibition with cysteine. It was previously reported by us [6] that cysteine did not inhibit rhodanese but on the contrary could prevent the inhibition with cyanide. These findings were in disagreement with the results obtained by Saunders and Himwich [14] who observed a strong inhibition with cysteine. That this compound failed to inhibit the enzyme in our experiments could be due to a too high proportion between substrate and inhibitor (0.04 and 0.02 *M* resp.), but it was then to be expected that an inhibition should be obtained if the substrate concentration was lowered sufficiently. This is in fact the case, as shown in Table 1. If only the thiosulfate concentration was lowered, no inhibition was observed, presumably because the inhibitory effect was cancelled by the protective effect of the cysteine against cyanide. The demonstration of a cysteine inhibition supports the theory of a disulfide bond as the active group in rhodanese. As previously [7] pointed out, the complex formed between rhodanese and cysteine must be freely dissociable and the cysteine easily displaced by thiosulfate, as preincubation of the enzyme and cysteine has no apparent effect on the inhibition [6]. It must be mentioned that there is still an unexplained discrepancy between our results and those of Saunders and Himwich, as they found 83 % inhibition with 0.04 *M* cysteine using the high substrate concentration of 0.42 *M* thiosulfate and 0.14 *M* cyanide.

Table 1. Inhibition with cysteine. The test system contained except indicated constituents 0.05 M phosphate and 4.3 μg rhodanese in a final volume of 2.5 ml. pH 7.4.

Cysteine *M*	Thiosulfate *M*	Cyanide *M*	CNS^- formed. μ-eq.	Inhibition %
—	0.005	0.005	3.10	—
0.05	0.005	0.005	2.81	9.4
—	0.0025	0.0025	1.74	—
0.10	0.0025	0.0025	1.13	35
—	0.0025	0.05	1.29	—
0.10	0.0025	0.05	1.31	0

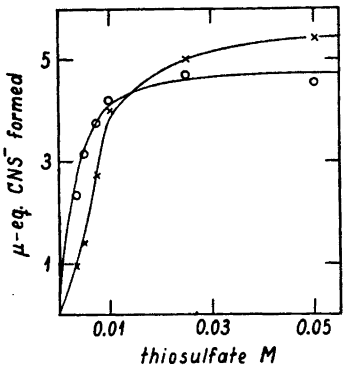

Fig. 5. Relation of thiosulfate concentration to activity at pH 7.4. × 0.05 M cyanide, 0.05 M phosphate. ⊙ 0.01 M cyanide, 0.05 M phosphate. Final volume 2.5 ml, 5 minutes incubation at 20° C.

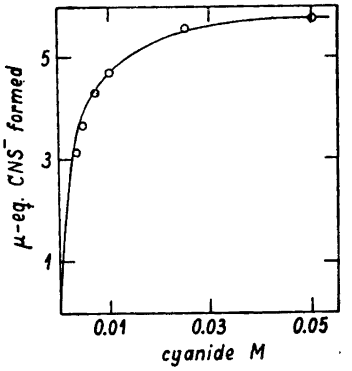

Fig. 6. Relation of cyanide concentration to activity at pH 7.4. 0.05 M thiosulfate, 0.05 M phosphate, final volume 2.5 ml. 5 minutes incubation at 20° C.

Effect of temperature. The relation between reaction rate and temperature was studied between 0° and 55° C (Fig. 7). A temperature optimum of about 50° C was observed (the value 38—40° C was reported by Saunders and Himwich [14]). When the logaritm of initial rate was plotted against the reciprocal of temperature according to Arrhenius [18], a straight line was obtained between 0° and 45° C, Fig. 8. Above this temperature inactivation occurs [6]. The corresponding value for the apparent activation energy was 7 900 cal.

Reversibility. Lang [2] could not demonstrate any reversibility of the rhodanese reaction, but Rosenthal [3] attributed the decrease of thiocyanate formation obtained with excess sulfite to a reversibility effect. As sulfite is a strong inhibitor of the enzyme [6] this conclusion was probably erroneous, and Goldstein and Rieders [4] reported also recently that the reaction was irreversible. Similar investigations had independently been carried out by us, and since we used the crystalline enzyme and a more sensitive method for determining cyanide than the one used by Goldstein and Rieders, our results are reported here. When rhodanese (90 RU or a 100-fold excess of the amounts used in following the forward reaction) was incubated with thiocyanate and sulfite, as described in "Methods", about 0.001 μ-equivalents of cyanide, corresponding to a concentration of $0.5 \cdot 10^{-6}$ *M* in the test, were obtained. The equilibrium constant $K = \frac{[CNS^-][SO_3^{2-}]}{[CN^-][S_2O_3^{2-}]}$ is then of the magnitude $1 \cdot 10^{10}$ and the reaction thus practically irreversible.

Substrate specifity. The ability of different thiosulfonates to replace thiosulfate in the rhodanese reaction is shown in Table 2. The results indicate that the activity of the thiosulfonate is roughly correlated with the electropositivity of the radical attached to the hexavalent sulfur atom, as the activity is increased when the hydroxyl group in the thiosulfate is replaced by an aromatic radical and further increased, when instead an aliphatic radical is introduced.

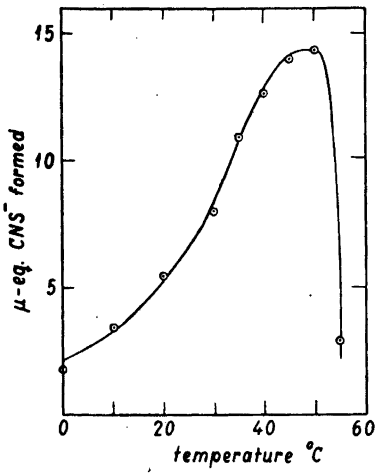

Fig. 7. Effect of temperature on rhodanese activity. 0.05 M thiosulfate and cyanide, 0.04 M phosphate, pH 8.5, final volume 2.5 ml. 5 minutes incubation at temperature indicated. At 0—30° C 3 μg rhodanese, at 35—55° C 1.5 μg rhodanese in the test. Activity values from the latter temperature interval corrected to correspond with the higher enzyme concentration.

Substitution in the benzene ring of the aromatic thiosulfonates seems on the other hand to have only a small effect on the activity. The difference between the previously reported value for *p*-toluene thiosulfonate versus thiosulfate and the value now obtained (457 and 389 % resp.) is ascribable to the change in the test system. It must be added that no studies on the kinetics of the thiosulfonate reactions have been carried out, and the values presented in Table 2 merely indicate the magnitude, but may not represent the initial reaction velocity in each case.

Sulfur groups in the enzyme. The native enzyme (recrystallized and in the form of crystals from ammonium sulfate) gave a strong positive reaction

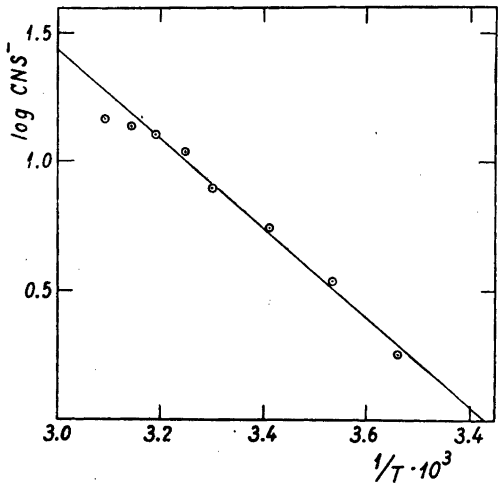

Fig. 8. Arrhenius equation plot of activity values from Fig. 7. Inactivation occurs above 45° C.

Table 2. Thiosulfonates as substrates for rhodanese. The test system as described in the text contained 1.5 μg crystalline rhodanese, except in the case of butane thiosulfonate, where the test contained 0.75 μg enzyme.

Compound			CNS^- formed μ-eq.	Activity of thiosulfate system %
Thiosulfate			1.46	100
Benzene thiosulfonate			4.44	304
p-Chlorobenzene	»		5.59	383
p-Bromobenzene	»		4.80	329
p-Toluene	»		5.67	389
(1)-Naphtalene	»	*	1.64	113
(2)-Naphtalene	»	*	1.93	132
Etane	»		15.4	1 054
n-Butane	»		10.2	1 396

* Partial decomposition occurred when the compound was dissolved.

with nitroprusside + cyanide, but with nitroprusside + ammonia a negative reaction appeared. Rhodanese thus contains disulfide bonds, but no "unmasked" sulfhydryl groups. A negative sulfhydryl reaction was however obtained with the enzyme in the presence of thiosulfate. This is apparently not in agreement with the reaction mechanism proposed for the enzyme [7], as the active disulfide bond in the enzyme is here supposed to react with thiosulfate under formation of a sulfhydryl group. Control experiments showed that thiosulfate had no inhibiting effect on the nitroprusside reaction given by cysteine. It is of interest in this connection that papain, which is activated by cyanide and then gives a positive nitroprusside reaction [19] is also activated by thiosulfate, but then gives a negative nitroprusside reaction [20]. Heat denatured rhodanese gave a strong positive reaction with nitroprusside and ammonia, demonstrating the presence of "masked" sulfhydryl groups in the enzyme. This may be related to the inhibition observed with certain sulfhydryl reagents [6,14].

DISCUSSION

The reaction mechanism for rhodanese referred to before was developed from the fact that rhodanese is inhibited by cyanide in the absence of thiosulfate, which makes the assumption of a primary enzyme-thiosulfate complex more likely than a primary enzyme-cyanide complex. As inhibition data indicated that rhodanese contained an active disulfide group, it was consequently assumed that thiosulfate reacts with the enzyme through this group under the formation of a sulfenyl thiosulfate according to

$$\left[\text{Enzyme}\right]\begin{matrix}-S\\ |\\ -S\end{matrix} + S_2O_3^{2-} \rightarrow \left[\text{Enzyme}\right]\begin{matrix}-S-S_2O_3^-\\ \\ -S^-\end{matrix} \qquad (1)$$

This compound then decomposes in the presence of cyanide according to

$$\left[\text{Enzyme}\right]\begin{matrix}-S-S_2O_3^-\\ \\ -S^-\end{matrix} + CN^- \rightarrow \left[\text{Enzyme}\right]\begin{matrix}-S\\ |\\ -S\end{matrix} + SO_3^{2-} + CNS^- \qquad (2)$$

The latter reaction probably takes place in two steps as

$$\left[\text{Enzyme}\right]^{-S-S_2O_3^-}_{-S^-} + CN^- \rightarrow \left[\text{Enzyme}\right]^{-S-SCN}_{-S} + SO_3^{2-} \qquad (2a)$$

giving a sulfenyl thiocyanate, which then reacts according to

$$\left[\text{Enzyme}\right]^{-S-SCN}_{-S^-} \rightarrow \left[\text{Enzyme}\right]^{-S}_{-S} + CNS^- \qquad (2b)$$

if the free enzyme is regenerated, or according to

$$\left[\text{Enzyme}\right]^{-S-SCN}_{-S^-} + S_2O_3^{2-} \rightarrow \left[\text{Enzyme}\right]^{-S-S_2O_3^-}_{-S^-} + SCN^- \qquad (2c)$$

if the enzyme-thiosulfate complex is regenerated. Sulfenyl thiocyanates do in fact rapidly condense with thiols [21] under formation of thiocyanate and a disulfide in support of equation 2 b (or 2 c). When analogies are sought for equation 2 a, one objection against the proposed reaction mechanism arises. Cyanide does not split off sulfite from sulfenyl thiosulfates. Instead thiosulfate[22] is formed according to

$$RS-S_2O_3^- + CN^- \rightarrow RSCN + S_2O_3^- \qquad (3)$$

If reaction 3 occurred instead of reaction 2 a, the enzyme would be without catalytical effect. The activation of thiosulfate brought about by combination with rhodanese may however weaken the sulfur-sulfur bond in thiosulfate, with the result that sulfite instead of thiosulfate is split off from the intermediate compound. The disulfide bond in rhodanese must be endowed with unique properties in this respect, as no other disulfide containing protein has the catalytical properties of rhodanese. Another example of such specific catalytic properties of a protein bound group is afforded by the sulfhydryl group in different dehydrogenases [23].

SUMMARY

The effect of different factors on the rhodanese-catalyzed reaction between thiosulfate and cyanide has been studied. From the relation between temperature and activity the apparent heat of activation was calculated to 7 900 cal. The reaction is inhibited by cysteine if the concentration of substrate is sufficiently low. The irreversibility of the reaction was confirmed. Different thiosulfonates were investigated with respect to their ability to function as sulfur donors in the enzyme reaction. The crystalline enzyme contains disulfide and sulfhydryl groups, as shown with the nitroprusside reaction. The reaction mechanism is discussed.

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The Complexity Constants of Cadmium Chloride and Bromide

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In a previous article [1] it was shown that ligand concentrations and hence consecutive complexity constants can be determined polarographically with the aid of suitable indicator ions even though the complex itself is not reduced at the electrode. It is, of course, necessary that the indicator ion is reversibly reducible; according to the literature, only a few ions — *e.g.* cadmium, lead, thallium — fulfill this condition. Fortunately, these metal ions are reduced at relatively low negative potentials, and it is therefore possible to use them in the investigation of the complex compounds of most other metals which are reducible at higher negative potentials.

When using the indicator method, it is very important to know accurately the values of the equilibrium constants of the complexes formed by the indicator ion, and in polarographic measurements it seems most appropriate to use polarographically determined values of the constants. Therefore, series of measurements have been started in order to determine the equilibrium constants of various cadmium complexes; in this article the results of measurements of the complexity constants of cadmium chloride and cadmium bromide are reported. A redetermination of these constants also seemed desirable in view of the fact that the values reported in literature differ greatly.

EXPERIMENTAL

A manual apparatus was used. The voltage applied to the cell circuit was regulated by means of a rheostat (total resistance 1 000 ohms) and was accurate to $\pm$ 0.1 mV. The rather high bridge resistance did not cause any appreciable errors (*cf.* Kolthoff and Lingane [2]), since only potential *differences* were measured and the currents were very small. The sensitivity of the "Multiflex" galvanometer used was $5.65 \cdot 10^{-8}$ A/scale unit.

The polarographic cell was connected to the calomel reference electrode by a 3 C sodium perchlorate — 3 C sodium chloride bridge. The resistance of the galvanic cell was *ca.* 1 000 ohms. All the experiments were performed at a temperature of 25° C $\pm$ 0.03°. Purified nitrogen containing less than 0.1 % oxygen was used to remove oxygen from the solutions.

Sodium perchlorate was added to maintain constant activity conditions. All measurements were conducted with a constant ionic strength of 3. The procedure followed in the

Table 1. The cadmium-chloride system at an ionic strength of 3.0. Series A and B: $c_{Cd^{2+}} = 1$ *mC;* $E_{\frac{1}{2}} = -549.6$ *mV (SCE) for* $c_{Cl^-} = 0$. *Series C and D:* $c_{Cd^{2+}} = 0.3$ *mC;* $E_{\frac{1}{2}} = -546.9$ *mV (SCE) for* $c_{Cl^-} = 0$. *The difference in the* $E_{\frac{1}{2}}$ *values is due to the high internal resistance of the cell.*

		$c_{Cl}-$ mC	$-\Delta E_{\frac{1}{2}}$ mV	(4) $[Cl_0^-]$ mC	(3) $\bar{n}$	(6) $\frac{c_{oCd^{2+}}}{[Cd_0^{2+}]}$	(10) F_1 1/mole	(10) F_2 (1/mole)^2	(10) F_3 (1/mole)^3	
A	1	11.54	4.4	11.38		1.41	36.1	3 170		
					0.37					
	2	19.62	6.9	19.32		1.71	36.8	1 889		
					0.53					
	3	27.33	9.2	27.05		2.05	38.8	1 433		
					0.57					
	4	36.84	11.4	36.50		2.42	38.9	1 064		
					0.81					
	5	50.00	14.6	49.60		3.11	42.5	858		○
					0.90					
	6	60.00	16.7	59.56		3.67	44.8	752		
					1.02					
	7	77.79	20.1	77.29		4.77	48.8	630	8 150	
					1.02					
	8	100.0	23.4	99.4		6.18	52.1	524	5 265	
					1.24					
	9	150.0	29.9	149.3		10.23	61.8	413	2 765	
B	1	29.7	9.2	29.4		2.05	35.7	1 215		
					0.76					
	2	58.8	15.9	58.4		3.45	42.0	720		
					1.10					
	3	115.4	25.5	114.8		7.26	54.6	475	4 140	
					1.25					
	4	196.2	34.0	195.4		14.09	67.0	343	1 752	
					1.66					
	5	273.3	41.1	272.4		24.4	85.8	314	1 151	
					1.92					
	6	368.4	48.5	367.4		43.5	115.8	315	857	
					2.04					●
	7	500.0	56.5	498.9		81.0	160.6	322	645	
					2.30					
	8	600.0	61.9	598.8		123.2	204.5	341	570	
					2.23					
	9	777.9	69.4	776.7		221	283	364	468	
					2.59					
	10	1 000	77.8	999		425	424	425	425	
					2.78					
	11	1 235	85.3	1 234		760	615	498	403	
					2.78					
	12	1 500	92.2	1 499		1 300	866	577	385	
C	1	11.68	4.5	11.63		1.42	36.1	3 100		
					0.35					
	2	19.62	6.8	19.55		1.70	35.8	1 835		

	3	27.33	8.7	27.24	0.44	1.97	35.6	1 304	
	4	36.84	11.3	36.74	0.68	2.41	38.4	1 044	
	5	50.00	14.6	49.88	0.84	3.11	42.4	851	
	6	69.24	17.8	69.10	0.76	3.99	43.2	625	9 040
	7	100.0	23.2	99.8	1.15	6.08	50.9	510	5 100
	8	150.0	29.5	149.8	1.23	9.91	59.6	398	2 656
D	1	29.7	9.0	29.6		2.01	34.1	1 152	
	2	58.8	15.3	58.7	0.71	3.29	38.4	641	
	3	115.4	24.6	115.2	1.04	6.77	50.1	434	3 760
	4	196.2	34.4	196.0	1.44	14.51	69.0	352	1 796
	5	273.3	41.4	273.0	1.64	25.0	87.9	322	1 179
	6	368.4	48.5	368.1	1.85	43.5	115.2	313	850
	7	500.0	57.4	499.7	2.28	86.8	171.9	344	687
	8	692.4	66.6	692.0	2.19	177.8	255	368	531
	9	931.0	76.1	930.6	2.49	373	399	429	460
	10	1 235	85.7	1 235	2.66	785	635	514	416
	11	1 500	92.5	1 500	2.76	1 330	885	590	394

experiments was analogous to that used by Leden [3] in his potentiometric measurements. The polarographic vessel was filled with a solution containing cadmium and sodium perchlorate. The cadmium concentration was usually very low, 1 mC or less. The ligand solution, which contained cadmium perchlorate in the same concentration as the sample solution and sodium perchlorate in such a concentration that the ionic strength was 3, was added from a burette.

The results of the measurements are presented in Tables 1 and 2. The figures above the columns refer to the equations used in the calculation of the values given in the columns.

THE CALCULATION OF THE COMPLEXITY CONSTANTS

The calculations were essentially analogous to those given by Leden [3]. In the derivation of the equations the fundamental differences between potentiometric and polarographic methods must, however, be taken into account.

The symbols used are the same as those employed in the previous paper [1]. The subscript o always refers to concentrations at the electrode surface. The constants are calculated as follows:

1. From polarograms taken at various ligand concentrations, values of ΔE, the difference between the potential of a cadmium perchlorate solution

Table 2. The cadmium-bromide system at an ionic strength of 3.0. Series A and B: $c_{Cd^{2+}} = 1$ *mC;* $E_{\frac{1}{2}} = -549.6$ *mV (SCE) for* $c_{Br^-} = 0$. *Series C:* $c_{Cd^{2+}} = 0.3$ *mC;* $E_{\frac{1}{2}} = -546.9$ *mV (SCE) for* $c_{Br^-} = 0$. *The difference in the* $E_{\frac{1}{2}}$ *values is due to the high internal resistance of the cell.*

		c_{Br^-} mC	$-\Delta E_{\frac{1}{2}}$ mV	(4) $[Br_o^-]$ mC	(3) $\bar{n}$	(6) $\frac{c_{oCd^{2+}}}{[Cd_o^{2+}]}$	(10) F_1 l/mole	(10) F_2 (l/mole)^2	(10) F_3 (l/mole)^3	(10) F_4 (l/mole)^4	
A	1	2.97	2.0	2.89		1.17	58.8	20 350			
					0.18						
	2	5.88	3.9	5.74		1.36	62.7	10 930			
					0.34						
	3	11.54	6.9	11.32		1.71	62.6	5 530			
					0.48						
	4	19.62	10.2	19.32		2.21	62.6	3 240			
					0.74						
	5	27.33	13.4	26.97		2.84	68.2	2 530	93 800		
					0.86						□
	6	41.36	18.0	40.90		4.06	74.9	1 831	44 800		
					1.04						
	7	60.00	23.0	59.44		6.00	84.2	1 419	23 900		
					1.30						
	8	85.72	29.0	85.01		9.56	100.9	1 184	13 920		
					1.66						
	9	100.0	32.3	99.2		12.35	114.2	1 151	11 610		
					1.55						
	10	123.5	36.5	122.6		17.2	132.1	1 078	8 800	71 900	
					2.01						
	11	150.0	41.5	149.0		25.4	163.9	1 099	7 380	49 500	
B	1	29.7	13.5	29.3		2.86	63.5	2 165	73 900		
					1.03						
	2	58.8	22.6	58.2		5.80	82.5	1 416	24 300		
					1.37						
	3	115.4	34.5	114.5		14.70	119.6	1 043	9 120	79 600	
					2.04						
	4	222.2	51.7	220.9		56.0	249	1 126	5 100	23 100	
					2.86						
	5	391.3	72.6	389.7		285	729	1 872	4 805	12 330	■
					3.37						
	6	600.0	91.1	598.3		1 205	2 015	3 370	5 630	9 410	
					3.50						
	7	857.2	107.1	855.4		4 195	4 900	5 720	6 690	7 810	
					3.68						
	8	1 235	124.4	1 233		16 140	13 090	10 600	8 590	6 960	
					3.61						
	9	1 500	133.5	1 498		32 720	21 800	14 580	9 730	6 500	
C	1	29.7	13.9	29.6		2.95	65.9	2 225	75 200		
					0.95						
	2	58.8	22.2	58.6		5.64	79.0	1 347	22 950		
					1.43						
	3	142.9	38.6	142.6		20.2	134.9	945	6 640	46 550	
					2.49						
	4	275.2	59.5	274.8		102.9	370	1 348	4 900	17 820	◪
					3.11						
	5	500.0	83.4	499.5		662	1 328	2 660	5 320	10 650	
					3.53						
	6	692.4	98.1	691.9		2 082	3 005	4 340	6 260	9 050	
					3.36						
	7	1 000	114.0	999		7 155	7 160	7 170	7 175	7 180	
					3.62						
	8	1 500	132.8	1 499		31 050	20 750	13 820	9 230	6 155	

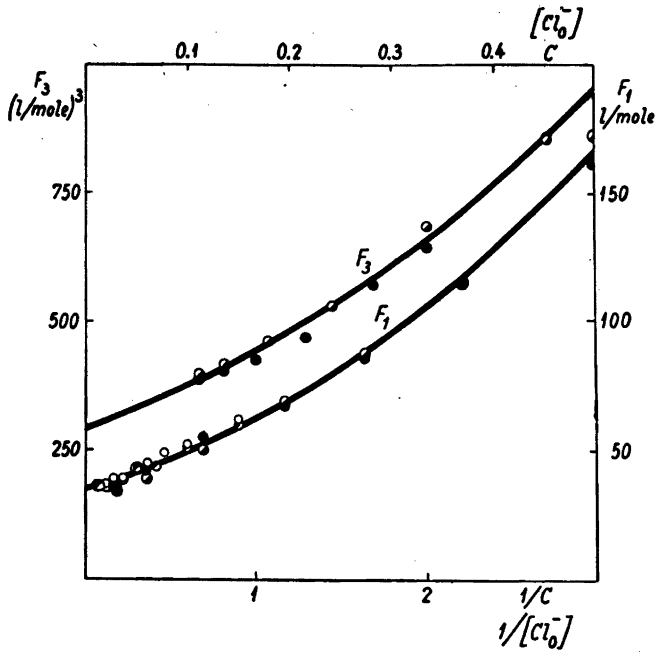

Fig. 1. F_1 as a function of $[Cl_0^-]$ and F_3 as a function of $1/[Cl_0^-]$. The curves have been drawn according to the graphically determined β values. The points are measured values.

containing ligand and the potential of a solution without any ligand, are read; the potentials refer to a selected current, expressed as a relative current $\frac{i}{i_d}$ (i_d = diffusion current). Usually the half-wave current $\frac{1}{2}i_d$ is used.

2. The average ligand number $\bar{n}$ of the system defined by

$$\bar{n} = \frac{\text{number of bound ligands}}{\text{total number of metal atoms}} \tag{1}$$

is computed from the equation of Bodländer

$$\bar{n} = -\frac{dE}{d\ln A} \cdot \frac{mF}{RT} \tag{2}$$

which, when cadmium polarograms taken at 25°C are considered, takes the form

$$\bar{n}_0 = -\frac{d\Delta E}{d\log A_0} \cdot \frac{1}{29.58} \tag{3}$$

$\bar{n}_0$ can be determined from this equation by using differences instead of differentials. As, however, A_0 is not known, the value of c_A is used as a first

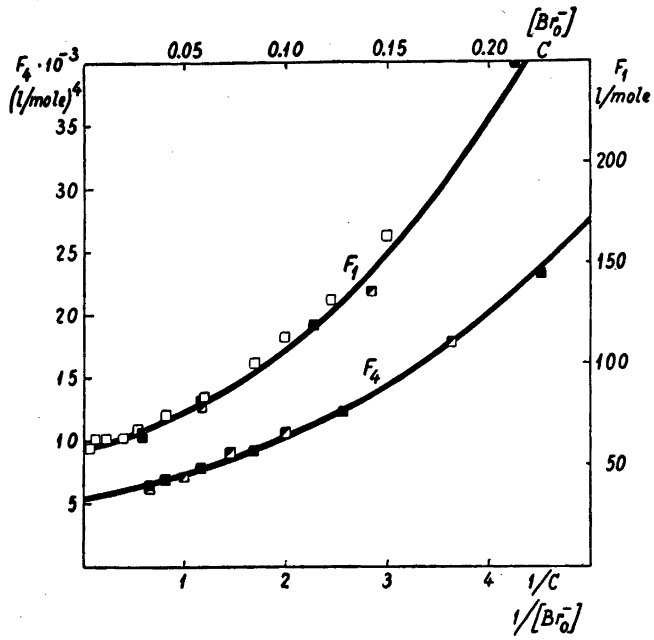

Fig. 2. F_1 as a function of $[Br_0^-]$ and F_4 as a function of $1/[Br_0^-]$. The curves have been drawn according to the graphically determined β values. The points are measured values.

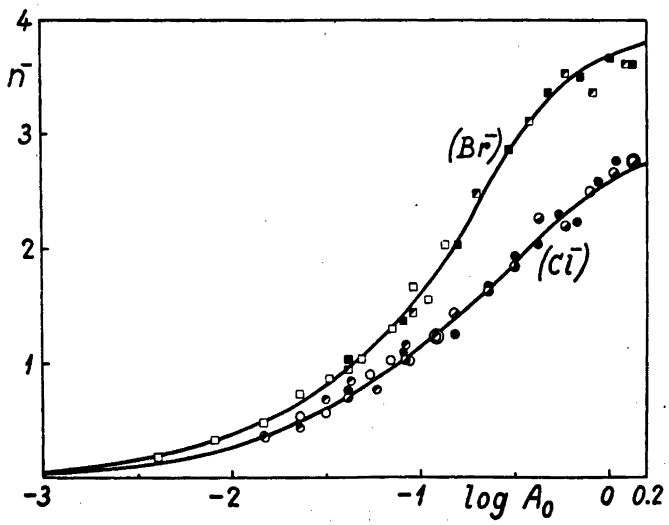

Fig. 3. $\bar{n}$ as a function of the ligand concentration. The curves have been drawn according to the graphically determined β values. The points are measured values.

approximation instead of A_0. When determining complexity constants of cadmium chloride and bromide, the differences between c_A and A_0 are very small.

3. Using these approximate $\bar{n}_0$ values, values of A_0 are then calculated from the equation

$$A_0 = c_A - \bar{n}_0 \cdot \frac{i_d - i}{i_d} \cdot c_M \tag{4}$$

Employing these approximate A_0 values, new $\bar{n}_0$ values are computed using equation (3) and more accurate A_0 values from equation (4).

4. The concentration M_0 of metal ion at the electrode surface is calculated from the equation

$$\Delta E = -29.58 \cdot \log \left(\frac{i_d - i}{i_d} \cdot \frac{c_M}{M_0} \right) \tag{5}$$

or, if the total metal concentration at the drop surface $\frac{i_d - i}{i_d} \cdot c_M$ is designated by c_{0M}, from the equation

$$\Delta E = -29.58 \cdot \log \frac{c_{0M}}{M_0} \tag{6}$$

In this equation all quantities are known except M_0.

5. The best way of calculating the β constants [1] seems to be a partly graphical method. For this purpose, we introduce functions F_n defined as follows

$$F_1 = \beta_1 + \beta_2 \cdot A_0 + \beta_3 \cdot A_0^2 + \ldots\ldots \tag{7}$$

$$F_2 = \frac{F_1}{A_0} = \frac{\beta_1}{A_0} + \beta_2 + \beta_3 \cdot A_0 + \ldots\ldots \tag{8}$$

or generally

$$F_n = \frac{F_1}{A_0^{(n-1)}} \tag{9}$$

On the other hand, it can easily be shown that

$$F_n = \frac{c_{0M} - M_0}{M_0 \cdot A_0^n} = \frac{\frac{c_{0M}}{M_0} - 1}{A_0^n} \tag{10}$$

As all the quantities on the right side of equation (10) are known, values of F_n corresponding to various values of A_0 can be determined.

F_1 is then plotted as a function of A_0. The point of intersection of the extrapolated curve with the F axis gives β_1 and the slope of the curve at this point β_2.

β_4 and β_3 can be determined in an analogous way by plotting F_4 as a function of $\frac{1}{A_0}$.

The values calculated from the slopes, *i.e.* β_2 and β_3, are, however, not very accurate. They can be more accurately determined on the basis of equations which use A_0 and F values corresponding to minimum values of F_2 and F_3 where the concentrations of MA_2 and MA_3 attain their maximum values. Graphically determined values of β_1 and β_4 have been used in this paper.

The existence of $CdCl_4^{2-}$ complexes has not been proved. F_4 is relatively small and is measured at rather high chloride concentrations which can affect activities of the species.

The F curves used in the calculations of various β values are shown in Figs. 1 and 2. In Fig. 3 the relationship between $\bar{n}$ and ligand concentration is presented graphically. It is assumed that only mononuclear complexes are formed.

COMPARISON OF THE VALUES OF COMPLEXITY CONSTANTS OBTAINED BY DIFFERENT AUTHORS

The cadmium chloride and bromide systems have been investigated by several authors. The papers published before 1943 have been critically reviewed by Leden [3]. In recent years the two systems have been investigated by Robinson and Wallace [4], Davies [5], Korenman [6], King [7], Strocchi [8,9,10], Vasil'ev and Proukhina [11], Korshunov, Malyugina and Balabanova [12], Ermolenko and Makkaveeva [13].

The results of many of these investigations are very approximate, and it seems that the values of Leden [3] and King [7] are the only ones of satisfactory accuracy. In Table 3 the values obtained by three different methods are compared.

Table 3. Complexity constants of cadmium chloride and bromide.

System	Author	Method *	β_1 1/mole	β_2 (1/mole)^2	β_3 (1/mole)^3	β_4 (1/mole)^4
$Cd^{2+}-Cl^-$	Leden [3]	Potent.	38.5	170	260	
	King [7]	Solubility	25	154	250	
	This paper	Polarogr.	35 ± 1	115 ± 5	290 ± 30	
$Cd^{2+}-Br^-$	Leden [3]	Potent.	57	220	2 100	5 000
	This paper	Polarogr.	58 ± 2	275 ± 25	1 600±200	5 400±400

* In all these experiments the ionic strenght was adjusted to 3 by means of sodium perchlorate.

SUMMARY

The consecutive complexity constants of the cadmium chloride and bromide systems have been calculated using polarographic data. The values given in Table 3 were obtained.

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Studies on Aspartase

II. On the Chemical Nature of Aspartase

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Our knowledge of the chemical nature of aspartase is negligible [1]. In 1938 Gale [2] notised that aspartase is composed of two enzymes, aspartase I and aspartase II, the latter requiring a coenzyme for its action. However, this observation has not been confirmed. Later Lichstein [3–6] and his collaborators have been able to show a biotin-linked deamination of aspartic acid with aged cells of different bacteria. The connection of this reaction with aspartase is unkown.

The present study has been undertaken in order to elucidate the chemical nature of aspartase. The enzyme was prepared from propionic acid bacteria. Different inhibitors were used, which in many cases have given results making possible a deeper understanding of the mode of action of the enzyme.

EXPERIMENTAL

Enzyme material: A finely ground dry preparation of *Propionibacterium peterssonii* (strain of this laboratory) was used as the enzyme preparation. The procedure was the same as was used earlier in cultivating bacteria mass [7].

Activity determinations: The activity of the preparations was determined by the rate at which aspartic acid was deaminated. The most convenient method for this purpose is to determine the liberated ammonia. The experiments were performed in 10 ml measuring cylinders. The following test solution was incubated in the cylinders at 37° C for 24 hours:

100	mg	dry bacteria mass
26.6	»	aspartic acid (in 2 ml, pH 7.2)
2	ml	phosphate buffer *M*/15 (pH 7.2)
6	»	distilled water
10	ml	total volume (0.3 ml toluene was added as antiseptic)

During the incubation, samples of 2 ml were taken for the determination of liberated ammonia, which was distilled after alkalization with a carbonate buffer [8] in a modified Pucher apparatus [9]. The receiver was charged with 5 ml 0.01 *N* sulfuric acid. Excess of acid was titrated iodometrically.

Inhibition experiments: Before adding the inhibitor solution to the enzyme the pH was adjusted to 7.0—7.5 with hydrochloric acid or potassium hydroxide. As phenylarsine oxide is insoluble in water a stock solution was prepared in *iso*-propanol. A stock solution of BAL (2,3-dimercaptopropanol) was prepared in ethyleneglycol monoethylether.

The dry bacteria mass and 5 ml inhibitor solution were incubated at 37° C for 30 minutes befor starting the reaction by adding aspartic acid (2 ml), phosphate buffer (2 ml) and distilled water (1 ml) to bring the total volume up to 10 ml.

In the experiments with acetonitrile, potassium cyanide and sodium sulfide the test cylinder was plugged with a rubber stopper.

RESULTS

Metal enzyme inhibitors: The effect of various metal inhibitors on aspartase is shown in Figures 1 to 3. Strong inhibition was produced by citrate, oxalate, versene (ethylenediamine tetraacetic acid) and pyrophosphate. The inhibition by cyanide, azide and acetonitrile is weak. No inhibition was seen with *ortho*phosphate and sodium sulfide. An apparent enzyme activation seems to take place in the presence of diethyldithiocarbamate. This reagent is not specific for copper only; silver mercury, nickel and manganese all give complex compounds. Hence the activation can be explained by removal of heavy metals from the enzyme surface.

These inhibitions point to the existence of an essential metal in the enzyme. The action of the four first-mentioned agents strongly suggest that one of the alkaline-earth metals is present, possibly magnesium.

The negligible inhibition produced by fluoride is surprising since fluoride has been regarded as a strong inhibitor of calcium and magnesium enzymes.

Thiol group inhibitors: Thiol groups essential to enzymes are usually detected through the inactivation of the enzyme by certain oxidizing and alkylating agents and mercaptide-forming compounds.

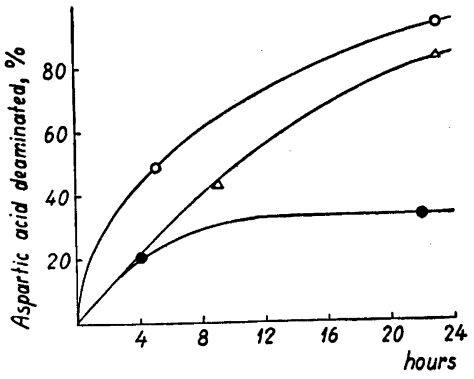


Fig. 1. Inhibitory effect of different anions on the deamination of aspartic acid:
○ *0.1* M *diethyldithiocarbamate*
● *0.1* M *ethylenediaminetetraacetic acid*
△ *control without addition of anions*

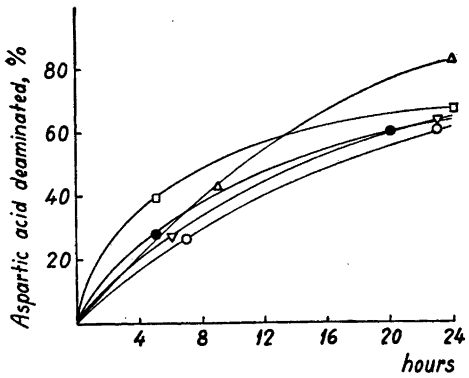


Fig. 2. Inhibitory effect of different metal reagents on the deamination of aspartic acid:
○ *0.1 M sodium azide*
□ *0.1 M sodium sulfide*
● *0.1 M potassium cyanide*
▽ *0.1 M acetonitrile*
△ *control without addition of reagent*

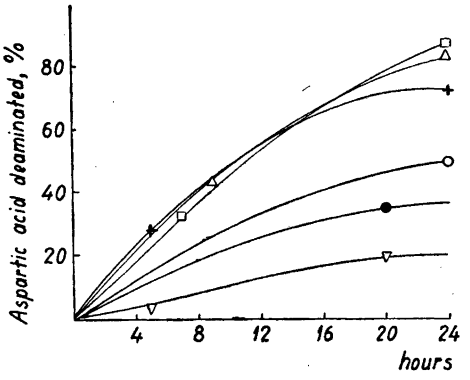


Fig. 3. Inhibitory effect of different anions on the deamination of aspartic acid:
○ *0.1 M citric acid*
● *0.1 M oxalic acid*
□ *0.1 M orthophosphate*
▽ *0.1 M pyrophosphate*
\+ *0.1 M potassium fluoride*
△ *control without addition of anions*

Barron and Singer [10] showed that *o*-iodosobenzoate was the most powerful of the oxidizing agents used in their studies. The effect of the reagent is based on the reaction:

$$\text{Protein}\begin{matrix}\diagup\text{SH}\\ \diagdown\text{SH}\end{matrix} + \text{IOC}_6\text{H}_4\text{COONa} = \text{Protein}\begin{matrix}\diagup\text{S}\\ \diagdown\text{S}\end{matrix} + \text{IC}_6\text{H}_4\text{COONa} + \text{H}_2\text{O}$$

Accordingly, the oxidizing agents act only on sulfhydryl groups close enough to allow disulfide formation. Fig. 4 shows the effect of different oxidizing agents. Iodine is the least specific and many groups in the protein molecule react with it either by being oxidized or by forming iodinated compounds. Ferricyanide has been extensively used for the determination of sulfhydryl groups in proteins. Only the sulfhydryl groups seem to reduce ferricyanide [11]. In metal-proteins, however, competition with the active metal may produce an inhibitory effect.

As detectors of sulfhydryl groups the alkylating agents are the least reactive of the commonly used agents. It is assumed that the agents react by replacing the hydrogen of the sulfhydryl group by the carboxymethyl group:

$$\text{Protein}-\text{SH} + \text{CH}_2\text{I}\cdot\text{COONa} = \text{Protein}-\text{S}-\text{CH}_2\cdot\text{COONa} + \text{HI}$$

The effect of iodoacetamide on aspartase is shown in Fig. 5. However, the action of iodoacetic acid or its amide is not confined to the sulfhydryl groups but may involve other groups in the protein molecule. Evidence of the

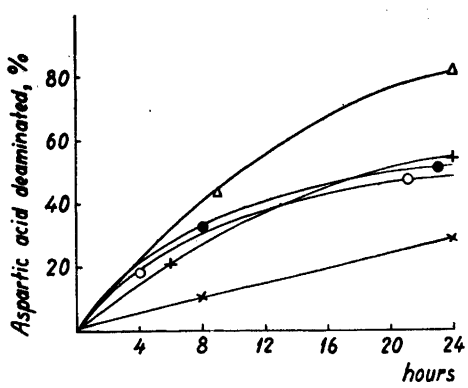


Fig. 4. Inhibitory effect of oxidizing thiol group reagents on the deamination of aspartic acid:
● *0.01* M *ferricyanide*
○ *0.01* M *o-iodosobenzoate*
\+ *0.0001* M *iodine*
× *0.001* M *iodine*
△ *control without addition of reagents*

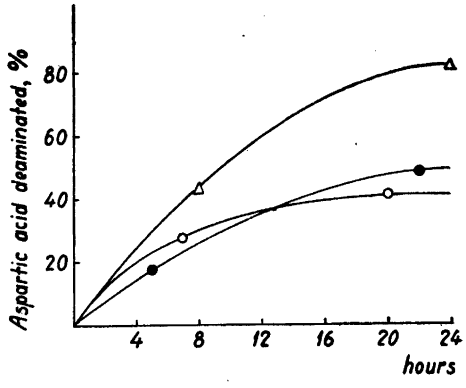


Fig. 5. Inhibitory effect of alkylating thiol group reagents on the deamination of aspartic acid:
○ *0.01* M *iodoacetamide*
● *0.05* M *iodoacetamide*
△ *control without reagent addition*

fact that iodoacetate inhibition, even in thiol enzymes, is produced by combination with groups other than sulfhydryl was given by Barron and Singer [10].

Among the mercaptide-forming agents *p*-chloromercuribenzoic acid has the advantage of combing with single sulfhydryl groups which makes possible the inhibition of sulfhydryl groups too far apart to form the disulfide linkage:

$$\text{Protein}-\text{SH} + \text{Cl}\cdot\text{Hg}\cdot\text{C}_6\text{H}_4\text{COONa} = \text{Protein}-\text{S}-\text{Hg}\cdot\text{C}_6\text{H}_4\cdot\text{COONa} + \text{HCl}$$

Thiol substances have a protecting effect against inhibition of this kind in the following way:

$$\text{Protein}-\text{S}-\text{Hg}\cdot\text{C}_6\text{H}_4\cdot\text{COONa} + \text{R}\cdot\text{SH} = \text{Protein}-\text{SH}+\text{R}-\text{S}-\text{Hg}\cdot\text{C}_6\text{H}_4\cdot\text{CCONa}$$

Fig. 6 shows the effect of *p*-chloromercuribenzoate and two arsenicals on aspartase. The antidotal effect of BAL (2,3-dimercaptopropanol) is shown in the same figure.

A possible protecting effect of aspartic acid, the natural substrate of the enzyme, against sulfhydryl reagents was tested with *p*-chloromercuribenzoate in the following way: Aspartic acid was added to the enzyme and incubated for 10 minutes before the mercurial was added to a concentration of $5 \cdot 10^{-3}$ *M* in 5 ml (the concentration was the same as in the experiment with BAL). No protection was noticed, at least not in this concentration, and the enzyme was completely inactivated.

Inhibitory effect of cations: Because an inactivation of aspartase by heavy metals can be assumed from the activation shown by diethyldithiocarbamate it was decided to investigate the influence of some metals on the activity of aspartase. Fig. 7 and 8 show the influence of different cations on the activity

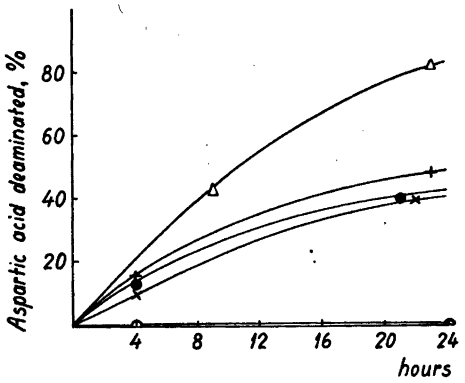


Fig. 6. Inhibitory effect of mercaptide-forming compounds on the deamination of aspartic acid:
- ○ *0.001* M p-*chloromercuribenzoate*
- ● *0.005* M p-*chloromercuribenzoate* + *0.01* M *BAL (2,3 dimercaptopropanol)*
- \+ *0.004* M *phenylarsine oxide*
- × *0.01* M *3-amino-4-hydroxydichlorarsine hydrochloride*
- △ *control without addition of mercaptide-forming compounds*

of the enzyme. Diethyldithiocarbamate gave the enzyme full protection against a mercurichloride concentration sufficient to inactivate it completely. The activation by the carbamate observed earlier may therefore be a protection against metals, as assumed above.

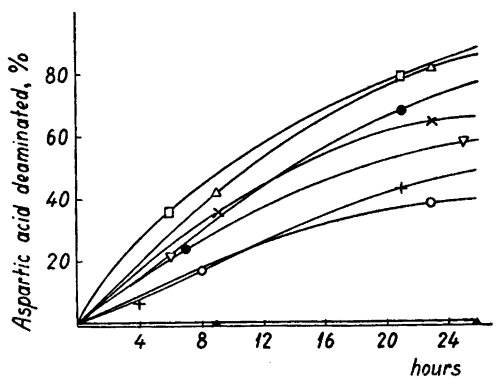


Fig. 7. Inhibitory effect of different metals on the deamination of aspartic acid:
- ● *0.001* M VCl_2
- ○ *0.001* M $AgNO_3$
- ▲ *0.001* M $HgCl_2$
- ■ *0.001* M SeO_2
- ▽ *0.001* M $CdCl_2$
- × *0.001* M $CuCl_2$
- \+ *0.001* M $ZnCl_2$
- △ *control without metal addition*

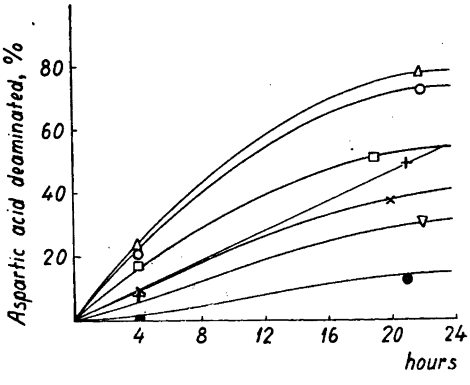


Fig. 8. Inhibitory effect of different metals on the deamination of aspartic acid:
- ● *0.01* M $CoCl_2$
- \+ *0.01* M $NiCl_2$
- ○ *0.01* M $MnCl_2$
- □ *0.01* M $FeCl_2$
- × *0.01* M $PbCl_2$
- ▽ *0.01* M $CdCl_2$
- △ *control without metal addition*

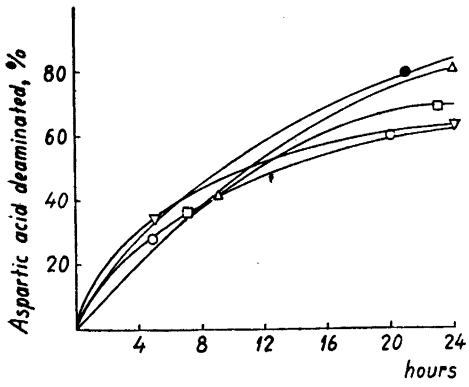


Fig. 9. The inhibitory effect of different carbonyl group reagents on the deamination of aspartic acid:
○ *0.01* M *potassium cyanide*
□ *0.01* M *semicarbazide*
▽ *0.01* M *hydrazine*
● *0.01* M *hydroxylamine*
△ *control without addition of reagents.*

Carbonyl group reagents: As cyanide is also known to react with carbonyl groups the effect of carbonyl group reagents on aspartase was examined. The results in Fig. 9 seem to indicate that the presence of an active carbonyl group in the enzyme can be excluded.

DISCUSSION

In the enzyme preparations used in this work fumarase was regularly present. Therefore the following reactions took place:

1) aspartic acid ⇌ fumaric acid + ammonia
2) fumaric acid + H_2O ⇌ malic acid

Accordingly, the velocity of the deamination of aspartic acid by the preparations used depends on the velocity of these two reactions. In the inhibition studies with aspartase the inhibitory effect observed may at least partially be explained as an inhibition of reaction 2, *i.e.* as an inhibition of fumarase. According to Massey [12], however, we know that crystalline fumarase is not inhibited by diethyldithiocarbamate, ethylenediamine tetraacetic acid, and citric acid, and hence fumarase cannot possibly be a metal protein nor responsible for the inhibitions with metal poisons used in this investigation. The inhibitions are limited, consequently, to the inhibition of aspartase, *i.e.* reaction 1.

On the basis of these studies it may be suggested that aspartase contains a metal ion essential for its activity. The metal in question seems to be one of the alkaline-earth metals, possibly magnesium. This being the case it is somewhat surprising to notice that aspartase was not inhibited by fluoride, although several experiments were performed with lengthening of the incubation time.

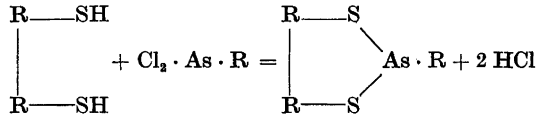
Warburg and Christian [13] have shown that enolase is a dissociating metal protein with magnesium as its activator. The protein component is inactive as such. The mechanism of the fluoride inhibition is not the binding of the magnesium of the enzyme but the displacement of the magnesium in the protein by a magnesium fluorophosphate molecule. It seems that the more readily the metal dissociates from the enzyme the stronger the effect of fluoride.

There are magnesium enzymes on which fluoride has no inhibitory effect at all *e.g.* carboxypeptidase [14] which is inhibited by cyanide. Alkaline phosphomonoesterase [15], a magnesium protein, is inhibited by fluoride in special experimental conditions only, whereas cyanide has a strong effect.

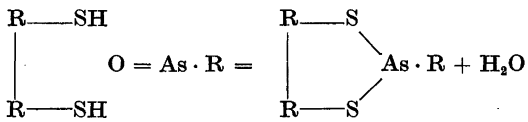
Aspartase apparently contains sulfhydryl groups. This is suggested by the complete inhibition achieved with some sulfhydryl reagents, which cannot be explained as an inhibition of fumarase.

Under the conditions of the experiments the inhibitory effect of the arsenicals and *o*-iodosobenzoic acid was weak compared with the effect of *p*-chloromercuribenzoic acid. The difference between the mercurial and the arsenicals and oxidizing agents may be explained in part from the manner in which they combine with sulfhydryl groups.

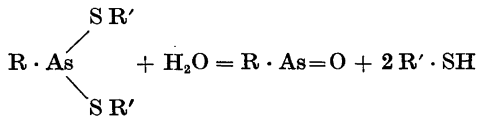
p-Chloromercuribenzoate reacts with one sulfhydryl group only. The arsenicals, on the contrary, react probably with two groups, in the following way:



or



Stocken and Thompson [16] have shown that a cyclic compound prepared by reacting arsenical with a dithiol is markedly more stable than the non-cyclic thioarsenites formed by interaction with monothiols, which undergo dissociation at physiological pH according to the reaction found by Cohen *et al.*[17]



Hence it is assumed that the high inhibitory effect produced with the arsenicals can be due to their ability to combine with two essential sulfhydryl groups forming a stable arsenical ring. A certain consonance between the effects of the arsenicals and *o*-iodosobenzoate can therefore be expected though it is known that arsenicals are able to react with sulfhydryl groups that are not attacked by oxidizing agents.

It is understandable that with only one sulfhydryl group and an inhibitor a reaction might occur more rapidly than with two groups. This, however, cannot provide a satisfactory explanation of all the facts, particularly as Barron and Singer [10] have shown with succinoxidase that the arsenicals and *p*-chloromercuribenzoate had about the same capacity to combine with the sulfhydryl groups in the protein *i.e.* 50 % inhibition was obtained with 3.2 $\times 10^{-5}$ *M* *p*-chloromercuribenzoate and 3.15 $\times 10^{-5}$ *M* 3-amino-4-hydroxy-

phenyldichlorarsine hydrochloride. However, in the experiments described above the effect of the arsenicals is only a fraction of that of the mercurial.

The evidence available is not sufficient to enable us to decide whether aspartase contains one or two sulfhydryl groups essential for its activity. If the enzyme contains only one essential group the weak inhibition obtained by the arsenicals and *o*-iodosobenzoate can be explained by assuming a reaction with two sulfhydryl groups from different molecules, an assumption that may explain the weak effect of the arsenicals. Hence it seems more likely that the activity of the enzyme depends on the presence of one sulfhydryl group or several sulfhydryl groups not situated close enough to permit formation of rings with arsenicals or disulfide linkages by oxidizing agents.

No protecting ability against *p*-chloromercuribenzoate could be proved with aspartic acid, which seems to indicate that no hindrance is produced by the substrate against attack of the inhibitor. Hopkins *et al.* discovered this protecting phenomenon in succinic dehydrogenase when the sulfhydryl reagent was added after malonate or succinate. The Hopkins phenomenon has since been shown to include several different enzymes *e.g.* carboxylase [19] and alcohol dehydrogenase [20].

Aspartase was strongly inactivated by metals. The toxicity of heavy metals seems largely to be due to their combining with sulfhydryl groups forming mercaptides or to their acting as oxidizing agents, as has been shown by Barron and Kalnitsky [21]. The inactivation of aspartase can be explained at least partially as an action on the sulfhydryl groups.

If, however, the action of different metals on aspartase is compared with the action of the same metals on urease [22], known as a sulfhydryl enzyme, a clear difference can be observed. Cobalt and nickel have a strong inhibitory effect on aspartase, on urease their inhibiting power is rather weak. The complete inhibition of urease with cobalt and nickel requires concentrations (molarities) thousands of times as great as with cadmium and zink [23]. Aspartase, on the contrary is inhibited more strongly with cobalt than with cadmium in the same concentration.

Accordingly, it is reasonable to assume still another inhibiting mechanism in the case of aspartase. The transition elements are known as strong formers of complex compounds. In their investigations of metal complex compounds Pfeiffer *et al.*[24] have shown that some metals are able to replace other metals in a metallo-organic complex compound. Pfeiffer obtained the following sequence, where the metal on the left was able to replace that on the right in a complex compound:

$$Cu \rightarrow Ni \rightarrow V, \quad Fe \rightarrow Zn \rightarrow Mg$$

In aspartase, cobalt and nickel replaced the alkaline-earth metal assumed to be responsible for the activity, and an inactivation was produced.

The negative results with the carbonyl group reagents show that these groups play no part in the activity of aspartase. Accordingly, the transfer of the amino group to an aldehyde group similar to that in the transaminase enzyme [25] can be excluded in the case of aspartase.

SUMMARY

1) The inhibition data obtained with the metal inhibitors show that in all probability aspartase is a metal-protein in which the metal is essential to its activity. The metal seems to be one of the alkaline-earth metals, possibly magnesium.

2) The activity of aspartase depends on thiol groups, which are sensitive to heavy metals and their compounds but less reactive to other thiol detectors like trivalent arsenicals, alkylating and oxidizing agents.

3) No evidence could be presented to justify the assumption of an active carbonyl group in aspartase.

4) In the discussion the assumption is made that aspartase contains only one active sulfhydryl group. To explain the strong inhibitory effect of cobalt and nickel, competition between these strong complex formers and the active alkaline-earth metal in the enzyme is assumed.

The author is greatly indebted to Professor A. I. Virtanen for his kind interest and for the facilities put at the author's disposal.

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Crystal Structure of *trans* 1,4-Chlorobromo*cyclo*hexane

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It has been known for many years that dibromo*cyclo*hexane m.p. 112° C and diiodo*cyclo*hexane m.p. 142° C are the 1,4-*trans* compounds [1,2]. The dichloro*cyclo*hexane m.p. 102° C corresponds to these compounds [1,3], and the modification of the latter, stable below 12° C, is isomorphous with them [3]. A further substance, chlorobromo*cyclo*hexane m.p. 102° C has recently been prepared and shown to be isomorphous with these three 1,4-dihalogeno*cyclo*hexanes [3]. The space group of all the substances was found to be $P2_1/a$ and the unit cell contains *two* molecules, implying that the molecules themselves have a center of symmetry, a requirement which may easily be fullfilled in the cases where the halogen atoms are both of the same kind, but not in the case of the chlorobromo compound. The crystallographic data of the four substances are as follows (Table 1):

Table 1. Crystallographic data of the trans 1,4-dihalogenocyclohexanes.

	a	*b*	*c*	*β*
$C_6H_{10}Cl_2$	11.58	5.36	5.80 Å	105°
$C_6H_{10}ClBr$	11.81	5.52	5.95 Å	103°
$C_6H_{10}Br_2$	11.92	5.56	6.02 Å	103°
$C_6H_{10}I_2$	12.50	5.72	6.20 Å	98°

Although no X-ray reflexions from crystals of the chlorobromo compound have been observed which would indicate that the true lattice constants are larger than those corresponding to the three other dihalogeno compounds, two heavily overexposed rotation diagrams were taken in order to prove the correctness of the unit cell deduced from the existing X-ray data. No indications of additional layer lines could be detected. It therefore appears probable that the structure is of a disordered type, the orientation of the molecules being statistically distributed in the crystal. Earlier examples of the same kind have been reported by Hendricks in the case of *p*-chlorobromobenzene [4] and Hassel, Wang Lund and Lunde [5] in the case of 1*α*,2*α*-dichloro-4*α*,5*α*-dibromo*cyclo*hexane.

As Fourier maps had already been worked out for the dibromo and dichloro compounds, approximate coordinates for the carbon and halogen atoms were

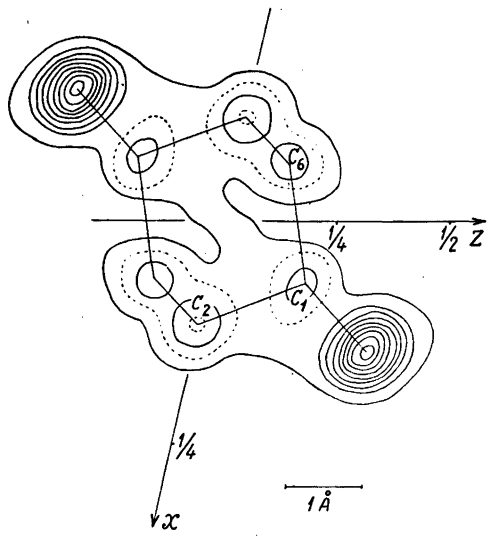

Fig. 1. $C_6H_{10}ClBr$. *Electron density map projected along the b axis. Contours at intervals of* 2 e. Å^{-2} *for the* C-*atoms, at intervals of* 4 e. Å^{-2} *for the* Cl-Br-*atoms. The first contour line is* 2 e.Å^{-2}.

available and thus the signs of the greater part of the amplitudes could be easily obtained. Fourier maps could therefore be worked out without much trouble. In Figures 1 and 2 these electron density maps corresponding to projection along the *b* and *c* axes, respectively, are reproduced. The atomic

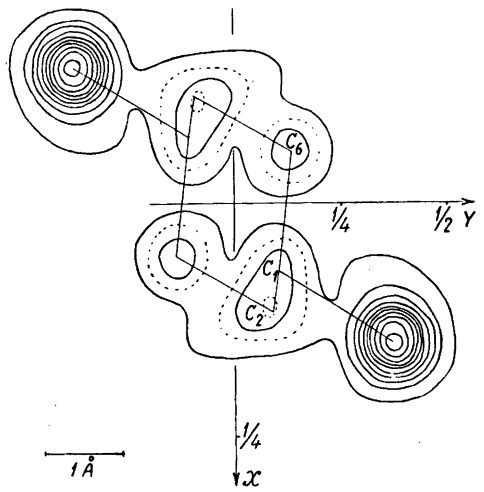

Fig. 2. $C_6H_{10}ClBr$. *Electron density map projected along the c axis. Contours at intervals of* 2 e. Å^{-2} *for the* C-*atoms, at intervals of* 4 e. Å^{-2} *for the* Cl-Br-*atoms. The first contour line is* 2 e. Å^{-2}. *Scale as in Fig. 1.*

coordinates (*cf.* Table 2) are generally in good agreement with coordinates earlier found in this laboratory for the dibromo compound (A. Bergskaug) [6], the dichloro compound (O. Bowitz) [7], and the iodine coordinates of the diiodo compound (Halmøy and Hassel) [1]. Some deviations are observed in the y-coordinate of the second carbon atom (C_2), but the overlapping of the C_1 and C_2 atoms makes an exact determination of the y-coordinates rather difficult in any case.

Table 2. a. Atomic coordinates as fractions of the corresponding cell edge, of the trans 1,4-dihalogenocyclohexanes.

	Atom	x	y	z
	Cl	0.148	0.369	0.378
$C_6H_{10}Cl_2$	C_1	0.071	0.099	0.205
	C_2	0.123	0.106	0.002
	C_6	−0.061	0.134	0.118
	Cl, Br	0.148	0.370	0.381
$C_6H_{10}ClBr$	C_1	0.072	0.101	0.201
	C_2	0.117	0.089	−0.013
	C_6	−0.060	0.134	0.119
	Br	0.148	0.371	0.383
$C_6H_{10}Br_2$	C_1	0.075	0.113	0.223
	C_2	0.116	0.108	−0.024
	C_6	−0.060	0.134	0.139
$C_6H_{10}I_2$	I	0.150	0.365	0.385

b. Bond lengths in chlorobromocyclohexane as derived from the coordinates given above.

Cl,Br−C_1	1.93 Å
C_1−C_2	1.49 Å
C_1−C_6	1.54 Å
C_2−C_6,	1.48 Å

Table 3 contains observed and calculated F-values. The calculated values were obtained on the basis of Thomas-Fermi and Hartree f-curves for bromine and chlorine atoms using a curve for all halogen atoms which is the arithmetic mean of the two f-curves. In general, the agreement between calculated and observed values is satisfactory, the reliability factor being 0.15 ($h0l$) and 0.14 ($hk0$). Still, it may be questioned if the way in which the f-values have been derived can be regarded as fully satisfactory. The procedure adopted implies that all halogen atoms have the same scattering power, a condition which is fulfilled only if the halogen atoms are quite irregularly distributed over the available positions. It might happen that halogen atoms in direct contact are to some extent preferably either of the same or of the opposite kind, two atoms belonging to the same molecule being necessarily always of different kinds. The reflexions which should be expected to be most sensitive to effects of the kind just mentioned are those whose amplitudes have opposite signs in the dichloro and the dibromo compound. Only *two* such reflexions have been found, namely 200 and 110. As a matter of fact the relative agreement between

Table 3. Comparison of observed and calculated structure factors.

hkl	F_{obs}	F_{calc}	*hkl*	F_{obs}	F_{calc}
2 0 0	3.3	+0.1	$\bar{2}$ 0 4	<2.1	−0.7
4 0 0	20.4	−18.3	$\bar{4}$ 0 4	4.5	+5.3
6 0 0	5.9	+6.1	$\bar{6}$ 0 4	3.7	−4.8
8 0 0	2.7	+2.4	$\bar{8}$ 0 4	<2.5	−1.3
10 0 0	6.1	−5.9	$\bar{10}$ 0 4	3.9	+5.1
12 0 0	<2.3	+0.6	$\bar{12}$ 0 4	<2.1	−0.1
14 0 0	1.6	+2.3	$\bar{14}$ 0 4	<1.2	−2.0
0 0 1	4.3	−7.2	0 0 5	6.3	+1.6
2 0 1	7.7	−6.6	2 0 5	2.5	+1.6
4 0 1	12.7	+12.0	4 0 5	6.0	−5.0
6 0 1	3.5	−3.1	6 0 5	<2.2	+0.3
8 0 1	7.7	−6.7	$\bar{8}$ 0 5	1.6	+2.0
10 0 1	4.4	+3.5	$\bar{2}$ 0 5	3.9	−4.6
12 0 1	<2.2	+1.6	$\bar{4}$ 0 5	2.4	−2.7
14 0 1	<1.2	−1.8	$\bar{6}$ 0 5	4.4	+5.4
$\bar{2}$ 0 1	20.1	+22.7	$\bar{8}$ 0 5	<2.3	−0.3
$\bar{4}$ 0 1	<1.4	+1.9	$\bar{10}$ 0 5	2.2	−2.9
$\bar{6}$ 0 1	15.5	−14.9	$\bar{12}$ 0 5	<1.7	+1.2
$\bar{8}$ 0 1	2.2	+2.5	0 0 6	<2.4	−0.3
$\bar{10}$ 0 1	5.0	+4.5	2 0 6	3.2	−3.2
$\bar{12}$ 0 1	3.8	−3.3	4 0 6	<1.9	+0.8
$\bar{14}$ 0 1	<1.7	−0.6	$\bar{6}$ 0 6	<1.3	+0.4
0 0 2	2.4	+2.0	$\bar{2}$ 0 6	4.2	+4.5
2 0 2	12.6	+14.4	$\bar{4}$ 0 6	<2.4	−0.8
4 0 2	8.4	−7.7	$\bar{6}$ 0 6	2.3	−2.3
6 0 2	5.3	−4.4	$\bar{8}$ 0 6	2.0	+2.5
8 0 2	8.2	+7.1	$\bar{10}$ 0 6	<1.7	+0.2
10 0 2	<2.4	+0.5	0 0 7	<1.7	−0.9
12 0 2	2.7	−3.1	$\bar{2}$ 0 7	<1.3	+1.7
$\bar{2}$ 0 2	14.6	−19.3	$\bar{2}$ 0 7	2.4	−1.5
$\bar{4}$ 0 2	3.2	+4.3	$\bar{4}$ 0 7	1.8	+2.4
$\bar{6}$ 0 2	8.9	+8.4	$\bar{6}$ 0 7	<1.7	+0.9
$\bar{8}$ 0 2	4.9	−5.7	$\bar{8}$ 0 7	<1.4	−1.6
$\bar{10}$ 0 2	<2.4	−0.6	1 1 0	3.0	−0.4
$\bar{12}$ 0 2	2.4	+3.3	2 1 0	17.0	−16.4
$\bar{14}$ 0 2	<1.7	−1.1	3 1 0	12.6	+12.2
0 0 3	7.8	+7.0	4 1 0	8.3	+7.3
2 0 3	10.2	−9.6	5 1 0	3.4	−2.7
4 0 3	<2.2	+0.0	6 1 0	8.0	+7.6
6 0 3	8.1	+7.8	7 1 0	9.3	−8.9
8 0 3	<2.5	−1.0	8 1 0	6.0	−5.8
10 0 3	2.2	−2.2	9 1 0	3.0	+2.7
12 0 3	<1.3	+0.9	10 1 0	<2.2	−0.5
$\bar{2}$ 0 3	3.1	+3.5	11 1 0	<2.2	+2.3
$\bar{4}$ 0 3	13.2	−14.5	12 1 0	2.3	+3.3
$\bar{6}$ 0 3	<2.1	+0.2	13 1 0	2.0	−2.8
$\bar{8}$ 0 3	8.5	+10.0	14 1 0	<1.4	−1.5
$\bar{10}$ 0 3	<2.5	−1.2	0 2 0	<1.3	+0.4
$\bar{12}$ 0 3	<2.3	−2.5	1 2 0	14.1	12.2
$\bar{14}$ 0 3	<1.6	+1.5	2 2 0	<1.3	+0.7
0 0 4	8.4	−8.2	3 2 0	5.0	+3.3
2 0 4	4.0	+4.1	4 2 0	<1.6	−0.2
4 0 4	4.9	+4.8	5 2 0	11.4	−11.6
6 0 4	4.3	−3.7	6 2 0	<1.8	−1.2
8 0 4	<2.3	−0.4	7 2 0	4.3	+4.7
10 0 4	9.1	+2.2	8 2 0	<2.1	+0.1

hkl	F_{obs}	F_{calc}	*hkl*	F_{obs}	F_{calc}
9 2 0	5.9	+6.8	8 4 0	<2.2	−1.1
10 2 0	<2.2	+0.8	9 4 0	<2.1	−0.5
11 2 0	3.3	−4.7	10 4 0	2.9	+4.9
12 2 0	<1.9	−0.2	11 4 0	<1.5	+0.4
13 2 0	<1.6	−1.8	12 4 0	<1.1	−0.6
14 2 0	<1.1	−0.2	1 5 0	<2.2	+0.4
1 3 0	5.5	+5.2	2 5 0	4.6	+4.5
2 3 0	9.7	−10.0	3 5 0	2.8	−3.4
3 3 0	9.7	−9.1	4 5 0	3.3	−3.7
4 3 0	3.0	+3.0	5 5 0	<2.2	+1.1
5 3 0	<2.0	+0.2	6 5 0	2.7	−3.0
6 3 0	4.3	+4.8	7 5 0	2.5	+3.3
7 3 0	6.9	+6.7	8 5 0	2.3	+3.2
8 3 0	3.0	−3.2	9 5 0	<1.6	−1.1
9 3 0	<2.2	−1.8	10 5 0	<1.2	+0.2
10 3 0	<2.1	−0.3	0 6 0	<2.0	+0.2
11 3 0	<2.0	−2.4	1 6 0	2.5	−3.8
12 3 0	<1.7	+2.2	2 6 0	<2.0	−0.5
13 3 0	<1.2	+2.3	3 6 0	<1.9	−1.5
0 4 0	13.1	−13.0	4 6 0	<1.8	−0.0
1 4 0	3.0	−2.4	5 6 0	2.1	+3.7
2 4 0	<2.1	+1.1	6 6 0	<1.5	+1.1
3 4 0	2.3	−2.3	7 6 0	<1.3	−1.2
4 4 0	6.6	+7.6	8 6 0	<0.8	−0.0
5 4 0	<1.7	+0.8	1 7 0	<1.0	−1.6
6 4 0	3.6	−4.1	2 7 0	<0.9	+2.1
7 4 0	<2.2	+0.3			

observed and calculated intensities is not good for these reflexions; the calculated intensity of 200 would be in much better agreement with the observed value if the *f*-curve of either only chlorine or only bromine had been employed. The same holds true for the 110 reflexion. (Table 4.)

Table 4. Calculated and observed F-values for 200 and 110 reflexions, the former in the case of two Cl, two Br and two "Artificial" atoms with a diffraction power midway between Cl *and* Br.

		Calculated:	Observed:
200	F_{Cl}	+ 2.63	
	$F_{\frac{Cl+Br}{2}}$	+ 0.12	3.28
	F_{Br}	− 2.37	
110	F_{Cl}	+ 3.03	
	$F_{\frac{Cl+Br}{2}}$	− 0.41	3.00
	F_{Br}	− 3.86	

The problem just mentioned might perhaps be attacked with success by studying corresponding intensities from mixed crystals containing nearly equimolecular amounts of the two dihalogeno compounds. Owing to lack of material such investigations have so far not been started. Corresponding studies in the case of *p*-dichlorobenzene, *p*-dibromobenzene and *p*-chlorobromobenzene have also been planned.

SUMMARY

The crystal structure of *trans* 1,4-chlorobromo*cyclo*hexane [3] has been determined and electron density projections along two of the principal axes worked out. The apparent crystallographic equivalency of chlorine and bromine atoms in the crystal suggested that structure factors could be evaluated on the basis of a single *f*-curve for all halogen atoms: the arithmetic mean of curves for chlorine and bromine atoms. The reliability factors thus obtained were actually satisfactory for both projections. However the intensity of two reflections — 200 and 110 — were much stronger than calculated. For both these reflexions the structure amplitude of the dichloro and dibromo compound is nearly the same, but the sign is reversed. These findings seem to indicate that an evaluation of the structure amplitudes of the chlorobromo compound assuming a true statistical distribution of chlorine and bromine atoms over all available halogen positions is not strictly correct.

We wish to express our gratitude towards Cand. real. Per Andersen, now at Cornell University, U.S.A., who prepared the first Weissenberg photographs and determined the space group of the chlorobromo*cyclo*hexane before he left this country.

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On the Intracellular Distribution of Catalase and Alcohol Dehydrogenase in Horse, Guinea Pig and Rat Liver Tissues

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These investigations have been made preliminary to isotopic studies on the velocity of amino acid incorporation in catalase and alcohol dehydrogenase (ADH) in liver.

The intracellular distribution of catalase in rat liver has been studied by H. von Euler and L. Heller [1]. They used the differential centrifugation method developed by Hogeboom, Schneider and Pallade [2] but did not isolate the submicroscopical particles. Their final supernatant thus contained the ground substance of the cytoplasm as well as the submicroscopical particles. They found that the catalase activity per mg nitrogen was almost equally distributed between the mitochondria and the final supernatant. Our investigation concerns the same question in some other species.

No studies of this kind have been reported on ADH.

EXPERIMENTAL

Methods: In our preparations we have followed the method described by Hogeboom, Schneider and Pallade [2]. Fresh liver was forced through a masher in order to remove as much as possible of the connective tissue. 5 – 20 g of the mash was used in each preparation. In the following the figures given for the volumes of sucrose solution added before the homogenisation and added for the washings are calculated per g liver mash. The mash was ground for 10 minutes in a Potter-Elvehjem homogenizer together with 8 ml of 0.25 *M* sucrose solution. The big particles consisting chiefly of nuclei were separated by centrifugation for 10 min. at 700 g(I). The sediment (nuclei) was washed once with 4 ml of 0.25 *M* solution and centrifuged under the same conditions. The two supernatants were combined and centrifuged for 10 min. at 20 600 g(II) to isolate the mitochondria. They were washed twice with 4 ml of 0.25 *M* sucrose solution and then centrifuged at the same speed. The submicroscopical particles were isolated from the combined supernatants by centrifugation for 60 min. at 70 000 g(III). This fraction was washed once with 4 ml of 0.25 *M* sucrose solution and centrifuged for 60 min. at 140 000 g(IV). All operations were performed at 0 – 4° C. The sediments were suspended in 0.01 *M* phosphate buffer (pH 7) in order to cause lysis of the cell components.

(I) 2 000 r.p.m. with rotor No. 269 of the International refrigerated centrifuge PR-1.
(II) 16 300 r.p.m. with multispeed attachment No. 296 of the International refrigerated centrifuge PR-1.
(III) 27 690 r.p.m. with the preparative K rotor of the Spinco centrifuge model E.
(IV) 39 460 r.p.m. with the preparative K rotor of the Spinco centrifuge model E.

Table 1. Horse. 5 g liver tissue.

Fraction	Nitrogen	Catalase			ADH		
	mg	mg	%	Catalase / Nitrogen	mg	%	ADH / Nitrogen
Homogenate	106	32.5	100	0.307	25.7	100	0.242
Nuclei	21.7	0.4	1.2	0.018	1.6	6.2	0.074
Mitochondria	17.7	1.2	3.7	0.068	0.3	1.2	0.017
Microsomes	14.4	0.1	0.3	0.007	2.1	8.2	0.146
Supernatant	49.2	27.2	83.7	0.553	20.1	78.3	0.408
Sum	103	28.9	88.9		24.1	93.9	

The catalase activities were calculated into mg by using Kat.f. = 60 000.
The alcohol dehydrogenase activities were calculated into mg by considering 1 mg alcohol dehydrogenase to give $\Delta\Sigma = 40$ in three minutes.

Table 2. Guinea pig.

Fraction	Nitrogen mg	Catalase		
		mg	%	Catalase / Nitrogen
Homogenate	—	233.0	100	—
Nuclei	463	16.2	6.9	0.035
Mitochondria	215	8.6	3.7	0.040
Microsomes Supernatant	203	170.5	73.0	0.861
Sum	881	195.3	83.9	—

Table 3. Rat. 8.0 g liver tissue.

Fraction	Nitrogen	Catalase			ADH		
	mg	mg	%	Catalase / Nitrogen	mg	%	ADH / Nitrogen
Homogenate	220	5.4	100	0.025	2.1	100	0.010
Nuclei	62	1.0	18.6	0.016	0.4	19.0	0.006
Mitochondria	44	3.3	61.0	0.075	0.3	14.3	0.007
Microsomes	26	0.2	3.7	0.008	0.0	0.0	0.000
Supernatant	84	1.9	35.2	0.023	2.0	95.2	0.024
Sum	216	6.4	118.5		2.7	128.5	

The catalase activity was measured according to the rapid titration method described by Bonnichsen, Chance and Theorell [3]. The ADH activity was determined according to the spectrophotometrical method described by Theorell and Bonnichsen [4]. The nitrogen determinations were made with the micro Kjeldahl method.

Materials: Horse, guinea pig and rat liver tissue has been investigated. The microsomes were not isolated from guinea pig liver.

RESULTS

Six series of experiments were made, two in each of the species. The results from one of the series for each kind of animal are given in Tables 1—3. The other series essentially agreed with those given in the tables.

In guinea pig liver most of the catalase, about 70 %, is found in the fraction containing microsomes and ground substance and only minor quantities are present in the other fractions. This fraction (microsomes + ground substance) shows an extremely high catalase/nitrogen ratio.

The final supernatant of the horse liver preparations contains about 80 % of the catalase and has a very high catalase/nitrogen ratio compared with those of the other fractions.

The distribution of catalase in rat liver shows a different pattern. In essential agreement with von Euler and Heller [2] we found about 50 % of the catalase activity in the mitochondrial fraction, 30 % in the final supernatant and the rest in the other two fractions. The catalase/nitrogen ratio of the mitochondria is about 4 times as big as those of the other fractions.

Guinea pig liver did not give measurable quantities of ADH. In horse liver most of the ADH is located in the ground substance. The final supernatant shows a high ADH/nitrogen ratio.

Rat liver contains very small quantities of ADH, so the accuracy of the activity determinations was low. Most of the ADH activity was found in the supernatant.

SUMMARY

The intracellular distribution of catalase and alcohol dehydrogenase in horse, guinea pig and rat liver tissues has been investigated by using the differential centrifugation method.

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Über *cis*- und *trans*-2,3-Dimethoxy-α-phenylzimtsäure

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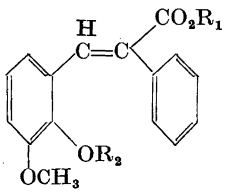
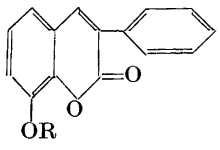
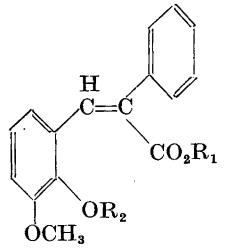
Bei der Kondensation von Benzaldehyd mit Phenylessigsäure entstehen bekanntlich *cis*- und *trans*-Form der α-Phenylzimtsäure nebeneinander, wobei die Menge der letzteren stark überwiegt [1]. Aus *o*-Oxybenzaldehyden und Phenylessigsäuren entstehen dagegen unter den üblichen Kondensationsbedingungen substituierte Cumarine d.h. die *cis*-Form der α-Phenylzimtsäure wird durch den Ringschluss stabilisiert. Die normalerweise begünstigte *trans*-Form ist unsres Wissens bisher nicht aufgefunden worden. Für die von Oglialoro [2] bei der Kondensation von Salicylaldehyd und Phenylessigsäure isolierte 2-Acetoxy-α-phenylzimtsäure ist die Zugehörigkeit zur *trans*-Reihe nicht bewiesen.

Anlässlich synthetischer Versuche in der Phenylcumaranreihe benötigten wir die *trans*-Form der 2-Oxy-3-methoxy-α-phenylzimtsäure (I) und wir versuchten, diese durch Kondensation von *o*-Vanillin mit dem Natriumsalz der Phenylessigsäure in Essigsäureanhydrid darzustellen. Um die Bildung des gleichzeitig zu erwartenden 3-Phenyl-8-methoxycumarins (II) [3] möglichst zu verhindern, wurde die Phenolgruppe des *o*-Vanillins vor der Kondensation acetyliert. Man erhält dann als Kondensationsprodukt ein Gemisch aus 2-Acetoxy-3-methoxy-α-phenylzimtsäure (III) und II. Das Verhältnis III : II hängt von den Kondensationsbedingungen ab und war bei den Kondensationstemperaturen 100°, 130° und 160° 1:1,7, 1:1,7 und 1:3,5. Die Gesamtausbeute an beiden Verbindungen lag bei 24, 36 und 41 % der Theorie.







I: $R_1 = H$ $R_2 = H$
III: $R_1 = H$ $R_2 = COCH_3$
IV: $R_1 = H$ $R_2 = CH_3$
V: $R_1 = CH_3$ $R_2 = CH_3$
VI: $R_1 = CH_3$ $R_2 = H$

II: $R = CH_3$
IX: $R = H$

VII: $R_1 = H$ $R_2 = CH_3$
VIII: $R_1 = CH_3$ $R_2 = CH_3$

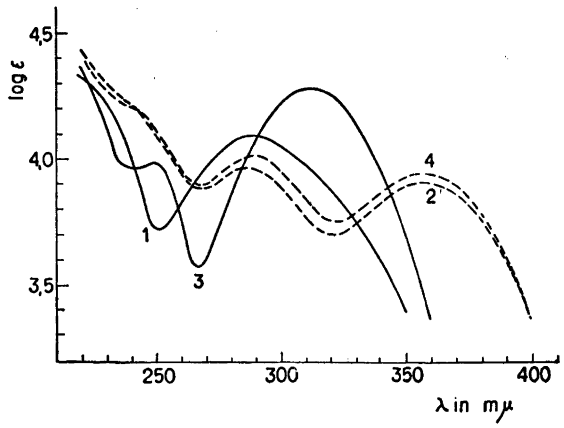


Fig. 1. UV-Spektra von 2-Oxy-3-methoxy-α-phenylzimtsäure (I) (in Alkohol: Kurve 1, in 0,1 N NaOH: Kurve 2) und 3-Phenyl-8-methoxycumarin (II) (in Alkohol: Kurve 3, in 0,1 N NaOH: Kurve 4).

Die aus III durch Verseifung der Acetylgruppe entstehende 2-Oxy-3-methoxy-α-phenylzimtsäure (I) erleidet in saurer Lösung keinen Ringschluss zum Cumarin, muss also die *trans*-Verbindung sein. Durch Methylierung mit Dimethylsulfat erhält man aus ihr *trans*-2,3-Dimethoxy-α-phenylzimtsäure (IV) sowie deren Methylester (V). Diazomethan reagiert rasch mit der Carboxylgruppe von I unter Bildung des Esters (VI)[3], methyliert dagegen die freie Phenolgruppe nur sehr träge. Aus dem Cumarin II lässt sich durch Methylierung mit Alkali und Dimethylsulfat die *cis*-2,3-Dimethoxy-α-phenylzimtsäure VII sowie deren Methylester VIII darstellen.

Die beiden der *trans*-Reihe angehörenden Verbindungen IV und V schmelzen erwartungsgemäss höher (154° bzw. 84°) als die entsprechenden *cis*-Verbindungen VII und VIII (144° bzw. 70°).

I und II zeigen auf Filtrierpapier unter der Quarzlampe kräftige hellblaue Fluorescenz, während IV und VII nur nach Zusatz von Ammoniak schwach fluorescieren.

Die Ultraviolettabsorptionsspektren der Verbindungen I und II — gemessen in alkoholischer Lösung — sind erwartungsgemäss stark verschieden. (Fig. 1.) In alkalischer Lösung (0,1 *N* Natronlauge) besitzen dagegen beide Verbindungen ein sehr ähnliches Spektrum und unterscheiden sich nur dadurch, dass II etwas kräftiger als I absorbiert. Die Spektren der beiden isomeren Säuren IV und VII (Fig. 2) zeigen ein Maximum bei 285 bzw. 293 mμ, sowie übereinstimmend ein Minimum bei 250 mμ. In Übereinstimmung mit bekannten *cis*- und *trans*-Stilbenen absorbiert VII als Derivat eines *trans*-Stilbens kräftiger als die *cis*-Verbindung IV. Der Unterschied zwischen diesen beiden Stilbencarbonsäuren ist jedoch nicht ganz so gross wie bei dem unsubstituierten Stilben [4].

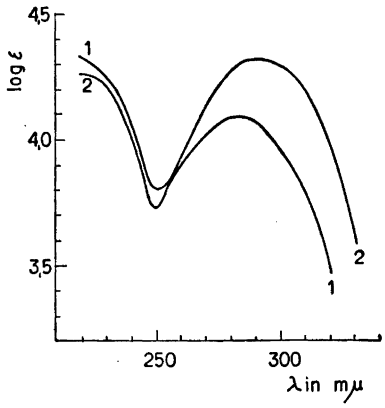


Fig. 2. UV-Spektra von trans-*2,3-Dimethoxy-α-phenylzimtsäure (IV) (Kurve 1) und* cis-*2,3-Dimethoxy-α-phenylzimtsäure (VII) (Kurve 2).*

Die beiden Säuren IV und VII lassen sich mit 67-proc. Jodwasserstoffsäure nicht decarboxylieren wie die 2-Methoxy-α-phenylzimtsäure [5]. Schon bei gewöhnlicher Temperatur wird die Methoxylgruppe in 2-Stellung abgespalten, wobei u.a. II entsteht. Bei höherer Temperatur konnte nur 3-Phenyl-8-oxycumarin (IX) isoliert werden.

VERSUCHSTEIL

Kondensation von o-Vanillinacetat mit Phenylessigsäure. 9,2 g *o*-Vanillinacetat werden mit 7,9 g phenylessigsaurem Natrium und 40 ml Essigsäureanhydrid 72 Stdn. in einem Ölbad von 100° erhitzt. Die Hauptmenge des Essigsäureanhydrids wird im Vakuum abdestilliert, der Rest mit Wasser zersetzt. Das rasch krystallisierende Reaktionsprodukt wird in Chloroform gelöst. Der Chloroformlösung wird durch Ausschütteln mit Natriumbicarbonatlösung die Säure III entzogen, welche beim Ansäuern der Bicarbonatlösung krystallin ausfällt. Nach einmaligem Umkrystallisieren aus Alkohol werden 1,4 g III in farblosen Nadeln erhalten. Schmp. 224°. (Gef. C 69,12; H 5,16; OCH_3 10,24; $C_{18}H_{16}O_5$. Ber. C 69,20 H 5,17 OCH_3 9,93.)

Der nach Eindunsten der Chloroformlösung hinterbleibende Rückstand liefert nach einmaligem Umkrystallisieren aus Alkohol 1,9 g II. Farblose Nadeln vom Schmp. 158°. Bei zwei weiteren Ansätzen (24 Stdn. bei 130° bzw. 24 Stdn. bei 160°) wurden 2,1 g III und 2,95 g II bzw. 1,4 g III und 4,15 g II erhalten.

Aus III entsteht durch Erhitzen mit Natriumacetat und Essigsäureanhydrid über 160° eine violett gefärbte, nicht näher untersuchte Verbindung.

trans-2-Oxy-3-methoxy-α-phenylzimtsäure (I). 2 g III werden mit 10 ml 10 % Natronlauge und 40 ml Methanol am Wasserbad 3 Stdn. erhitzt. Nach Abdestillieren des Methanols wird der Rückstand angesäuert und die ausfallende Säure aus Alkohol umkrystallisiert. Man erhält lange, seidenglänzende Nadeln vom Schmp. 198–200°. Mit Eisenchlorid entsteht eine rotbraune, allmählich rotviolett werdende Färbung. (Gef. C 71,27; H 5,21; OCH_3 11,33; $C_{16}H_{14}O_4$ Ber. C 71,08; H 5,22; OCH_3 11,74.)

Mit ätherischer Diazomethanlösung entsteht der Methylester VI. Aus Alkohol farblose verfilzte Nadeln vom Schmp. 160–161°. (Gef. C 71,51; H 5,82; OCH_3 21,80; $C_{17}H_{16}O_4$ Ber. C 71,80; H 5,68; OCH_3 21,82.)

trans-2,3-Dimethoxy-α-phenylzimtsäure (IV). Zu einer Lösung von 10 g I in 100 ml 30 % Natronlauge wird unter Rühren langsam Dimethylsulfat bis zur sauren Reaktion eingetropft. Die Reaktionsmischung enthält neben der mit Natriumbicarbonat abgetrennten Säure IV noch wenig Ester V. IV schmilzt nach Umkrystallisieren aus Alkohol oder Benzol bei 154°. (Gef. C 72,11; H 5,80; OCH_3 21,73; $C_{17}H_{16}O_4$ Ber. C 71,80; H 5,68; OCH_3 21,82.)

V krystallisiert aus Cyclohexan in derben Krystallen vom Schmp. 84°. (Gef. C 72,72; H 6,23; OCH_3 30,78; $C_{18}H_{18}O_4$ Ber. C 72,45; H 6,09; OCH_3 31,22.)

cis-2,3-Dimethoxy-α-phenylzimtsäure (VII). Zu einer Lösung von 10 g II in 100 ml 30 % Natronlauge werden unter Umrühren bei 70° langsam Dimethylsulfat bis zur sauren Reaktion eingetropft. Aus der durch Zusatz von etwas Soda alkalisch gemachten Lösung lassen sich mit Äther 9,3 g des Esters VIII abtrennen. Aus Cyclohexan derbe, rautenförmige Krystalle vom Schmp. 70°. (Gef. C 72,14; H 6,32; OCH_3 31,14; $C_{18}H_{18}O_4$ Ber. C 72,45; H 6,09; OCH_3 31,22.)

Aus der angesäuerten Lösung sowie durch Verseifung von VIII erhält man die Säure VII, welche nach Umkrystallisieren aus Alkohol oder Benzol bei 144° schmilzt. (Gef. C 71,79; H 5,63; OCH_3 21,92; $C_{17}H_{16}O_4$ Ber. C 71,80; H 5,68; OCH_3 21,82.)

3-Phenyl-8-oxycumarin (IX). Durch Erhitzen von II oder III mit 57-proc. Jodwasserstoffsäure. Aus Alkohol farblose kubische Krystalle vom Schmp. 203—204°. (Gef. C 75,45; H 4,07; $C_{15}H_{10}O_3$ Ber. C 75,60; H 4,23.)

Durch Acetylierung mit Essigsäureanhydrid in Pyridin erhält man 3-Phenyl-8-acetoxycumarin. Aus Alkohol farblose Stäbchen vom Schmp. 142°. (Gef. C 72,66; H 4,40; $C_{17}H_{12}O_4$ Ber. C 72,83; H 4,32.)

ZUSAMMENFASSUNG

Die Kondensation von *o*-Vanillinacetat mit Phenylessigsäure führt zu 3-Phenyl-8-methoxycumarin und *trans*-2-Acetoxy-3-methoxy-*α*-phenylzimtsäure. Durch Methylierung wurden aus diesen Verbindungen *cis*- und *trans*-2,3-Dimethoxy-*α*-phenylzimtsäure sowie deren Ester dargestellt. Die Ultraviolettabsorptionsspektren von *cis*- und *trans*-2,3-Dimethoxy-*α*-phenylzimtsäure wurden in alkoholischer Lösung, diejenigen von 3-Phenyl-8-methoxycumarin und *trans*-2-Oxy-3-methoxy-*α*-phenylzimtsäure auch in alkalischer Lösung gemessen.

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Über den Abbau von Lignin und Ligninmodellsubstanzen mit Hypochlorit. I. Mitteilung

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Die Bleichung des Zellstoffs, d.h. die Entfernung des restlichen Lignins durch Oxydation mit Hypochlorit oder Chlordioxyd ist eine technisch ausserordentlich wichtige und daher auch viel untersuchte Reaktion. Über den Ablauf dieser Oxydation des Lignins herrscht jedoch noch keine völlige Klarheit. Aus einer sehr gründlichen Untersuchung von Hibbert und Mitarbb.[1] geht hervor, dass bei der Einwirkung von unterchloriger Säure im wesentlichen 3 Reaktionen stattfinden, nämlich eine Chlorierung, eine Entmethylierung und ein oxydativer Abbau des Lignins. Definierte Abbauprodukte wurden bisher jedoch nicht isoliert. Es wurde daher eine Untersuchung begonnen mit dem Ziel, zunächst die Bedingungen für den Abbau von Lignin und von Ligninmodellsubstanzen vergleichend zu studieren und dann, worüber später berichtet werden soll, die entstehenden Abbauprodukte zu isolieren.

Da aus den Versuchen von Hibbert bekannt war, dass in alkalischer Lösung die Chlorierung des Lignins am geringsten ist, wurden alle Abbauversuche bei einem pH von etwa 11 durchgeführt. Für die Abbauversuche wurden folgende Ligninpräparate benützt: eine als Bariumsalz isolierte, nicht dialysierbare Ligninsulfosäure aus der Ablauge eines starken Fichten-Sulfitzellstoffs, ein aus Schwarzlauge ausgefälltes, nicht dialysierbares Sulfatlignin, ein nach Hibbert u. Mitarbb.[2] dargestelltes Äthanollignin, ein nach Brauns [3] dargestelltes »natives Lignin» und ein mit überkonzentrierter Salzsäure bei tiefer Temperatur dargestelltes Salzsäurelignin [4]. Für sämtliche Ligninpräparate wurde der Hypochloritverbrauch pro Lignineinheit d.h. pro Äquivalent Methoxyl unter verschiedenen Bedingungen bestimmt. Gleichzeitig wurde untersucht, wie das UV-Spektrum der Präparate im Verlauf der Hypochloritbehandlung verändert wird.

Bei sämtlichen Ligninpräparaten beobachtet man übereinstimmend, dass zunächst in einer sehr raschen, nur wenige Minuten dauernden Reaktion die Hauptmenge des reagierenden Hypochlorits verbraucht wird. Darnach findet nur noch eine wesentlich langsamere Reaktion statt, die einige Stunden dauern kann. Die in rascher Reaktion verbrauchte Hypochloritmenge beträgt für lösliche Ligninpräparate bei Temperaturen zwischen −4 und 20° etwa 2–3

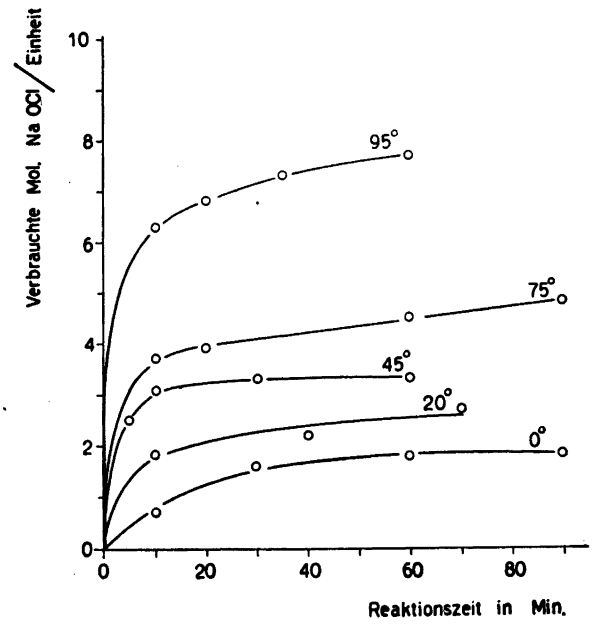

Fig. 1. Hypochloritverbrauch von Ligninsulfosäure bei verschiedenen Temperaturen.

Mol pro Lignineinheit, während das unlösliche Salzsäurelignin in der Kälte langsamer reagiert. Bei etwa 95° werden rasch durchschnittlich 7 Mol Hypochlorit, von Sulfatlignin sogar 10—11 Mol verbraucht. In Fig. 1 und 2 ist der Hypochloritverbrauch von Ligninsulfosäure (charakteristisch auch für Alkohollignin und »natives Lignin») bzw. Sulfatlignin bei verschiedenen Temperaturen dargestellt. Das Verhältnis Hypochlorit : Lignin wurde in der Regel so gewählt, dass nur 20—50 % des angesetzten Hypochlorits verbraucht wurden.

Das UV-Spektrum der löslichen Ligninpräparate, z.B. das in Fig. 3 dargestellte von Ligninsulfosäure, zeigt nach einem Verbrauch von 2 Mol Hypochlorit pro Einheit noch keine besonders charakteristische Veränderung. Nach einem Verbrauch von 6 und 8 Mol Hypochlorit verschwindet jedoch das ursprüngliche Maximum bei ca. 280 mμ rasch und die Absorption geht im gesamten Bereich kräftig zurück. Das abgebaute Salzsäurelignin zeigt ein gleichartiges Spektrum.

Wird Ligninsulfosäure bzw. Sulfatlignin mit 2 bzw. 2,7 Mol Hypochlorit bei gewöhnlicher Temperatur oxydiert, so nehmen die Präparate nur 3,3 bzw. 4,5 % Chlor auf. Der Methoxylgehalt sinkt dagegen von 13,3 bzw. 12,7 % auf 9,1 bzw. 11 % (asche- und chlorfrei gerechnet). Ein merklicher Abbau zu kleinen Bruchstücken scheint noch nicht erfolgt zu sein, denn die oxydierten Präparate sind nicht in nennenswertem Umfang dialysierbar. Nach Verbrauch von etwa 7 Mol Hypochlorit sind jedoch die allem Anschein nach noch Sulfogruppen enthaltenden Abbauprodukte der Ligninsulfosäure zum grössten Teil

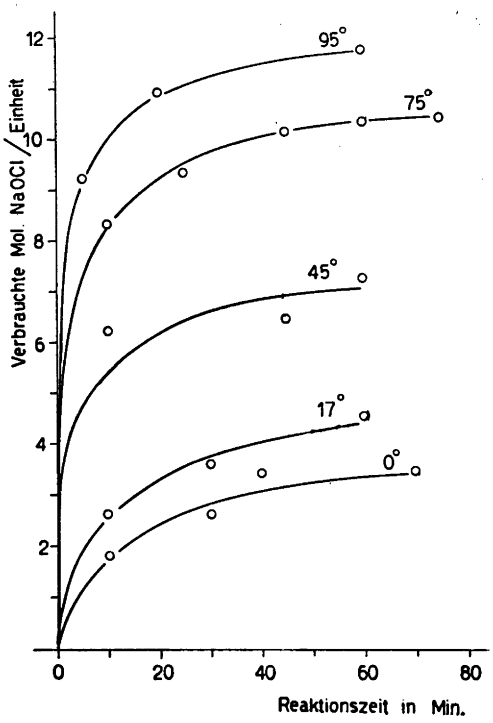

Fig. 2. Hypochloritverbrauch von Sulfatlignin bei verschiedenen Temperaturen.

dialysierbar. Ob der nicht dialysierbare Anteil noch aromatisch ist, sei vorerst dahingestellt.

Der Einfluss der Methylierung auf den Abbau wurde an folgenden Präparaten untersucht: Salzsäurelignin (15,5 % OCH_3), das mit Diazomethan bzw. Alkali-Dimethylsulfat methyliert war (21 bzw. 29 % OCH_3), analog behandelte Ligninsulfosäure mit 13,3 % OCH_3 vor und 20,5 bzw. 23,6 % OCH_3 nach der Methylierung, sowie Sulfatlignin mit 12,7 % OCH_3 vor und 24,6 bzw. 26,4 % OCH_3 nach der Methylierung. Fig. 4 zeigt die bei 95° verbrauchten Hypochloritmengen vor und nach der Methylierung. Man ersieht daraus deutlich, dass der Verbrauch durch die Methylierung sehr kräftig gesenkt wird. Die rasche Reaktion mit Hypochlorit wird, wie man am Beispiel des Salzsäurelignins wohl am besten sieht, schon durch eine relativ kleine Steigerung des Methoxylgehalts weitgehend verhindert. Dies kan nur darauf beruhen, dass freie Phenolgruppen des Lignins methyliert worden sind.

Dass Phenole im Gegensatz zu Phenoläthern mit Hypochlorit rasch reagieren, geht aus Abbauversuchen mit zahlreichen Modellsubstanzen klar hervor. Im 1. Teil der Tabelle 1 ist für eine Reihe von Phenolen und Phenoläthern die ungefähre Dauer der raschen Reaktion mit Hypochlorit bei 95° sowie die in dieser Zeit verbrauchte Hypochloritmenge pro Mol Substanz angegeben. Der

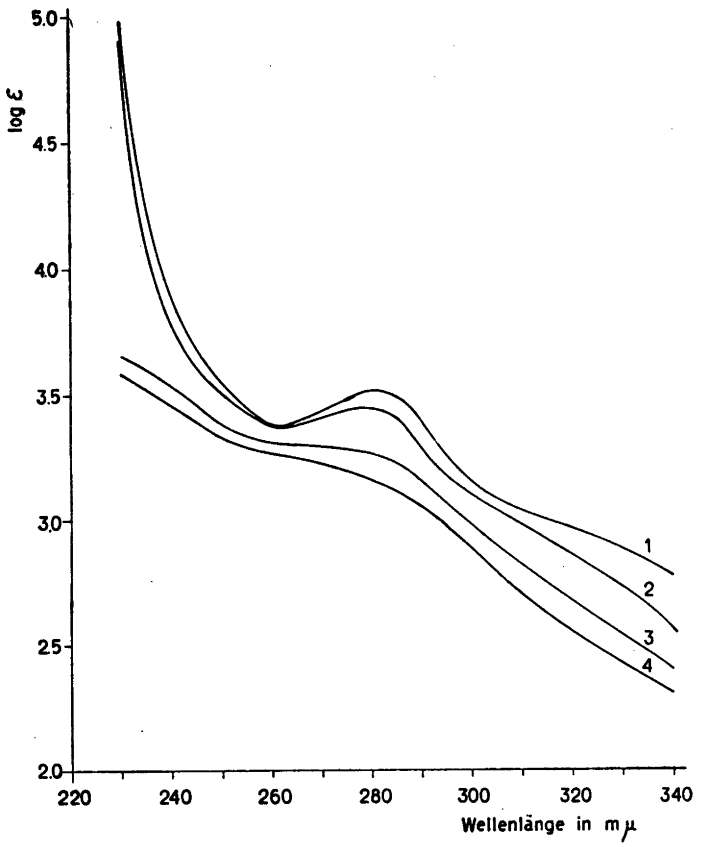

Fig. 3. Änderung des Ultraviolettspektrums von Ligninsulfosäure nach Verbrauch verschiedener Hypochloritmengen.
Kurve 1: vor der Oxydation
Kurve 2, 3 und 4: nach Verbrauch von 2, 6 und 8 Mol Hypochlorit/Einheit

2. Teil der Tabelle bringt die entsprechenden Angaben über einige aliphatische Verbindungen, welche teils als Abbauprodukte denkbar, teils im Hinblick auf die Konstitution der Seitenkette des Lignins von Interesse waren. In Fig. 5 ist der zeitliche Verlauf des Abbaus einiger der untersuchten Phenole und Phenoläther wiedergegeben.

Aus dem vorliegenden Material ergibt sich recht klar, dass für den Abbau eines phenolischen Ringes durchschnittlich 5—7 Mol Hypochlorit verbraucht werden (Phenol, Guajacol, Brenzcatechin). Für einen Abbau von Brenzcatechin oder Guajacol zu einem Mol Oxalsäure und einem Mol Maleinsäure würden z.B. theoretisch 6 Mol Hypochlorit benötigt. (Beim Guajacol wird dabei vorausgesetzt, dass aus der Methoxylgruppe nur Methanol entsteht, welches ziemlich stabil gegen Hypochlorit ist.) Bei gleichzeitigem Vorhandensein

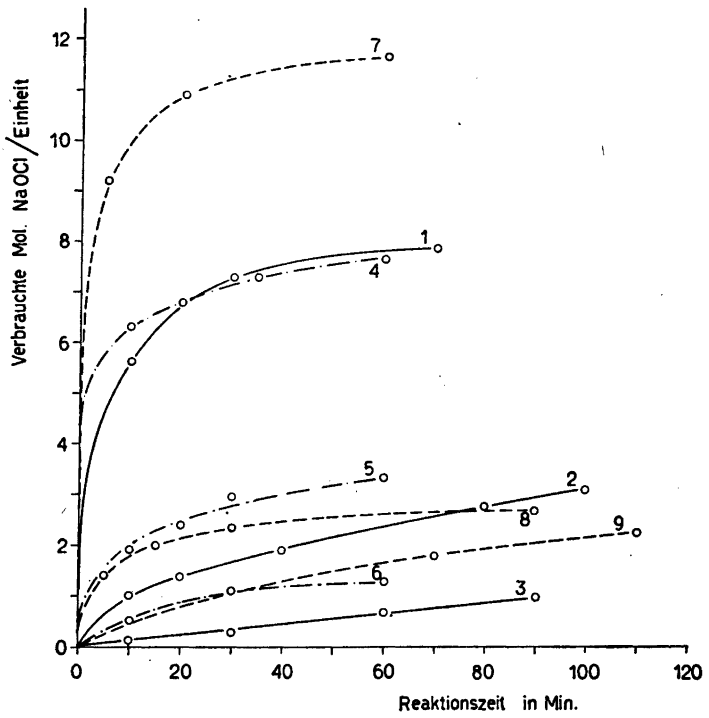

Fig. 4. Einfluss der Methylierung auf den Hypochloritverbrauch verschiedener Ligninpräparate.

——— *Salzsäurelignin:* *1 unmethyliert, 2 methyliert (21 % OCH_3), 3 methyliert (29 % OCH_3).*

—.—.—. *Ligninsulfosäure:* *4 unmethyliert, 5 methyliert (20,5 % OCH_3) 6 methyliert (23,6 % OCH_3).*

— — — *Sulfatlignin:* *7 unmethyliert, 8 methyliert (24,6 % OCH_3) 9 methyliert (26,4 % OCH_3).*

oxydationsempfindlicher Seitenketten kann der Verbrauch bis auf etwa 10 Mol (Eugenol) ansteigen. Ohne hier auf den Abbaumechanismus schon näher einzugehen, kann gesagt werden, dass die erste Reaktion dieser Phenole eine Chlorierung ist, falls eine *o*- oder *p*-Stellung zur Phenolgruppe frei ist. Beim Guajacol erhält man z.B. nach Verbrauch von nur 2 Mol Hypochlorit bei 20° in guter Ausbeute ein Gemisch chlorierter Verbindungen. Verbindungen ohne Phenolgruppen oder Phenoläther verbrauchen nur dann nennenswert Hypochlorit, wenn sie oxydationsempfindliche Seitenketten besitzen. Eine Ringsprengung in grösserem Ausmass konnte bei den in der Tabelle aufgeführten Phenoläthern nicht beobachtet werden. Aus Veratrylalkohol und dem relativ viel Hypochlorit verbrauchenden Isoeugenolmethyläther entsteht erwartungsgemäss Veratrumsäure. Phenoläther werden demnach in alkalischem Hypochlorit nicht chloriert.

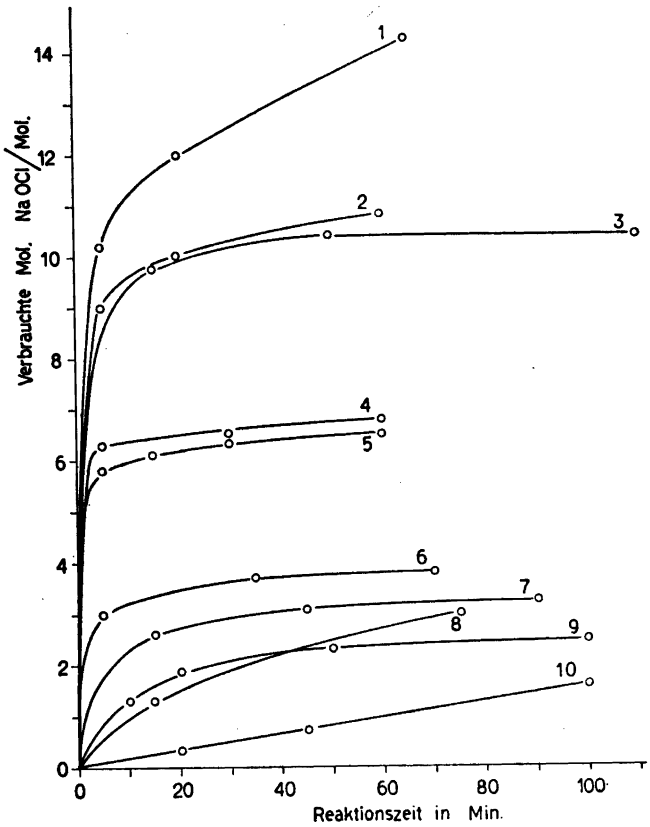

Fig. 5. Hypochloritverbrauch einiger Phenole und Phenoläther: 1. Pinoresinol, 2. Guajacylglycerin-α-sulfonsäure, 3. Isoeugenol, 4. Vanillinsäure, 5. Guajacol, 6. Veratrylglycerin, 7. 3,4-Dimethoxyzimtalkohol, 8. Isoeugenomethyläther, 9. Veratrylakohol, 10. Eugenolmethyläther.

Vergleicht man den Abbau der in der Tabelle 1 aufgeführten Phenole und Phenoläther mit dem der Ligninpräparate, so zeigen sich folgende Parallelen: 1) Im Durchschnitt verbrauchen Salzsäurelignin, Ligninsulfosäure, »natives Lignin» und Alkohollignin ebensoviel Hypochlorit pro Einheit wie die untersuchten Phenole. Beim Sulfatlignin liegt der Verbrauch dagegen höher. (Hierbei ist allerdings zu berücksichtigen, dass der Methoxylgehalt dieses Lignins in Folge von partieller Entmethylierung während der Sulfatkochung niedriger ist, als der der anderen Präparate. Die angegebene Hypochloritmenge wird in Wirklichkeit für den Abbau von etwa 1,1—1,2 Einheiten verbraucht.) 2) Methyliertes Lignin ist ebenso stabil gegen Hypochlorit wie die meisten Phenoläther. Der noch vorhandene Hypochloritverbrauch dürfte auf einer Oxydation der Seitenkette beruhen. 3) Die Spektra der abgebauten Lignin-

Tabelle 1.

Substanz	Ungefähre Dauer der raschen Reaktion in Min.	Pro Mol Substanz verbrauchte Mol Hypochlorit
1. Phenole u. Phenoläther		
Phenol	25	5
Anisol	—	0
Brenzcatechin	10	7
Guajacol	5	6
Veratrol	—	0
Benzylalkohol	60	<1
3,5-Dimethyl-4-oxy-benzylalkohol	10	8— 9
Vanillylalkohol	15	9
Veratrylalkohol	20	2
Diveratryläther [6]	—	0
Vanillin	10	7— 8
Veratrumaldehyd	10	1— 2
Syringaaldehyd	10	9
p-Oxybenzoesäure	40	6
Vanillinsäure	5	6
Veratrumsäure	—	0
o-Vanillinsäure	10	6
4-Oxy-5-methoxyisophthalsäure	10	6
Acetovanillon	5	9
Acetoveratron	10	3— 4
p-Oxypropiophenon	20	11
4-Propylguajacol	5	9— 8
Eugenol	10	10—11
Eugenolmethyläther	80	<1
Isoeugenol	15	9—10
Isoeugenolmethyläther	45	5
3,4-Dimethoxyzimtalkohol	15	2— 3
Ferulasäure	20	9—10
Dihydroferulasäure	10	10
Guajacylglycerin-α-sulfonsäure [7]	5	9
α-Veratrylglycerin [8]	10	3— 4
Pinoresinol	20	12
α,α'-Diäthyl-α-α'-di-(4-oxyphenyl)-äthylenglykol [9]	10	20
2. Aliphatische Verbindungen		
Oxalsäure	—	0
Fumarsäure	—	0
Maleinsäure	—	0
Crotonsäure	100	<1
Äthylenglykol	85	<1
Allylalkohol	45	2
1,2-Propylenglykol	55	4
Propylalkohol	65	<1
Isopropylalkohol	110	<1
Propionaldehyd	5	2—3
Oxyaceton	10	2—3
Milchsäure	25	2—3
Brenztraubensäure	5	2

präparate gleichen vielfach denen der abgebauten Phenole. Sie deuten auf einen starken Abbau der Ringe hin, ohne jedoch besonders charakteristisch zu sein.

Dass die untersuchten Ligninpräparate genau so rasch abgebaut werden wie Phenole war überraschend, denn der Gehalt der Ligninpräparate an freien Phenolgruppen ist recht verschieden. Das Cuproxamlignin der Fichte enthält nach K. Freudenberg [10] etwa eine freie Phenolgruppe auf 4 Einheiten, was auch angenähert für das Salzsäurelignin gelten dürfte. G. Aulin-Erdtman schliesst aus spektrophotometrischen Messungen, dass die Ligninsulfosäure höchstens 1 Phenolgruppe auf 4—6 Einheiten enthält [11].* Im Alkohollignin fanden E. Hägglund u. H. Richtzenhain 1 Phenolgruppe auf 2 Einheiten [12], während im Sulfatlignin nach T. Enkvist [13] sogar 1 Phenolgruppe auf 1—1,5 Einheiten kommt. Ob sich die nach verschiedenen Methoden an verschiedenartigen Ligninpräparaten erhaltenen Werte für den Gehalt an Phenolgruppen exakt vergleichen lassen, soll hier nicht diskutiert werden. Setzt man dies voraus, so sollte eigentlich nur bei dem sehr phenolreichen Sulfatlignin ein den Phenolen einigermassen analoger Abbau möglich sein. Bei den anderen Präparaten, besonders Salzsäurelignin und Ligninsulfosäure sollte jedoch nur die phenolische Einheit rasch abgebaut werden, während die übrigen Einheiten mit verätherter Phenolgruppe — abgesehen von Veränderungen in der Seitenkette — nicht oder doch nur wesentlich langsamer angegriffen werden sollten. Dies ist jedoch nicht der Fall. Der hohe Hypochloritverbrauch sowie das kräftige Absinken der UV-Absorption zeigen, dass ausser der phenolischen Einheit auch noch der grösste Teil der anderen Einheiten rasch abgebaut werden muss.

Hierfür sind 2 Erklärungen denkbar. Die eine ist, dass in den verätherten Lignineinheiten Gruppierungen vorliegen können, die auch ohne freie Phenolgruppen von Hypochlorit angegriffen werden. Derartige Gruppierungen können aber — wenn überhaupt — nur in beschränktem Umfang vorkommen. Für den Abbau solcher Einheiten sollte es nämlich belanglos sein, ob die phenolischen Gruppen frei oder methyliert sind. Tatsächlich wird aber der Abbau durch die Diazomethanbehandlung weitgehend verhindert. Wahrscheinlicher ist es deshalb, dass durch den raschen Abbau der im Lignin vorhandenen phenolischen Einheiten ursprünglich verätherte Phenolgruppen freigelegt und dann ebenfalls rasch oxydiert werden. Für Phenolätherbindungen, welche vom γ-Kohlenstoffatom der Ligninseitenkette ausgehen, ist ein derartiger Abbau schon von H. Hibbert u. Mitarbb. in Betracht gezogen, aber nicht experimentell gestützt worden.

In Kürze wird über Abbauversuche von Modellsubstanzen berichtet werden, in welchen Guajacyleinheiten ätherartig an das α-, β- oder γ-Kohlenstoffatom der Seitenkette gebunden sind.

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* Anm. bei der Korrektur: In einer inzwischen publizierten Arbeit (*Ber.* **86** (1953) 757) weisen Freudenberg und Rasenack darauf hin, dass nach unveröffentlichten Versuchen von Boesenberg und Schotte mindestens jede zweite Einheit der Ligninsulfosäure eine freie Phenolgruppe enthält.

ZUSAMMENFASSUNG

Die Einwirkung von alkalischer Hypochloritlösung auf Ligninpräparate wurde bei verschiedenen Temperaturen untersucht. Bei 95° werden im Durchschnitt sehr rasch ca. 8 Mol Hypochlorit pro Lignineinheit verbraucht. Die UV-Spektra wurden nach Verbrauch verschiedener Hypochloritmengen gemessen. Sie deuten auf einen weitgehenden Abbau der aromatischen Ringe hin. Durch Methylierung wird der Abbau der Ligninpräparate stark gehemmt.

Hypochloritoxydationen einer grossen Zahl von einfachen Phenolen und Phenoläthern zeigten, dass nur Verbindungen mit freien Phenolgruppen rasch abgebaut werden.

Der freien Phenolen analoge, rasche Abbau derjenigen Ligninpräparate, welche nur wenige freie Phenolgruppen enthalten, wird durch eine während des Abbaus erfolgende Freilegung von ursprünglich ätherartig gebundenen Phenolgruppen erklärt.

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Studies on the Extraction of Metal Complexes

VIII. The Extraction of La, Sm, Hf, Th, and U(VI) with Oxine and Cupferron

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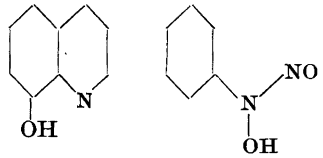
Solvent extraction has proved to be an important method for the separation and determination of metals in analytical chemistry. The procedures worked out in that field should be applicable to chemical industry as well as in preparative laboratory work since extraction is a very practical unit operation. With a multistage countercurrent extraction process a practically complete fractionation of materials which are closely related may be obtained.

The importance of solvent extraction led our group to study the extraction of lanthanide and actinide complexes with organic solvents. For this purpose, the following metal ions were chosen: La^{+3} (radius * = 1.15 Å), Sm^{3+} (r = 1.07 Å), Th^{4+} (r = 1.02 Å), Hf^{4+} (r = 0.86 Å) and UO_2^{2+}. As the behavior of these elements to some extent is determined by the ion charges and sizes it might be feasible to predict the behavior of other rare earth metals as of the transuranium elements, *e.g.* Am^{3+} (r = 1.11 Å) or Pu^{4+} (r = 0.92 Å).

It was further considered as favorable if the distribution of the metals between the two phases could be measured radiometrically; suitable radioisotopes were available for La (La^{140}), Sm (Sm^{153}), Hf (Hf^{181}), and Th (Th^{234}, UX_1). Two organic solvents were used, chloroform and hexone (methyl *iso*butyl ketone). Chloroform is known as a very good solvent for metal chelates and hexone was chosen as representative for oxygen-containing solvents.

The ionic strength in the aqueous phase was kept constant at 0.1 *M* using $HClO_4$, $NaClO_4$ and NaOH, and all experiments were carried out at 25° C. In the following part the extraction of the oxinates and cupferrates are reported.



Oxine Cupferron

* The radii are approximately given in the Pauling scale by comparison with data by Zachariasen [1].

INTERPRETATION OF DATA

Data on metal extraction with complex forming agents (HA) such as dithizone and oxine are usually given in diagrams plotting the per cent metal extracted against the pH for a given reagent concentration. The theoretical understanding of these curves have been based on the following reaction

$$M^{N+}\ (aq) + N \cdot HA\ (org) \rightleftharpoons MA_N\ (org) + N \cdot H^+\ (aq)$$

with the equilibrium constant

$$K = \frac{[MA_N]_{org} \cdot [H^+]^N}{[M^{N+}] \cdot [HA]^N_{org}}$$

This equation is however only valid when the water-soluble M—A complexes may be neglected.

From the very beginning of our work we looked upon this problem of metal extraction as a stepwise complex formation of M^{N+} with a ligand A^- giving among other complexes MA_n, an uncharged extractable complex MA_N. With the use of radioactive tracers we extended the measurable range of the net distribution ratio of M,

$$q = \frac{[M]_{total,\ org}}{[M]_{total,\ aq}}$$

from about 100—0.01 to about 1 000—0.001. In doing so we found in most cases that q approached a maximum value (see Fig. 1). This could be explained by assuming the presence of MA_N in the aqueous phase. The distribution constant of this complex is

$$\lambda_N = \frac{[MA_N]_{org}}{[MA_N]_{aq}}$$

Furthermore the data could only be explained if all the complexes MA, MA_2, ... MA_N were considered. In the mathematical treatment [2,3] of our data we have found it most useful to plot log q against log $[A^-]$. Calculations on these curves have enabled the more or less independent determination of λ_N and the various complexity products $\varkappa_n$.

$$\varkappa_n = \frac{[MA_n]}{[M][A]^n} = k_1 \cdot k_2 \ldots\ldots k_n$$

Most of the experimental data in this work is therefore found in other articles [4,5]. where the complex formation constants and distribution constants are calculated. The log q curves in those papers are here transformed to curves where the ordinate is $100 \cdot q/1 + q$, *i.e.* per cent metal extracted. Figure 1 shows an evenly spaced family of log q curves with different values of λ_N and how it spreads out around $q = 1$ in the $100 \cdot q/1 + q$ interpretation. The characteristic type of curves in Figure 6 are obtained when log $\lambda_N \geq 2$.

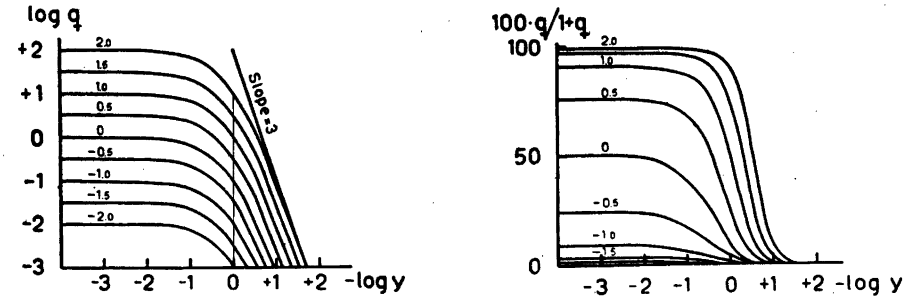


Fig. 1. Variation of the net distribution ratio q in two different graphical representations for various values for the logarithm of the partition coefficient λ_3 of the uncharged complex MA_3. Abscissa: — log y = — log [A⁻] — a (cf. part V[3], the parameter b is 0.25, N = S = 3).

As stated above the abscissa chosen is generally pH while we prefer to use —log [A⁻]. With this representation the points for different values of the initial reagent concentration in one phase, C_A, will usually coincide. In Figures 2 and 3 the relations between pH (-log [H⁺]) and —log [A⁻] for the oxine-chloroform and cupferron-hexone, cupferron-chloroform systems are shown.

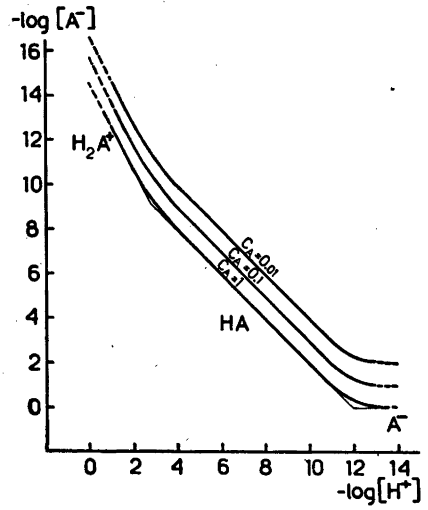


Fig. 2. The oxinate ion concentration (— log [A⁻]) as a function of the hydrogen ion concentration for different values of the initial oxine concentration in the chloroform phase C_A (in moles/lit.). The volume of the aqueous phase is equal to the volume of the organic phase.

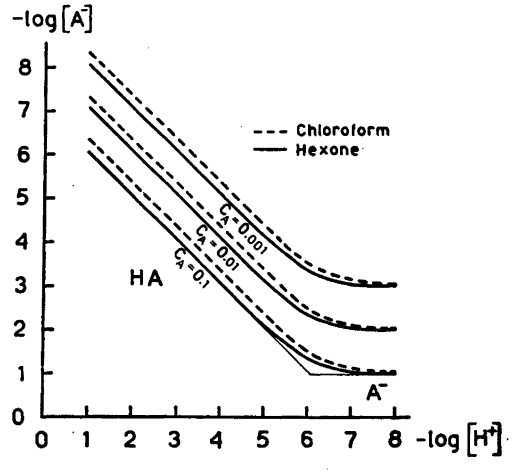


Fig. 3. The cupferrate ion concentration (— log [A⁻]) as a function of the hydrogen ion concentration for different values of the initial Na-cupferrate concentration in the aqueous phase C_A (in moles/lit.). The volume of the aqueous phase is equal to the volume of the organic phase.

Table 1. Distribution of UO_2^{2+}. *The oxine-chloroform system.*

Initial conc. of oxine in the $CHCl_3$ phase M	Initial conc. of U in the aq. phase M	$\frac{100 \cdot q}{1+q}$	log q	$-\log [H^+]$	$-\log [A^-]$
0.100	0.001	0.1	(−3.00)	1.91	11.95
0.100	0.001	0.5	(−2.30)	2.05	11.70
0.100	0.001	2.8	(−1.54)	2.21	11.42
0.100	0.001	9.4	−0.98	2.34	11.21
0.100	0.001	32.1	−0.32	2.46	11.03
0.100	0.001	36.2	−0.24	2.48	11.00
0.100	0.001	55.5	+0.11	2.57	10.87
0.100	0.001	57.6	+0.13	2.59	10.84
0.100	0.001	55.5	+0.11	2.68	10.71
0.100	0.001	79.2	+0.59	2.71	10.67
0.100	0.001	85.2	+0.77	2.73	10.64
0.100	0.0001	4.6	(−1.32)	2.23	11.39
0.100	0.0001	16.2	−0.70	2.32	11.25
0.100	0.0001	40.4	−0.16	2.46	11.03
0.100	0.0001	56.6	+0.11	2.58	10.85
0.100	0.0001	69.2	+0.35	2.59	10.84
0.100	0.0001	77.4	+0.54	2.63	10.78
0.100	0.0001	81.6	+0.65	2.67	10.72
0.100	0.0001	90.5	+0.97	2.72	10.66
0.100	0.0001	94.0	(+1.20)	2.79	10.56
0.100	0.0001	94.5	(+1.24)	2.85	10.49
0.100	0.0001	99.0	(+1.99)	3.53	9.69
0.100	0.0001	84.4	+0.73	10.38	2.57
0.100	0.0001	77.5	+0.54	10.55	2.41
0.100	0.0001	68.2	+0.33	10.77	2.20
0.100	0.0001	58.2	+0.14	10.90	2.08
0.100	0.0001	47.5	−0.05	11.17	1.84
0.050	0.0001	12.1	−0.86	2.40	11.42
0.050	0.0001	18.8	−0.64	2.45	11.34
0.050	0.0001	22.3	−0.54	2.49	11.29
0.050	0.0001	29.1	−0.39	2.53	11.23
0.050	0.0001	39.3	−0.19	2.59	11.14
0.050	0.0001	46.6	−0.06	2.71	10.97
0.050	0.0001	67.9	+0.40	2.85	10.79
0.050	0.0001	72.5	+0.42	2.88	10.75
0.050	0.0001	79.7	+0.59	2.98	10.61
0.050	0.0001	83.8	+0.71	3.04	10.54
0.020	0.0001	6.7	(−1.14)	2.69	11.40
0.020	0.0001	6.7	(−1.14)	2.75	11.31
0.020	0.0001	10.8	−0.87	2.79	11.26
0.020	0.0001	15.9	−0.72	2.78	11.27
0.020	0.0001	19.1	−0.63	2.86	11.17
0.020	0.0001	35.3	−0.26	3.04	10.95
0.020	0.0001	53.1	+0.05	3.19	10.77
0.020	0.0001	76.1	+0.51	3.43	10.49
0.020	0.0001	86.5	+0.81	3.54	10.38
0.020	0.0001	86.5	+0.81	3.70	10.22

Table 2. Distribution of UO_2^{2+}. *The oxine-hexone system.*

Initial conc. of oxine in the $CHCl_3$ phase M	Initial conc. of U in the aq. phase M	$\frac{100 \cdot q}{1+q}$	log q	$-\log [H^+]$	$-\log [A^-]$
0.100	0.001	4.7	(−1.31)	2.32	11.17
0.100	0.001	12.7	−0.83	2.44	10.97
0.100	0.001	20.2	−0.59	2.53	10.81
0.100	0.001	27.5	−0.41	2.57	10.75
0.100	0.001	37.5	−0.21	2.63	10.65
0.100	0.001	48.8	−0.02	2.65	10.61
0.100	0.001	59.0	+0.16	2.74	10.47
0.100	0.001	66.0	+0.29	2.78	10.40
0.100	0.001	72.5	+0.43	2.82	10.35
0.100	0.001	80.5	+0.62	2.91	10.21
0.100	0.001	83.3	+0.70	2.93	10.17

These curves are calculated from data given in Part IV of this series [6]. It is seen from the curves that the same $-\log [A^-]$ value may be obtained for different C_A values simply by changing $-\log [H^+]$ in the aqueous phase. The figures also show that the oxine-chloroform systems has two buffer points at $-\log [H^+]$ 2.72 ($pk_1 - \log p$) and 11.94 ($pk_2 + \log p$) and that the cupferron-hexone system has one point at $-\log [H^+]$ 6.09 ($pk_a + \log p$).

Table 3. Distribution of UO_2^{2+}. *The cupferron-chloroform system.*

Initial conc. of Na-cupferrate in the aq. phase M	Initial conc. of U in the the aq. phase M	$\frac{100 \cdot q}{1+q}$	q *	$-\log [H^+]$	$-\log [A^-]$
0.01	0.0001	7.3		2.42	5.92
0.01	0.0001	14.3		2.86	5.48
0.01	0.0001	24.8		3.22	5.12
0.01	0.0001	36.1	0.57	3.58	4.76
0.01	0.0001	38.0	0.61	3.63	4.71
0.01	0.0001	35.3	0.55	3.78	4.56
0.01	0.0001	35.3	0.55	4.01	4.33
0.01	0.0001	28.2	0.39	4.35	3.99
0.01	0.0001	27.2	0.37	4.70	3.65
0.01	0.0001	27.5	0.38	5.14	3.22
0.01	0.0001	25.6	0.34	5.26	3.12
0.01	0.0001	30.6	0.44	5.69	2.76
0.01	0.0001	23.4	0.31	5.88	2.60

* 10 q-values chosen for the determination of $q_{max} = \lambda_2 = 0.45$

Table 4. Distribution of UO_2^{2+}. The cupferron-hexone system.

Initial conc. of Na-cupferrate in the aq. phase M	Initial conc. of U in the aq. phase M	$\frac{100 \cdot q}{1+q}$	q *	—log [H+]	—log [A⁻]
0.01	0.0001	17.5		1.30	6.75
0.01	0.0001	15.9		1.43	6.62
0.01	0.0001	42.3		1.84	6.21
0.01	0.0001	50.9		1.96	6.09
0.01	0.0001	67.3	2.97	2.12	5.93
0.01	0.0001	82.2	4.62	2.36	5.69
0.01	0.0001	69.0	3.29	2.42	5.63
0.01	0.0001	65.2	2.63	2.59	5.46
0.01	0.0001	50.7	1.03	2.61	5.44
0.01	0.0001	76.8	3.31	2.99	5.06
0.01	0.0001	42.5	0.74	3.57	4.48
0.01	0.0001	71.1	2.46	5.30	2.82

* 8 q-values chosen for the determination of $q_{max} = \lambda_2 = 2.63$

EXPERIMENTAL

All experiments were carried out as described earlier [4]. The volume of the two phases were equal. Only small amounts of the metals were used and the distribution between the organic and aqueous phases was measured radiometrically in the case of La, Sm, Hf, and Th and spectrophotometrically in the case of U (VI) [7]. The radioisotopes of La, Sm, and Hf were obtained by neutron irradiation of the corresponding oxides at AERE, Harwell; Th^{234} (UX_1) was used as a tracer for Th. It might be pointed out that the metals were not mixed, the experiments being run separately for each metal. The La and Sm data are given in Part XVII [5], the Th data have been given in Part VI [4] and the data on the uranyl ion are given in Tables 1—4 and in Figures 4 and 5. With Hf very few experiments were carried out and the results are only approximate.

It should be noted that we used no buffers such as phosphate and the pH in the 0.1 *M* $NaClO_4$ aqueous phase was adjusted with $HClO_4$ and NaOH. The reason for this is apparent as perchlorate ions are considered not to form metal complexes. Of course it was then somewhat difficult to obtain the right pH between 5 and 9 with the oxine system.

RESULTS

The uranyl-oxine system: Within the small log q range investigated it is not possible to calculate $\varkappa_1$, $\varkappa_2$ and λ_2 as the points fall on a straight line with a slope equal to 2. From Fig. 4 we may obtain at log $q = 0$ $\lambda_2 \cdot \varkappa_2 = 10^{2 \cdot 10.94}$ for the chloroform system and $\lambda_2 \cdot \varkappa_2 = 10^{2 \cdot 10.54}$ for hexone (equation 7 in Part V[3]). Figure 5 gives likewise $\lambda_2 \cdot \varkappa_2 \cdot \varkappa_3^{-1} = 10^{-1.88}$ for chloroform (equation 8 in Part V[3]). In this way

$$\varkappa_3 = \frac{[UO_2A_3^-]}{[UO_2^{2+}][A]^3} = 10^{23.76}$$

is obtained.

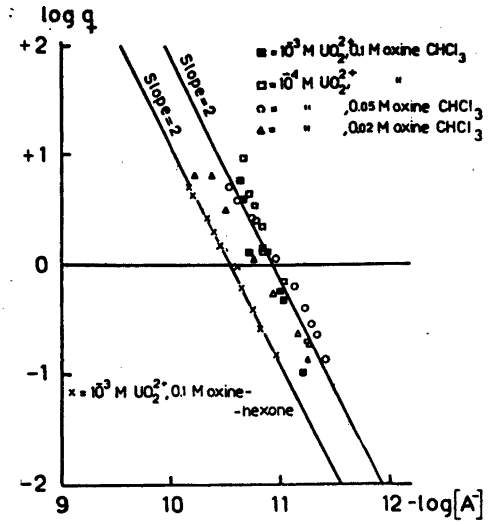


Fig. 4. The distribution of the uranyl ion between chloroform or hexone and acid perchlorate solutions as a function of the oxinate ion concentration (— log [A⁻]).

The oxine-system: The difference between chloroform and hexone as a solvent extraction liquid for oxine or the metal oxinates is very small, chloroform having a slightly better solving capacity. Fig. 6 shows the results with the chloroform-oxine system. It is seen that all of these metals can be extracted at different —log [A⁻] values, thus it is possible to separate them by means of controlled pH. The sequence between Hf^{4+}, Th^{4+}, Sm^{3+}, and La^{3+} is the one expected from the charges and sizes of the ions, the uranyl ion, however, seems to hold a unique position. It should also be noted that Th and U are re-extracted at high concentration of A^- (low —log [A⁻] values) probably because negative complexes are formed.

The cupferron system: For the metal cupferrates investigated here hexone is a better solvent, even though cupferron (HA) itself is more soluble in chloroform than hexone (*cf.* Part IV[6]). The extraction of Hf^{4+} and Th^{4+} is very good and takes place at low values of —log [A⁻] as seen from Fig. 7. The extraction of La^{3+} and especially UO_2^{2+} is poor, the solubility in the organic

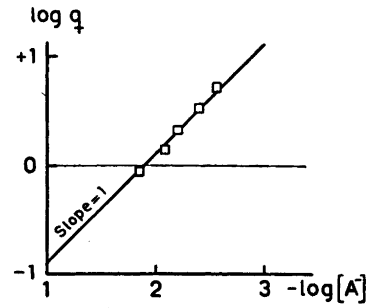


Fig. 5. The distribution of the uranyl ion between chloroform and alkaline perchlorate solutions as a function of the oxinate ion concentration (— log [A⁻]).

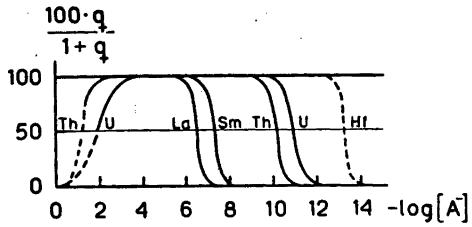


Fig. 6. The distribution of La (III), Sm (III), Hf (IV), Th (IV), and U (VI) between chloroform and perchlorate solutions as a function of the oxinate ion concentration (— log [A⁻]).

phase being only slightly greater than in the aqueous phase. The sequence between Hf, Th, Sm and La is the same as for oxine, but it may be noted that the position of U in relation to Th has changed. Also in this case it is possible to separate the metals at a controlled pH. However, it should be remembered that cupferron decomposes quite readily.

DISCUSSION

Although oxine and cupferron show excellent possibilities for separating the metals investigated by us, it should be pointed out that the total metal concentration always was low ($< 10^{-3} > 10^{-8}$ *M*). If we had used higher metal concentrations the metal oxinates and cupferrates would have precipitated at certain pH values. Some data on the solubilities that we have obtained are given in Table 5 together with Lacroix's [8] values of the Al, Ga and In oxinates.

Feigl [9] connects the solubility of the oxinates with their content of bound water or oxine (HA). We are of the same opinion as Pokras, Kilpatrick, and Bernay[10] that H_2O or HA in many cases is bound only by weak lattice for-

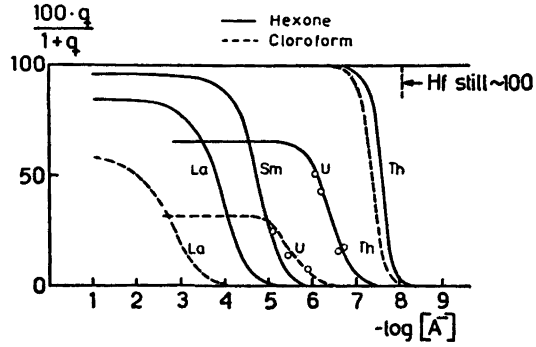


Fig. 7. The distribution of La (III), Sm (III), Hf (IV), Th (IV), and U (VI) between hexone or chloroform and perchlorate solutions as a function of the cupferrate ion concentration (— log [A⁻]). The plateau's for the uranyl curves are calculated from data in Tables 3 and 4.

Table 5. Solubilities of some metal oxinates and cupferrates.

Solvent / reagent	Metal ion	Temp. °C	Solubility moles / lit
Chloroform (aq.sat.) / oxine	Th^{4+}	25	$(6.4 \pm 1.7) \cdot 10^{-4}$
Hexone (») / »	Th^{4+}	25	$(5.4 \pm 1.0) \cdot 10^{-4}$
Chloroform (») / »	UO_2^{2+}	25	$7.76 \cdot 10^{-4}$
» (») / »	Sr^{2+}	25	$0.94 \cdot 10^{-4}$
Chloroform / oxine	Al^{3+}	18	0.045
» / »	Ga^{3+}	18	0.092
» / »	In^{3+}	18	0.24
Chloroform (aq.sat.) / cupferron	La^{3+}	25	$(5.6 \pm 0.6) \cdot 10^{-4}$
Hexone (») / »	La^{3+}	25	$(4 \pm 4) \cdot 10^{-4}$

ces. It is therefore questionable if this binding should be connected with the extraction of the metal oxinates. How irregular the H_2O or HA appears is shown by a few examples [9,11]:

Precipitates with HA: Sc^{3+}, Th^{4+}, UO_2^{2+}
» with 2 H_2O: Mg^{2+}, Mn^{2+}, Ni^{2+}, Cd^{2+}, Ce^{4+}
» without HA or H_2O: Al^{3+}, Fe^{3+}, Cu^{2+}, La^{3+}, WO_2^{2+}

As pointed out by Moeller [12] the pH of the extraction is closely related to the pH of the precipitation of the metal oxinates. This case may however not be taken as a rule, it should rather be taken as a coincidence of the factors that determine extraction and precipitation.

The pH at which the extraction begins depends on the reagent concentration, C_A, as seen from Figures 2, 3, 6 and 7. If the reagent concentration is too low the metal ions will be hydrolyzed before the complex formation and extraction with A^- takes place. With oxine a rather high concentration (0.1—0.5 *M*) is generally needed and with cupferron much lower concentrations can be used (0.005—0.010 *M*).

The lanthanides and actinides are of course not the only metals that are extractable as oxinates and cupferrates. Table 6 summarizes data found on oxine-chloroform extraction of different metals. The extraction of cupferrates has been extensively treated by Furman, Mason and Pekola [15] and qualitative data on the extraction of many metal oxinates and cupferrates with chloroform are given by Gorbach and Pohl [16].

SUMMARY

Extraction data on the oxinates and cupferrates of La(III), Sm(III), Hf(IV), Th(IV) and U(VI) are given. Two organic solvents have been used, chloroform and hexone. The ionic strength of the aqueous phase has been kept constant at 0.1 *M* using $HClO_4$, $NaClO_4$ and NaOH, and all experiments were carried out at 25° C. Curves are given showing the partition of the metals

Table 6. The pH for 50 % extraction of metal oxinates with chloroform.

Metal ion	pH	Procedure	Reference
Ga^{3+}	1.0	$V_{aq} = V_{org}$, 0.1 *M* total oxine.	8
In^{3+}	2.1	Anions in aq. sol.: chloride	
Al^{3+}	3.4		
Fe^{3+}	1.6	4 successive extractions with	12, 13
Cu^{2+}	2.0	0.01 *M* solution of oxine in	
In^{3+}	2.2	chloroform.	
Bi^{3+}	3.0	Anions in aq. sol.: sulfate,	
Al^{3+}	4.2	acetate, nitrate, chloride	
Ni^{2+}	6.1		
Co^{2+}	6.5		
Sn^{4+}	0.0	$V_{aq} = 5 \cdot V_{org}$, 0.07 *M* oxine.	14
Mo	1.0	Anions in aq. sol.: acetate,	
Fe^{3+}	2.0	chloride, tartrate	
Cu	2.1		
Ni	3.7		
Al	3.8		
Mn^{2+}	6.4		
Hf^{4+}	1.3 *	$V_{aq} = V_{org}$, 0.1 *M* total oxine.	this work
UO_2^{2+}	2.6	Anions in aq. sol.: perchlorate	
Th^{4+}	3.1		
Sm^{3+}	5.7		
La^{3+}	6.5		

* $pH = -\log [H^+] + 0.1$

between the organic phase and the aqueous phase as a function of the oxinate or cupferrate ion concentration.

From these curves it is evident that the metals can be separated by extraction with these systems.

The authors wish to thank the head of FOA 1, Professor G. Ljunggren for his interest in our work, which is part of a program supported by *Atomkommittén*.

We also wish to thank Professor L. G. Sillén for valuable advice, Fil.mag. S. Hultberg for aid in the preliminary experiments, and Fil.lic. J. Rydberg and Fil.mag. Brita Hök for a pleasant cooperation.

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The Interaction between Halogen Acids and Nitro-amines

III *. The Reactivity of Mono- and Dinitroanilines

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When treated with hydrochloric acid certain nitro-amines are transformed to chlorosubstituted diazonium salts. This reaction has been described and discussed in the preceding papers of this series [1,2]. The mono- and dinitroanilines have now been studied.

The mononitroanilines failed to yield any diazonium salts when heated to 100 °C in a mixture of glacial acetic and hydrochloric acids and were recovered unchanged.

Of the six dinitroanilines only 3,5-dinitroaniline failed to react; the others reacted quantitatively yielding large amounts of diazonium salts. In order to compare the reactivity of these dinitroanilines they were allowed to react at 40 °C and the amounts of diazonium salts present in the reaction solution were determined at suitable intervals (for 2,4-dinitroaniline a temperature of 80 °C was necessary for the reaction to proceed). The results are shown in Fig. 1 where the amount of diazonium salt is plotted against the time of reaction. The results show that, with regard to reactivity, the dinitroanilines may be arranged in the following order,

$$2,3- > 3,4- \geq 2,5- \quad \text{and} \quad 2,6- > 2,4\text{-dinitroaniline.}$$

Assuming the chain mechanism postulated for the reaction between 4,9-dinitro-3-aminoretene and hydrochloric acid [2] the formation of the diazonium salts is probably due to the following series of reactions

Initiation:	$C_6H_3(NO_2)_2NH_3^+$	$\xrightarrow{HCl}$	$C_6H_3(NO_2)ClNH_3^+$	$+ HNO_2$	(1)
Propagation:	$C_6H_3(NO_2)_2NH_3^+$	$\xrightarrow{HNO_2}$	$C_6H_3(NO_2)_2N_2^+$		(2)
	$C_6H_3(NO_2)_2N_2^+$	$\xrightarrow{HCl}$	$C_6H_3(NO_2)ClN_2^+$	$+ HNO_2$	(3)
	$C_6H_3(NO_2)ClN_2^+$	$\xrightarrow{HCl}$	$C_6H_3Cl_2N_2^+$	$+ HNO_2$	(4)
Termination:	$C_6H_3(NO_2)ClNH_3^+$	$\xrightarrow{HNO_2}$	$C_6H_3(NO_2)ClN_2^+$		(5)
	$C_6H_3(NO_2)_2N_2^+$	$\xrightarrow{HCl}$	$C_6H_3(NO_2)_2Cl$	$+ N_2$	(6)

* Part II. *Acta Chem. Scand.* 7 (1953) 790.

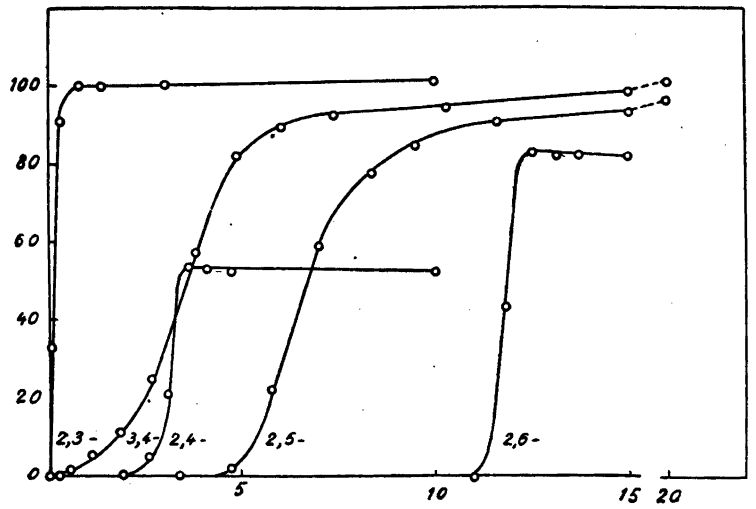
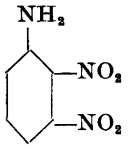
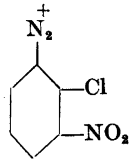
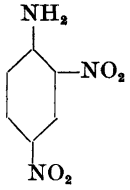
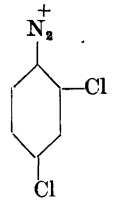
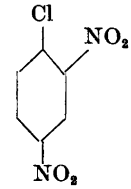
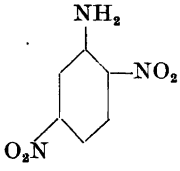
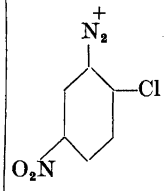
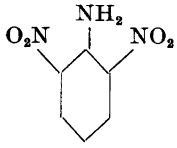
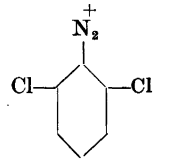
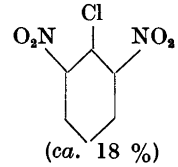
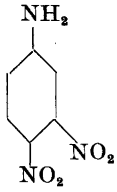
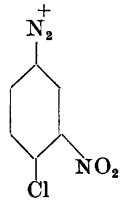
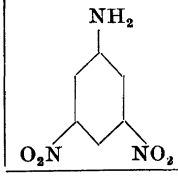


Fig. 1. Relation between yields of diazonium salts in per cent of the theoretical (ordinate) and time of reaction in hours (abscissa).

The three most reactive dinitroanilines (2,3-, 2,5-, and 3,4-) gave quantitative yields of the respective chloronitrobenzene diazonium chlorides. These are fairly stable and the second nitrogroup (situated in *m*-position to the diazonium-group) is not replaced by chlorine. The process takes place *via* reactions (1), (2), (3), and (5). The other isomers (2,4- and 2,6-) are transformed to the relatively stable dichlorobenzene diazonium chlorides and to chlorodinitrobenzenes. It is clear that reactions (4) and (6) also obtain for the latter transformations. The maxima of the curves for the reactions of these two dinitroanilines (see Fig. 1) represent the amounts of dichlorobenzene diazonium chlorides present in the reaction solutions. These dichloro-compounds are formed in yields of more than fifty per cent; thus the amount of nitrous acid produced exceeds that required for the diazotisation of the amino-group and nitric oxide is evolved. The amount of 2,4- and 2,6-dinitrobenzene diazonium chloride which decomposes during the reaction (with formation of chlorodinitrobenzene) has also been determined. For both isomers the sum of stable and unstable diazonium salts accounted for the total amount of the dinitroaniline consumed. The results are listed in Table 1. The methods used for the identification and quantitative determination of the reaction products are given in the experimental section.

The investigation shows that only nitro-groups in *o*- or *p*-position to the amino-group are displaced. The different reactivity of 2,3- and 3,4-dinitroaniline indicates that a nitro-group in *o*-position to the amino-group is more reactive than one in the *p*-position. Comparison of the reactivities of 2,3-, 2,4- and 2,5-dinitroaniline shows that the reacting nitro-group is more activated by an adjacent nitro-group than by a *p*-nitro-group. The latter in turn has a greater influence than a nitro-group in *m*-position.

Table 1.

Dinitro-aniline	Temp. °C	Time hours	Reaction products
	40	1	 (*ca.* 100 %)
	80	4	 and (*ca.* 52 %)  (*ca.* 48 %)
	40	20	 (*ca.* 100 %)
	40	16	 and (*ca.* 82 %)  (*ca.* 18 %)
	40	12	 (*ca.* 100 %)
	100	2	Unchanged material

In agreement with the assumed reaction mechanism, the reaction between dinitroanilines and hydrochloric acid was found to be catalyzed by small amounts of nitrite and retarded by compounds which rapidly consume the diazotising agent formed in the initiating reaction. It was also found that when slightly impure dinitroanilines were used a longer period of time elapsed before detectable amounts of diazonium salts appeared in the solution. However, once started, the diazotisation was generally not influenced by small amounts of impurities. It is probable that the diazotising agent is removed initially by interaction with the impurities and diazotisation of the dinitroaniline does not begin until they are consumed.

To show the velocity of the initiating reaction, the most reactive dinitroaniline (2,3-) was heated to 80 °C for 15 hours with addition of an easily diazotisable amine which rapidly removes the diazotising agent formed. The resulting reaction solution contained no stable diazonium salts and most of the dinitroaniline remained unchanged. Attempts to isolate 2-chloro-3-nitroaniline formed in the initiating reaction failed and, consequently, the initiating reaction must be very slow. By comparison 4,9-dinitro-3-aminoretene reacts quantitatively within 15 minutes under the same conditions [2].

Since the initiating reaction is very slow even for the most reactive dinitroaniline, it may be expected that when a new electron repelling substituent is introduced, no reaction will occur under the preceding conditions. On the other hand, the nitro-groups should be more reactive after the introduction of an electron attracting substituent and as a matter of fact 5-chloro-2,4-dinitroaniline, recently investigated by Dey, Krishna Maller and Pai [3], is more reactive than 2,4-dinitroaniline.

All these experiments were carried out with hydrochloric acid; in the presence of hydrobromic acid the dinitroanilines did not yield detectable amounts of diazonium salts but remained essentially unchanged. Various amounts of hydrobromic acid were used at temperatures varying from 15 to 100 °C and attempts were also made to initiate the reaction by the addition of sodium nitrite. An experiment carried out with 2,3-dinitroaniline at 60 °C showed that on the addition of a very small amount of nitrite (*ca.* 0.5 % of the theoretical) a chain reaction was started whereby dinitroaniline was converted to 2,3-dibromonitrobenzene. This reaction soon ceased, however, but could be started again with nitrite. After the addition of ten consecutive portions of nitrite (5 % of the theoretical amount) all the dinitroaniline had reacted. Experiments carried out at different temperatures showed that the higher the temperature the larger the amount of nitrite required for complete diazotisation. The termination of the reaction may be due to liberation of bromine by reaction of hydrobromic with nitrous acid. Indeed a mixture of these two acids can be used as a brominating agent. Thus Milton and Reade [4] have used such a mixture for bromination of *p*-nitrodimethylaniline, and following a method described by them the presence of free bromine could be demonstrated in a mixture of glacial acetic, hydrobromic, and nitrous acids.

Although the reaction between hydrobromic and nitrous acids undoubtedly is partly responsible for the termination, the greater instability of the diazo-

* Part II. *Acta Chem. Scand.* **7** (1953) 790.

nium ion in hydrobromic acid solution may also be a factor of importance. Assuming that the initiating reaction is also slow when hydrobromic acid is used, termination will occur though only a small fraction of the dinitrobenzene diazonium ion decomposes before the reactive nitro-group is displaced by bromine. Although no compounds, the presence of which would prove that termination takes place in this way, could be isolated, this possibility can not be excluded. It may be remembered that with hydrochloric acid, 2,4-dinitroaniline yields 4-chloro-1,3-dinitrobenzene (*ca.* 48 %) and 2,4-dichlorobenzene diazonium chloride (*ca.* 52 %). If more than 50 % of 2,4-dinitrobenzene diazonium chloride were converted to chlorodinitrobenzene, termination should have taken place also in this case.

As mentioned above, 2,3-dinitroaniline is quantitatively diazotised in the presence of hydrobromic acid by an amount of nitrite corresponding to *ca.* 5 % of the theoretical (the reaction product being 2,3-dibromonitrobenzene). If the initiating reaction takes place at all when hydrobromic acid is used, it might be expected that a certain amount of 2,3-dibromonitrobenzene would also be formed when the dinitroaniline is heated with glacial acetic and hydrobromic acids for a long time, in spite of the rapid termination of the expected chain reaction under these conditions. An experiment carried out at 100—110 °C for 15 hours showed that 2,3-dibromonitrobenzene is indeed formed (yield about 15 %), and unchanged dinitroaniline could also be isolated.

Dey *et al.*[3] in an investigation of the reaction between hydrochloric acid and 5-chloro-2,4-dinitroaniline found that this compound "splits off its nitro-groups on merely heating its solution in a mixture of glacial acetic and hydrochloric acids to 100 °C. Nitrous fumes were evolved with effervescence and this was observed to continue even after the heating was discontinued. In the resulting solution the presence of both 1,2,4,5-tetrachlorobenzene and 2,4,5-trichloroaniline were detected. It is therefore seen that mere heating of the compound is enough to eliminate both nitro-groups and replace them by chlorine, thereby giving rise to 2,4,5-trichloroaniline which in its turn is partly diazotised by the nitrous acid originating from the displaced nitro-groups, the resulting diazonium group being replaced by chlorine to yield 1,2,4,5-tetrachlorobenzene". The assumption that 2,4,5-trichloroaniline should be so easily formed under these conditions is very improbable and it is unlikely that this compound is only partly diazotised when nitrous acid is present in excess. The reaction mechanism postulated for 2,4-dinitroaniline (see p. 1197) seems to be more probable, *i. e.* the reaction takes place *via* 5-chloro-2,4-dinitrobenzene diazonium chloride. Part of the diazonium compound then gives rise to 2,4,5-trichlorobenzene diazonium chloride and another part to 1,5-dichloro-2,4-dinitrobenzene. Repetition of the experiment of Dey *et al.* afforded only these compounds; no trichloroaniline could be detected. Dey *et al.* obtained the latter compound after steam distillation of the reaction solution and treatment of the distillate with tin and hydrochloric acid. It is possible that the trichloroaniline was formed from 2,4,5-trichloronitrobenzene, although this derivative could not be found in the reaction solution by the present author.

In the first paper of this series [1] it was pointed out that acetic or other carboxylic acids are the only reaction media in which the reaction between halogen acids and nitro-amines takes place. In the case of the dinitroanilines,

however, it was found that the reaction also occurs in an aqueous hydrochloric acid solution. However, the reaction does not proceed as smoothly as in glacial acetic acid solution and 2,4-dinitroaniline remained unchanged even after heating to 100 °C for 15 hours in 2 *M* hydrochloric acid.

EXPERIMENTAL *

Preparation of the nitroanilines

The *mononitroanilines* were all commercially available in high purity and were not further purified.

2,4-Dinitroaniline. A commercial product was purified by crystallization from ethanol and from glacial acetic acid to m.p. 181—182 °C.

2,6-Dinitroaniline was prepared by the method described in Org. Syntheses [5]. The crude 2,6-dinitroaniline, crystallized twice from ethanol (charcoal), gave m.p. 141—142 °C.

3,5-Dinitroaniline was prepared by reducing 1,3,5-trinitrobenzene with ammonium sulphide by the method of Flürscheim [6]. The crude product was crystallized twice from water (charcoal) to m.p. 162—163 °C.

The syntheses of *2,3-*, *2,5-*, and *3,4-dinitroaniline* chiefly followed the method of van de Vliet [7]. In agreement with his description the mixture of the three isomeric dinitroacetanilides obtained by nitration of *m*-nitroacetanilide was separated into three fractions (I—III) from which the three dinitroanilines were prepared.

Fraction I consisting of pure 2,3-dinitroacetanilide was hydrolysed with conc. sulphuric acid at 110 °C. On crystallization from benzene pure 2,3-dinitroaniline, m.p. 126—127 °C, was obtained.

Fraction II, which van de Vliet reported to consist of almost pure 3,4-dinitroacetanilide, was hydrolysed as above. A sample of the hydrolysed product was chromatographed (Al_2O_3, benzene) and found to contain 2,3-dinitroaniline (3 %), 2,5-dinitroaniline (17 %), and 3,4-dinitroaniline (72 %). The main part of the hydrolysed product was crystallized from benzene giving pure 3,4-dinitroaniline, m.p. 157—158 °C.

Fraction III reported to be an eutectic mixture of 2,3- and 2,5-dinitroacetanilide was hydrolysed as above. After repeated extraction of the reaction product with hot water van de Vliet obtained a residue (60 %) consisting of rather pure 2,5-dinitroaniline. As only a small amount of material was required for the present investigation, a chromatographic separation (Al_2O_3, benzene) was preferred. In this way pure 2,5-dinitroaniline, m.p. 138—139 °C, was obtained from fraction III in a yield of about 70 %.

The reaction between hydrochloric acid and the mono- and dinitroanilines

o-Nitroaniline (0.50 g) was suspended in a mixture of glacial acetic (10 ml) and conc. hydrochloric acids (2 ml) and heated in a sealed glass tube for 2 hours at 100 °C. After dilution with water and neutralization with sodium carbonate unchanged starting material precipitated and was collected and washed with water. It was obtained in a yield of 0.35 g, m.p. 71—72 °C, undepressed on admixture with *o*-nitroaniline.

m-Nitroaniline (0.50 g) was treated in the way described above. Unchanged starting material (0.38 g), m.p. 114—115 °C, undepressed on admixture with *m*-nitroaniline, was obtained.

p-Nitroaniline (0.50 g) was treated in the same way and yielded unchanged starting material (0.41 g), m.p. 150—151 °C, undepressed on admixture with *p*-nitroaniline.

3,5-Dinitroaniline (0.50 g) was treated as described above, but after neutralization the reaction solution was extracted with ether. The residue (0.50 g, m.p. 143—148 °C) obtained on evaporation of the ether, was suspended in a small volume of ether. The insoluble material (0.03 g) which was collected, had m.p. 184—186 °C, undepressed on

* All melting points are corrected.

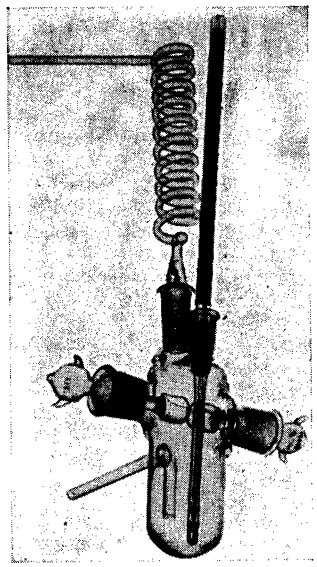


Fig. 2. Apparatus, used for quantitative determination of the diazonium salts.

admixture with 3,5-dinitroacetanilide. The soluble product melted at 156—157 °C, undepressed on admixture with 3,5-dinitroaniline.

On treatment with hydrochloric acid *2,3-*, *2,4-*, *2,5-*, *2,6-*, and *3,4-dinitroaniline* were quantitatively consumed and diazonium salts were present in the reaction solution. The following method was used to determine the reactivity of these dinitroanilines.

A sample of the dinitroaniline (1 g) was accurately weighed into a ground-stoppered Erlenmeyer flask and glacial acetic acid (40 ml) added. The flask was placed in a water bath (40 °C ± 0.2 °C) and the clear solution mixed with warm (40 °C) 12 *M* hydrochloric acid (8 ml). In the case of 2,4-dinitroaniline no reaction occurred at 40 °C even after 24 hours. This isomer was therefore allowed to react at 80 °C ± 1 °C whereupon diazonium salts appeared after *ca.* 2 hours. At suitable intervals samples (4 ml) were pipetted into the apparatus used for quantitative determination of the amount of diazonium salt (see Fig. 2). The apparatus was immersed in a water bath at 40° or 80 °C. When the air had been expelled with carbon dioxide, the diazonium salts were decomposed by the addition of cuprous oxide from one of the small containers and the nitrogen evolved was collected in an azotometer containing 50 % aqueous potassium hydroxide. From the amount of nitrogen, the yield of diazonium salts in each sample was calculated. In the curves of Fig. 1 the yields of diazonium salts are plotted against the time of reaction. The first point of each curve indicates the time when the presence of diazonium salts could just be demonstrated (reaction with 3-retenol).

The method described above allows a determination every 15 minutes. In the case of the most reactive isomer, 2,3-dinitroaniline, in order to make a determination after a shorter period of time, the following procedure was used.

A sample of 2,3-dinitroaniline (0.1 g) was weighed into the apparatus and dissolved in glacial acetic acid (4 ml). When the air had been expelled, 12 *M* hydrochloric acid (0.8 ml) was added from the one container and 4 minutes later cuprous oxide was added from the other. The amount of nitrogen was collected in the usual way.

In the investigation of 2,4- and 2,6-dinitroaniline the nitrogen collected in the azotometer was found to contain nitric oxide. In the first experiments the nitric oxide was removed by washing the nitrogen with an alkaline solution of potassium permanganate. However, it was found that the nitric oxide was more conveniently removed if the nitro-

gen (after washing with aqueous sodium carbonate) was passed through a hot (190 °C) tube charged with lead dioxide, before being collected in the azotometer.

The amount of relatively unstable diazonium salt formed when 2,4- and 2,6-dinitroaniline is treated with hydrochloric acid as described above was determined in the following way. As soon as diazonium salts could be detected in the reaction solution an aliquot of this solution was pipetted into the apparatus (Fig. 2) which had been cooled to 5—10 °C to avoid loss of hydrogen chloride while the air was expelled with carbon dioxide. The reaction was then allowed to proceed for 5 hours at the normal reaction temperature. Before entering the azotometer the gases evolved were passed over hot lead dioxide. The amount of diazonium salt which decomposed during the reaction was calculated from the amount of nitrogen collected and was found to be 49 % for 2,4-dinitroaniline and 19 % for 2,6-dinitroaniline.

The products formed when the reactive dinitroanilines were treated with hydrochloric acid were identified in the following way.

2,3-Dinitroaniline (0.50 g) was dissolved in glacial acetic acid (20 ml) and the solution was mixed with conc. hydrochloric acid (4 ml) and kept at 40 °C. After 1 hour the diazonium salt formed was decomposed by adding the solution to a hot (*ca.* 70 °C) solution of cuprous chloride (4 g) in conc. hydrochloric acid (40 ml). The reaction product was precipitated by addition of water (250 ml), collected, washed with water and dried (yield 0.35 g, 67 %). It melted at 60—61 °C, undepressed on admixture with 2,3-dichloronitrobenzene.

The experiment was repeated in the presence of *p*-chloroaniline. 2,3-Dinitroaniline (0.92 g) and *p*-chloroaniline (1.28 g) were dissolved in glacial acetic acid (40 ml). The solution was mixed with conc. hydrochloric acid (8 ml) and heated to 80 °C for 15 hours. The reaction solution was diluted with water, neutralized with sodium carbonate solution and extracted with ether. The ether solution was washed with water and dried. *p*-Chloroaniline was precipitated as its hydrochloride by the introduction of hydrogen chloride. The ether solution was washed free from hydrogen chloride and the ether evaporated. The residue was crystallized from water (200 ml) yielding yellow crystals (0.70 g), m.p. 126—127 °C, undepressed on admixture with 2,3-dinitroaniline. The mother liquor was extracted with ether yielding a crystalline product (0.10 g) which after crystallization from benzene had m.p. 182—184 °C, undepressed on admixture with 2,3-dinitroacetanilide.

2,4-Dinitroaniline (3.0 g) was dissolved in glacial acetic acid (120 ml) and the solution was mixed with conc. hydrochloric acid (24 ml) and kept at 80 °C for 5 hours. After cooling to room temperature the reaction solution was diluted with water (800 ml), extracted with ether, mixed with 50 % hypophosphorous acid (50 ml), kept at room temperature for 5 hours and again extracted with ether. Both ether solutions were washed with aqueous sodium carbonate and water, dried over Na_2SO_4 and decolourized with a little charcoal. The ether was then evaporated.

The first solution gave an oil (1.3 g) which soon solidified. After trituration with light petroleum light-yellow crystals (1.1 g, 33 %), m.p. 49—51 °C, undepressed on admixture with 4-chloro-1,3-dinitrobenzene, were obtained.

The second ether solution yielded a light-yellow liquid (0.9 g, 37 %), $n_D^{20°} = 1.538$. It distilled almost quantitatively in a vacuum (10—12 mm. Hg). The colourless distillate ($n_D^{20°} = 1.546$) crystallized on cooling and melted at *ca.* —25 °C, undepressed on admixture with *m*-dichlorobenzene.

2,5-Dinitroaniline (0.50 g) was dissolved in glacial acetic acid (20 ml), conc. hydrochloric acid (4 ml) was added and the solution was heated to 40 °C for 20 hours. The solution was then worked up as described for 2,3-dinitroaniline. 3,4-Dichloronitrobenzene, obtained in a yield of 0.38 g (72 %), had m.p. 40—42 °C, undepressed on admixture with an authentic sample.

2,6-Dinitroaniline (3.0 g) was dissolved in glacial acetic acid (120 ml), conc. hydrochloric acid (24 ml) was added and the solution heated to 40 °C. After 11 hours (the time varies considerably with the purity of the dinitroaniline) diazonium salts could be detected in the reaction solution. After another 5 hours the solution was worked up as described for 2,4-dinitroaniline.

From the first ether solution, obtained by extraction of the diluted reaction solution, light-yellow crystals (0.53 g, 16 %), m.p. 86—87° C, were obtained. [Calc. for $C_6H_3O_4N_2Cl$

(202.6): C 35.6; H 1.49. Found: C 35.9; H 1.65.] The product was aminated by heating a solution in alcoholic ammonia in a sealed tube to 115—120 °C for 3 hours (*cf.* the amination of 2-chloro-1,3-dinitrobenzene by Borsche and Rantscheff [8]). Pure 2,6-dinitroaniline was obtained, proving the extracted compound to be 2-chloro-1,3-dinitrobenzene.

The second ether solution yielded a light-yellow liquid (1.65 g, 68 %), $n_D^{20°} = 1.542$, which on cooling gave crystals, m.p. *ca.* —26 °C, undepressed on admixture with *m*-dichlorobenzene.

3,4-Dinitroaniline (0.50 g) was heated to 40 °C in a mixture of glacial acetic (20 ml) and conc. hydrochloric acids (4 ml). After 12 hours the reaction solution was worked up as described for 2,3-dinitroaniline. The crude product, m.p. 47—50 °C, obtained in a yield of 0.35 g (67 %), was recrystallized from ethanol to m.p. 54—55 °C, undepressed on admixture with 2,5-dichloronitrobenzene.

The reaction between hydrobromic acid and 2,3-dinitroaniline

2,3-Dinitroaniline (0.50 g) was dissolved in a mixture of glacial acetic (20 ml) and 40 % hydrobromic acids (4 ml). An aqueous solution of sodium nitrite at 60 °C was added to the mixture in portions containing about 0.5 % of the amount theoretically required. After the first addition diazonium salt could be detected in the reaction solution for 20—25 minutes after which time all the diazonium salt had decomposed. After another addition of nitrite more diazonium salt was formed but again decomposed within 20—25 minutes. This procedure was repeated until (after the eleventh addition) no diazonium salt was formed. This means that all the dinitroaniline was diazotised by an amount of nitrite corresponding to *ca.* 5 % of the theoretical. The reaction solution was then diluted with water and neutralized. The precipitate was collected, washed with water and dried. The crude product (0.60 g, 78 %; m.p. 77—79 °C) was crystallized from ethanol yielding light-yellow crystals, m.p. 85—86 °C, undepressed on admixture with 2,3-dibromonitrobenzene.

In another experiment 2,3-dinitroaniline (0.50 g) was heated to 100—110 °C in a sealed tube in a mixture of glacial acetic (20 ml) and 40 % hydrobromic acids (4 ml). After 15 hours the reaction solution was diluted with water, neutralized, and extracted with ether. The ether solution was washed with water, dried and the ether evaporated. The residue (0.45 g) so obtained yielded on steam distillation a light-yellow crystalline product (0.15 g, m.p. 60—65 °C). One crystallization from ligroin gave m.p. 82—83 °C, undepressed on admixture with, 2,3-dibromonitrobenzene. The residue from the distillation was recrystallized three times from benzene giving yellow crystals, m.p. 122—123 °C, undepressed on admixture with 2,3-dinitroaniline.

The reaction between hydrobromic and nitrous acids

Air was aspirated for 40 minutes through a cool (0—5 °C) mixture of glacial acetic (40 ml), propionic (5 ml) and 40 % hydrobromic acids (10 ml) into an acidified silver nitrate solution. A hardly detectable opalescence appeared in the silver nitrate solution. After the addition of sodium nitrite (30 mg), dissolved in a small volume of water, silver bromide precipitated within 5 minutes.

The reaction between hydrochloric acid and 5-chloro-2,4-dinitroaniline

5-Chloro-2,4-dinitroaniline (1.0 g) was suspended in a mixture of glacial acetic (10 ml) and conc. hydrochloric acids (10 ml) and heated. At 70 °C all the chlorodinitroaniline dissolved with formation of diazonium salts and the reaction solution turned reddish-brown. The solution was heated to 95—100 °C and then allowed to cool. After dilution with water (100 ml) it was extracted with ether, mixed with 50 % hypophosphorous acid (20 ml), kept at room temperature for 1 hour and then again extracted with ether. Both ether solutions were washed with aqueous sodium carbonate and water, dried over Na_2SO_4 and decolourized with a little charcoal. The ether was then evaporated.

The first ether solution gave a crystalline product (0.10 g) melting at 75—80 °C. After one crystallization from ligroin pure 1,5-dichloro-2,4-dinitrobenzene (0.07 g, *ca.* 6 %), m.p. 101—102 °C, undepressed on admixture with an authentic sample, was obtained.

The second ether solution yielded a light-yellow oil (0.50 g, 60 %; $n_D^{20°} = 1.568$) which on cooling gave crystals, m.p. 15—16 °C, undepressed on admixture with 1,2,4-trichlorobenzene.

The reaction solution was neutralized and again extracted with ether. No residue was obtained by evaporation of the ether.

SUMMARY

The reaction between hydrochloric acid and the mono- and dinitroanilines has been investigated and discussed. On treatment with hydrochloric acid 2,3-, 2,5-, and 3,4-dinitroaniline are quantitatively converted to chloronitrobenzene diazonium chlorides; 2,4- and 2,6-dinitroaniline yield dichlorobenzene diazonium chloride and chlorodinitrobenzene. The mononitroanilines and 3,5-dinitroaniline remain unchanged.

5-Chloro-2,4-dinitroaniline was found to react in the same way as the above dinitroanilines rather than by the mechanism assumed by Dey, Krishna Maller, and Pai.

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The Biogenesis of Orotic Acid in Liver Slices

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The biological existence of orotic acid was first demonstrated by Biscaro and Belloni [1], who isolated this substance from cow's milk in 1905. Orotic acid is the only pyrimidine carboxylic acid known to exist in nature and its possible significance has at different times evoked speculations. The finding of Loring and Pierce [2] and Rogers [3] that uracil can be replaced by orotic acid as a growth stimulant for certain bacteria suggested a connection between this acid and nucleic acid pyrimidine biogenesis. Mitchell and coworkers [4,5] extensively studied the function of orotic acid in the pyrimidine metabolism of different mutants of *Neurospora*. From genetic evidence Mitchell, Houlahan and Nyc [5] came to the conclusion that orotic acid is formed as a byproduct in a side reaction during pyrimidine synthesis in *Neurospora* and thus is not a normal intermediate in pyrimidine biogenesis. Instead it was proposed that pyrimidines are formed from oxaloacetic acid via aminofumaric acid, and that ribosidation of an acyclic pyrimidine precursor precedes ringclosure [4].

Interest in the metabolism of orotic acid was greatly stimulated by the finding of Arvidson *et al.*[6] that 3-N^{15}-orotic acid is utilized by the rat for the synthesis of pentose nucleic acid (PNA) pyrimidines. Even though no degradation of the pyrimidines was carried out the low dilution of the isotope seemed to justify the conclusion that the whole molecule of orotic acid was incorporated. Confirmation of that has come from the finding in different laboratories that orotic acids labeled in either the 2 or 6 positions are equally well utilized for pyrimidine synthesis by a great variety of different organisms [7–9].

The incorporation of orotic acid into polynucleotide pyrimidines *in vitro* has been demonstrated by Weed *et al.*[10] and Reichard and Bergström [11]. Our interest in the *in vitro* system arose from the hope to be able to decide if orotic acid is a "normal" intermediate in pyrimidine biogenesis. Reichard has shown that the presence of a pool of orotic acid greatly diminishes the incorporation of isotope from $N^{15}H_4Cl$ into uridine in liver slices [12]. Furthermore it was found that the reisolated orotic acid at the end of the experiment contained a high label of N^{15}. These findings agreed with the hypothesis that orotic acid is a direct intermediate in pyrimidine biogenesis.

The finding of a *de novo* synthesis of orotic acid in liver slices which was about ten times more rapid than the simultaneous synthesis of PNA pyri-

midines, made the liver slice system a very valuable tool for further investigations of pyrimidine biogenesis. In the present investigation the synthesis of orotic acid from various labeled precursors has been investigated. It is thought that this synthesis reflects the more general pathways of pyrimidine biogenesis in the rat.

Two experimental approaches were chosen. The first one consisted of incubation of the suspected labeled precursor together with unlabeled orotic acid and liver slices. After incubation orotic acid was reisolated from the medium and the distribution of the isotope within the molecule determined after a partial degradation.

The second approach consisted of the incubation of the suspected nonlabeled precursor in the liver slices together with $N^{15}H_4Cl$ and nonlabeled orotic acid. The effect of the presence of the nonlabeled substance on the incorporation and distribution of N^{15} in orotic acid after incubation was studied.

By a combination of these two methods it was possible to demonstrate that aspartic acid was a precursor of N_3 plus C_4, C_5, C_6 and C_7 of orotic acid and CO_2 a precursor of C_2. Ureidosuccinic acid was an intermediate in the synthesis of orotic acid.

EXPERIMENTAL

Incubation. Slices from nonregenerating livers were used in the present investigation. The livers were obtained from young albino rats weighing between 150 and 200 g. Slices from three livers in 50 ml of a Krebs-Henseleit substrate [13] + 15 mg non-labeled orotic acid + 0.1 m*M* − 2 m*M* of the different labeled or non-labeled precursors were incubated in a 300 ml Erlenmeyer flask from 80 minutes to 8 hours. When more material was needed in one experiment the contents of several flasks were combined after incubation. Details of the procedure and of the isolation of orotic acid after incubation have been described earlier [12].

Synthesis of isotopic compounds

N^{15}-L-*aspartic acid* (33 % excess N^{15}) was synthesized according to the method of Wu and Rittenberg [14].

1,4-C^{13}-L-*aspartic acid* (35 % excess) was synthesized from $KC^{13}N$, *via* succinic and fumaric acids [15]. The fumaric acid was converted to aspartic acid according to Wu and Rittenberg, though an excess NH_4Cl was used in the synthesis (2 g NH_4Cl per 1.2 g fumaric acid).

2,3-C^{14}-L-*aspartic acid* (8 100 c/min) was synthesized from C^{14}-acetylene, *via* ethylene, dibromoethylene, succinic acid and fumaric acid [15,16].

N^{15}-L-*ureidosuccinic acid* was synthesized from N^{15}-L-aspartic acid by condensation with KCNO [17]. It was found that the L-isomer unlike the racemic form of ureidosuccinic acid did not crystallize from the reaction mixture after acidification. Because of that a procedure had to be worked out for its isolation. The alkaline solution from condensation of 0.8 g N^{15}-L-aspartic acid with 1.2 g KCNO was adjusted to pH 4 − 5 by addition of small amounts of Dowex-50(H^+-form, 200 − 400 mesh). After filtration the combined filtrate and washings were brought to pH 10 − 11 and chromatographed on Dowex-2(Cl-form, 200 − 500 mesh), column 10 × 4 cm. Elution was first carried out with 1 400 ml of 0.005 *N* HCl, followed by 0.05 *N* HCl. The ureidosuccinic acid was completely eluted by 600 ml of 0.05 *N* HCl. The ureidosuccinic acid was localized in the chromatogram by a modified ninhydrine reaction [18]. For this purpose 0.2 ml of each chromatographic fraction was transferred into a test tube and after addition of 0.5 ml *N* H_2SO_4 was kept in an oven at 100° over night. After exact neutralisation with *N* NaOH a buffered ninhydrin solution was added and a violet colour developed after 10 minutes at 100°, when ureidosuccinic acid was present.

The solvent was evaporated *in vacuo* from the combined fractions containing ureidosuccinic acid. Great care was taken to ensure low temperature during the procedure. The ureidosuccinic acid was obtained as an oil. It was dissolved in 10 ml of water. A hot saturated solution of barium hydroxide was added to pH 8—9, and the barium salt of the acid was precipitated by addition of 4 volumes of alcohol. After filtration it was dissolved in a small amount of warm water. Alcohol was added to incipient crystallization, and crystallization was completed in the ice box over night. A first crop of 660 mg of a white crystalline powder was obtained. By further addition of alcohol a second crop of 230 mg was obtained. Isotope analysis showed the presence of 16.7 atom per cent excess N^{15} (calc. 16.5). The substance contained 9.30 % N (calc. * 9.10). $[\alpha]_D^{25} = +24.1°$ (c = 3 % in water). The substance did not melt below 300° C.

Immediately before use in an experiment the barium salt was converted to the free acid by dropwise addition of 0.1 *N* sulfuric acid to a water solution used directly in the experiment.

Reisolation of ureidosuccinic acid

In some experiments incubation with $N^{15}H_4Cl$ was carried out in the presence of a non-labeled hypothetical precursor. It was sometimes desirable to investigate whether the added substance had received some isotope at the end of the experiment. This was done with D,L-ureidosuccinic acid which was reisolated by a carrier technique from the same ion exchange column (Dowex-2, Cl^--form) used for the reisolation of orotic acid [12]. The effluent preceding the orotic acid peak was collected in three fractions and each fraction was treated separately. After evaporation of the HCl *in vacuo* 100 mg of non-labeled D,L-ureidosuccinic acid was added to each fraction and the mixture dissolved in a small amount of hot water. On standing in the ice box ureidosuccinic acid crystallized. A small part was analyzed for N^{15}, the rest was recrystallized twice and analyzed each time for N^{15}. Isotopic excess was found in the ureidosuccinic acid of only the last fraction, obtained from the 100 ml which immediately preceded the orotic acid on the column.

Degradation of orotic acid

The partial degradation of orotic acid was carried out by a method similar to that used by Lagerkvist [19] for the degradation of uracil. Orotic acid was thus hydrogenated with chloroplatinic acid as catalyst. Between 10 and 30 mg of orotic acid was dissolved by warming in 10 ml of a 0.5 % freshly prepared solution of gum arabic. Then 0.3 ml of a 10 % solution of catalyst in water was added and reduction carried out at 80° in a hydrogen atmosphere (pressure = 3 atmospheres) for one day. After that an aliquot was removed and the light absorption at 280 mμ was determined after proper dilution. Unless the light absorption was below 5 % of the original, another 0.3 ml of 10 % chloroplatinic acid solution was added and the reduction repeated. If necessary the process was repeated again.

When the light absorption at 280 mμ had reached a low enough value, the solution was centrifuged and the precipitate washed twice with a few ml of warm water. The combined supernatants were evaporated to dryness *in vacuo*. The residue was transferred to a bombtube with a total of 5 ml of concentrated HCl and hydrolyzed at 100° for 24 hours. After that the bomb tube was opened and the contents were centrifuged. The precipitate was twice washed with a few ml of warm water. The combined supernatants were evaporated *in vacuo*. The residue was dissolved in a few ml of water and transferred to the top of a Dowex-50 column (H^+-form, 200—400 mesh, 15 × 0.9 cm). Elution was carried out with 0.5 *N* HCl, the products were localized by treatment with ninhydrin as described by Moore and Stein [18]. Two peaks were observed in this way. The position of the first one corresponded to aspartic acid, the second one to ammonia. When the substance appearing in the first peak was mixed with 5 mg of aspartic acid and rechromatographed on a starch column with propanol — 0.5 *N* HCl, 2 : 1, as described by Moore and Stein [20] one single peak corresponding to aspartic acid was obtained. Furthermore, the same sub-

* Consideration was taken to the presence of N^{15} in the compound.

stance could be transformed to its crystalline copper salt immediately after Dowex-50 chromatography (see below). The evidence that this substance is aspartic acid seems us to be conclusive. The identity of the second substance with ammonia was established in the following way: After removal of solvent, the substance was dissolved in a small amount of water and distilled into 0.01 *N* HCl in a Kjeldahl apparatus after addition of borate buffer (pH = 10.5). All nitrogen could be distilled in this way. When alkaline hypobromite was added to the solution for mass spectrometer analysis gaseous nitrogen was obtained showing the original presence of ammonia.

The aspartic acid from the first peak was purified as its copper salt. For this purpose the solvent from the combined fractions was carefully removed by repeated evaporation *in vacuo*. The dry residue was transferred to a small centrifuge tube with a minimum amount of water (1—1.5 ml), and neutralized to pH 7—8 by addition of a few drops of *N* NaOH. A saturated solution of $CuSO_4$ (0.4 ml) was added. The pH of the solution was adjusted to 5—6 by dropwise addition of 0.2 *N* NaOH. Crystallization was allowed to take place in the ice box for 2—3 days. The crystals were then centrifuged and washed twice with 0.5 ml of ice cold water. The yield of aspartic acid after crystallization was about 40 % calculated on orotic acid. When the degradation was carried out in a N^{15} experiment the copper aspartate and the ammonia from the second peak were directly analyzed for N^{15}.

When isotopic carbon was used the copper aspartate was further degraded with ninhydrin. The copper aspartate was suspended in 5 ml hot water and treated with H_2S for 15 min. The CuS was centrifuged and the precipitate washed twice with a few ml hot water. The combined supernatants were transferred to a small flask and 100 mg citrate buffer pH 2.5 was added. A stream of nitrogen was passed through the solution for 5 minutes while it was warmed on a boiling water bath. After cooling 100 mg of ninhydrin was added and the solution was heated to 100° C for 15 min. During this periode nitrogen was passed through the solution and the CO_2 collected as $BaCO_3$.

The reliability of the method was tested by the degradation of 3-N^{15}-orotic acid. The results are summarized in Table 1 and show that the nitrogen of the copper aspartate was representative of N_3 of the orotic acid. Furthermore this must mean that the carbon dioxide obtained by ninhydrin decarboxylation of the copper aspartate represents C_6+C_7 of orotic acid. The results in Table 1 also show that ammonia is not derived to any significant amount from N_3. We do not feel that ammonia accurately represents N_1 during our experimental conditions, as no special care was taken to avoid the introduction of ammonia from the air, gum arabic, *etc.* However, the discrepancy between the isotope values obtained as ammonia and those calculated by difference are comparatively small. Therefore both values are given in the tables.

A diagram of the degradation is given in Fig. 1.

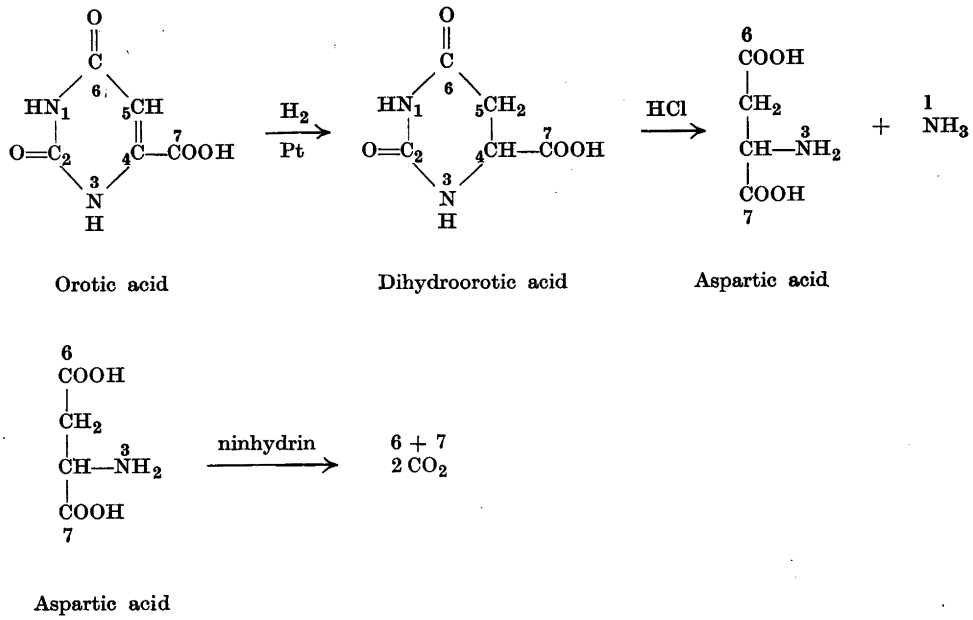
C_2 of orotic acid was obtained by the following degradation. 15—25 mg of orotic acid was dissolved in 3 ml of water by dropwise addition of 0.5 *N* NaOH and warming. A 5 % solution of $KMnO_4$ was added dropwise until the permanganate colour persisted [1]. Precipitation of MnO_2 was facilitated by addition of a few drops of *N* H_2SO_4. The remain-

Table 1. Degradation of 3-N^{15}-*orotic acid.*

Exp. number	Orotic acid	atom % excess N^{15} in N_1^a	N_1^b	N_3
16 [c]	0.415	0.03	0.00	0.83
17 [d]	0.415	0.03		0.74

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.
[c] The aspartic acid was crystallized as its copper salt.
[d] The aspartic acid was used directly after chromatography on Dowex-50.

Fig. 1. Degradation of orotic acid.



ing $KMnO_4$ was destroyed with H_2O_2 and the solution was centrifuged. The precipitate was washed twice with 2 ml of warm water. One ml of 5 *N* NaOH was added to the combined supernatants and the solution hydrolyzed at 100° for 15 minutes. One drop of phenol-red was added and the solution was acidified with 5 *N* sulfuric acid. A stream of nitrogen was passed through the solution, which was warmed to 100° C. After cooling 5 *N* NaOH was added until a slight pink colour developed. One ml urease solution [21] was introduced and the mixture kept at 38—42° for 30 minutes. The solution was then acidified with 5 *N* H_2SO_4, placed in a boiling water bath and the CO_2 driven over into a barium hydroxide solution with nitrogen gas. The yield calc. as $BaCO_3$ was 55—60 % of the theory.

Isotope measurements

N^{15} and C^{13} were determined as N_2 and CO_2 respectively in the mass spectrometer. C^{14} was measured as $BaCO_3$ at infinite thickness.

RESULTS

Ammonia as precursor

It has earlier been found that ammonia is a very effective precursor of orotic acid [12]. It was thought of interest to determine the distribution of isotope in orotic acid using this precursor. Slices from three livers were incubated in 50 ml Krebs-Henseleit substrate with 15 mg orotic acid and 1 m*M* of $N^{15}H_4Cl$. The results of the subsequent degradation of the orotic acids from the different experiments are summarized in Table 2.

Table 2. Incubation of liver slices + orotic acid with $N^{15}H_4Cl$ (32 % excess) and subsequent isolation and degradation of orotic acid.

Exp. number	Hours incubation	Atom per cent excess N^{15} in			
		Orotic acid	N_1^a	N_1^b	N_3
1.	1.33	0.67	0.69	0.69	0.65
2.	4	1.71	1.64	1.89	1.53
3.[c]	4	0.82			

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.
[c] No orotic acid was added at the beginning of the experiment. Instead 15 mg carrier orotic acid was added before the isolation procedure.

In experiment No. 1 the slices were first incubated with 15 mg orotic acid without $N^{15}H_4Cl$ for 80 minutes at 37°, after which time the isotope was added. This was done in order to get conditions comparable with experiment No. 7.

The degradations after both 80 minutes and 4 hours show an almost equal distribution of the isotope between N_1 and N_3. The distribution of the N^{15} between these two positions was not influenced by the presence of 1 m*M* of non-labeled L-glutamine in a 6 hours experiment.

Aspartic acid as precursor

N^{15}-aspartic acid, 1,4-C^{13}-aspartic acid and 2,3-C^{14}-aspartic acid were used as precursors.

One m*M* N^{15}-*aspartic acid* (33 atom per cent excess) and slices from three livers were incubated in 50 ml Krebs-Henseleit substrate with 15 mg orotic acid for 2 and 8 hours (Table 3).

Table 3. Incubation of liver slices + orotic acid with N^{15}-aspartic acid (33 % excess) and subsequent isolation and degradation of orotic acid.

Exp. number	Hours incubation	Atom per cent excess N^{15} in			
		Orotic acid	N_1^a	N_1^b	N_3
4.	2		0.012		0.106
5.[c]	2	0.093	0.030	0.011	0.175
6.[c]	8	0.638	0.523	0.491	0.785

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.
[c] 1.5 m*M* NH_4Cl was added to the substrate.

Both after 2 and 8 hours more isotope was incorporated into position 3 than 1. After 8 hours, however, the difference was much smaller than in the

short time experiments. The presence of non-labeled ammonium chloride did not affect the distribution of N^{15}.

The influence of the presence of non-labeled aspartic acid on the incorporation of isotope from $N^{15}H_4Cl$ is demonstrated by Table 4. Slices from 3 livers were incubated with 15 mg orotic acid and 1.5 m*M* non-labeled aspartic acid in 50 ml Krebs-Henseleit substrate for 80 minutes. After that time 1 m*M* $N^{15}H_4Cl$ + 1.5 m*M* L-aspartic acid were added and incubation allowed to proceed for another 80 minutes. For comparison an identical experiment was performed without aspartic acid.

Table 4. Influence of L-*aspartic acid on the incorporation of* $N^{15}H_4Cl$ *into orotic acid in rat liver slices.*

Exp. number	Isotopic compound	Non-isotopic compound	Atom per cent excess N^{15} in Orotic acid	N_1^a	N_1^b	N_3
1.	$N^{15}H_4Cl$	none	0.67	0.69	0.69	0.65
7.	»	1.5 m*M* L-aspartic acid	0.28	0.56	0.49	0.07

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.

The results indicate that the incorporation of isotope into position 3 of orotic acid is considerably diluted in the experiment with non-labeled aspartate.

The experiments with *carbon labeled aspartic acid* were conducted with 0.5 m*M* labeled aspartate + 1.5 m*M* NH_4Cl + 15 mg orotic acid per three rat livers. Incubation was carried out during 8 hours. The orotic acids from three and four experiments respectively were pooled and degraded, with the results shown in Table 5.

Table 5. 1,4-C^{13}-L-*aspartic acid (atom per cent excess = 18) and* 2,3-C^{14}-L-*aspartic acid (8 100 counts/min.) as precursors of orotic acid.*

Exp. number	Isotopic precursor	Amount isotope [a] in Orotic acid	C_2	$C_6 + C_7$	$C_4 + C_5$
8.	C^{13}-aspartic acid	0.25	0.04	0.53	0.04 [b]
9.	C^{14}-aspartic acid	129	2	56	265 [b]

[a] Atom per cent excess in exp. 8, counts/min. in exp. 9.
[b] Calculated by difference between orotic acid, C_2 and $C_6 + C_7$.

The results clearly indicate that the carboxyl groups of aspartic acid were utilized for the synthesis of $C_6 + C_7$ of orotic acid, while the methylene carbons donate their isotope mainly to $C_4 + C_5$. This together with the results obtained with N^{15}-aspartate indicates that the whole molecule of this amino acid was utilized for the synthesis of $N_3 + C_4 - C_7$ of the orotic acid.

Bicarbonate as precursor

From the results of Heinrich and Wilson [22] and of Lagerkvist [23], which showed that CO_2 by the rat was specifically utilized for the synthesis of the ureidocarbon from uracil, it was highly probable that a corresponding relation between bicarbonate and C_2 of orotic acid should exist. In an experiment 2 m*M* $NaHC^{13}O_3$ (atom per cent excess 36) + 1 m*M* of NH_4Cl + 0.5 m*M* L-aspartic acid + 15 mg orotic acid were incubated with slices from 3 rat livers for 6 hours. The isotope content of the orotic acid and that of C_2 of the orotic acid were determined. (Table 6.)

Table 6. $NaHC^{13}O_3$ (atom per cent excess = 36) as precursor of orotic acid.

Exp. number	Isotopic precursor	Atom per cent excess C^{13} in		
		Orotic acid	C_2	C_4, C_5, C_6 and C_7
10	$NaHC^{13}O_3$	0.146	0.534	0.049 [a]

[a] Calculated by difference between orotic acid and C_2.

Ureidosuccinic acid as precursor

The work of Wright [24,25] and coworkers has demonstrated that this acid is an intermediate in pyrimidine biogenesis in *Lactobacillus bulgaricus* 09. Weed and Wilson * have shown that ureidosuccinic acid is utilized for pyrimidine synthesis in liver slices. Because of this an investigation was started to find out, if ureidosuccinic acid was an intermediate in the present system. It was thought to be of advantage to test the L-isomer, especially since in an earlier work only the D,L-isomer had been used. Two types of experiment were carried out. First the N^{15}-L-ureidosuccinic acid was incubated with orotic acid and liver slices for 4 and 7 hours respectively. Second, $N^{15}H_4Cl$ was incubated together with orotic acid and liver slices in the presence of non-labeled D,L-ureidosuccinic acid. In both cases the incorporation of isotope into the orotic acid was determined with the results of Table 7.

Clearly ureidosuccinic acid was very effectively utilized for the synthesis of orotic acid. That the isotope incorporation did not take place after degradation of the acid was shown by the N^{15} values for N_1 and N_3; practically all isotope was located in N_3, as would be expected after direct ringclosure of the ureidosuccinic acid. The dilution experiment furthermore showed that the presence of non-labeled ureidosuccinic acid diminished the incorporation of $N^{15}H_3$ into orotic acid and the isotope content of the ureidosuccinic acid at the end of the experiment demonstrated a *de novo* synthesis of this acid. All this speaks in favour of L-ureidosuccinic acid as a normal intermediate in the biogenesis of orotic acid in liver slices.

* Personal communication from Dr. Wilson.

Table 7. Ureidosuccinic acid as precursor of orotic acid in rat liver slices.

Exp. number	Isotopic [a] precursor	None-isotopic addition	Hours incubation	Atom per cent excess N^{15} in Orotic acid	N_1	N_3
11	0.1 m*M* N^{15}-DL-ureido succinic acid	none	4	0.75		
12	0.2 m*M* N^{15}-DL-ureido succinic acid	none	7	2.70	0.10	5.76
2	1 m*M* $N^{15}H_4Cl$	none	4	1.71		
13	1 m*M* $N^{15}H_4Cl$	0.1 m*M* D,L-ureido succinic acid [b]	4	0.29		

[a] The amounts are given per slices from 3 rat livers. Atom per cent excess N^{15} : $N^{15}H_4Cl$ = 32, N^{15}-*L*-ureido succinic acid = 16.7.

[b] The ureido succinic acid was reisolated after the experiment by addition of 100 mg D,L-ureido succinic acid as carrier and subsequent crystallization. It contained 0.082 per cent excess N^{15}.

DISCUSSION

Some evidence has earlier been obtained by Lagerkvist, Reichard and Ehrensvärd [26] that the carbon chain of aspartic acid was used for the synthesis of the polynucleotide pyrimidines in rat liver slices. N^{15}-aspartic acid, however, donated very little of its isotope to uracil. In view of our present results we think that the earlier finding of the non-utilization of the N^{15} of aspartic acid probably arose from the rapid transamination of the aspartic acid in liver slices as compared to the slow synthesis of polynucleotide pyrimidines. Furthermore a relative permeability barrier has been demonstrated in liver cells for aspartic acid [27], which again would tend to give an erroneous low incorporation of the isotope. The synthesis of orotic acid in liver slices, however, proceeds at a much higher rate than that of polynucleotide pyrimidines, and it was therefore possible to demonstrate the specific incorporation of isotope from N^{15}-aspartic acid into position 3 of the orotic acid. Thus we do not believe that our present results show a difference between the biogenesis of polynucleotide uracil and orotic acid respectively.

The finding that CO_2 was incorporated specifically into position 2 of orotic acid shows the similarity between the synthetic pathway for this substance and for uracil in this respect.

The origin of N_1 is not clear. In earlier experiments *in vivo* Lagerkvist [28] has found that N^{15}-ammonia in rat liver is preferentially incorporated into this position of uracil. In the present system no preferential incorporation of the same precursor could be demonstrated. This is maybe not so surprising in view of the rapid transamination reactions in liver slices. If anything it might indicate that ammonium ions are not the immediate precursor of N_1. The amido group of glutamine is probably not involved as indicated by the fact that the presence of non-labeled glutamine together with $N^{15}H_4Cl$ did not affect the incorporation of N^{15}. This experiment, however, is not considered quite

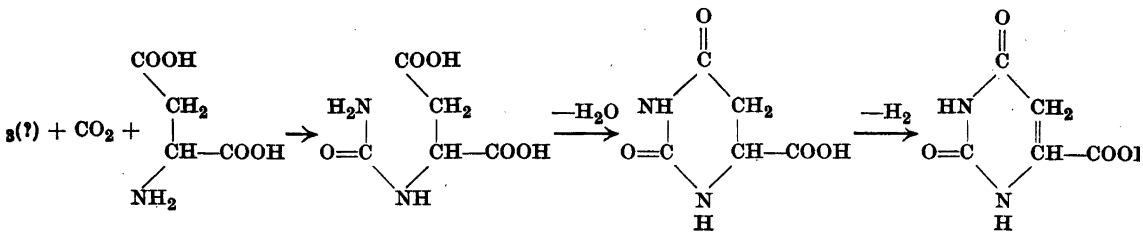
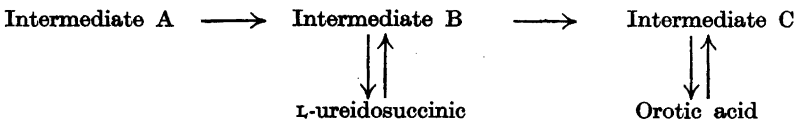


Fig. 2. Biogenesis of orotic acid in rat liver slices.

conclusive and more experiments — if possible in soluble enzyme systems — are needed to answer the question of the origin of N_1 of orotic acid.

Our experiments with L-ureidosuccinic acid are thought to indicate that this substance is an intermediate in the synthesis of orotic acid from aspartic acid. Another possible explanation is given below:



At the present time we cannot see any experimental evidence for this complicated explanation and prefer the more straightforward scheme represented by Fig. 2.

The transformation of ureidosuccinic acid to orotic acid requires at least two steps: ringclosure, and the introduction of a double bond. Depending on which of these reactions occurs first, either dihydroorotic acid or ureidofumaric acid might be an intermediate. Lieberman and Kornberg [29] have recently demonstrated that in an extract from an anaerobic bacterium the synthesis of orotic acid from ureidosuccinic acid proceeds *via* dihydroorotic acid. Spicer *et al.*[30] had earlier reported that dihydroorotic acid was not an intermediate in the formation of orotic acid from ureidosuccinic acid in *Lactobacillus bulgaricus 09*. In their investigation dihydroorotic acid was synthesized according to Bachstez and Cavallini [31]. However, it has recently been shown that dihydroorotic acid prepared in another way was as effective as orotic acid in promoting growth of *L. bulgaricus 09*.* We therefore consider it probable that dihydroorotic acid is an intermediate in orotic acid synthesis also in rat liver slices.

A summary of the synthetic reactions for the biogenesis of orotic acid as visualized by us is given in Fig. 2.

We wish to acknowledge the valuable technical assistance of Mr. K. Avots, Mr. J. Grabosz and Miss T. Koziorowska.

* Personal communication from Dr. L. D. Wright. This dihydroorotic acid was either prepared enzymatically (Lieberman and Kornberg) or by a new chemical synthesis.

SUMMARY

The biogenesis of orotic acid from different precursors in rat liver slices has been investigated. Using N^{15}-L-aspartic acid, 1,4-C^{13}-L-aspartic acid and 2,3-C^{14}-L-aspartic acid it was found that N_3 plus C_4, C_5, C_6 and C_7 of orotic acid were derived from aspartic acid.

CO_2 was preferentially incorporated into position 2 of orotic acid.

N^{15}-L-ureidosuccinic acid was prepared as a crystalline barium salt from N^{15}-L-aspartic acid. It was found to be transformed to orotic acid in liver slices. The presence of non-labeled D,L-ureidosuccinic acid considerably diluted the incorporation of isotope from $N^{15}H_4Cl$ into orotic acid. At the same time a *de novo* synthesis of ureidosuccinic acid could be demonstrated.

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Low-molecular Carbohydrates in Algae

III * Synthesis of 1-D-Mannitol β-Glucoside

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The isolation of 1-D-mannitol β-D-glucopyranoside from the brown alga *Fucus vesiculosus* was reported in Part I[1] of this series, and the synthesis of this substance has now been accomplished by conventional methods. 2,3,4,5-Mannitol tetra benzoate was reacted with an equimolecular amount of acetobromoglucose following Reynold's and Evan's modification [2] of the Koenigs-Knorr method. The reaction products were deacylated, and the mannitol monoglucoside isolated by chromatography on a charcoal-Celite column, using the gradient elution techniques. The substance, m.p. 138—139° and $[\alpha]_D^{20}$ −20° (water, $c = 2$) proved to be identical to that isolated from *Fucus vesiculosus*.

EXPERIMENTAL

1.6-Dityrityl-tetrabenzoyl-D-mannitol (10 g) was detritylated by hydrogen bromide in chloroform-acetic acid [3]. The sirup obtained (5.6 g) was not further purified but was dissolved in dry chloroform (14 ml). Freshly prepared silver oxide (3 g) and Drierite (10 g) were added and the mixture was shaken vigorously in a brown bottle for one hour. A solution of iodine (0.5 g) and acetobromoglucose (3.8 g) in dry chloroform (15 ml) was added in ten portions during the next hour and the shaking continued overnight. The mixture was then filtered through a layer of Celite and the filter washed with chloroform. The combined chloroform solutions were washed with a small amount of sodium thiosulphate solution and with water, dried over calcium chloride and concentrated to dryness under reduced pressure. The remaining sirup was dissolved in absolute ethanol, (50 ml) to which sodium (0.2 g) had previously been added, and was kept at 0° overnight. Water (50 ml) was added and ionic material removed by filtration through the Amberlite resins IR 120 and IR 4B. The solution was then concentrated to dryness under reduced pressure. Paper chromatographic investigation of the residue (2.6 g) revealed the presence of mannitol monoglucoside, small amounts of mannitol diglucoside, mannitol and reducing sugars. The residue was dissolved in 1 % ethanol (30 ml) and put on a carbon-Celite column (35 × 4.5 cm). The column was eluted with aqueous ethanol (4 000 ml), the concentration of the ethanol being continuously increased from 1 % to 25 %. The eluate was divided into fractions, which were investigated by means of paper chromatography. Those containing the monoglucoside (between 2 100 and 2 500 ml) were combined and the solvent removed under reduced pressure. The remaining sirup (0.9 g) was dissolved in

* Part II. *Acta Chem. Scand.* 7 (1953) 1123.

methanol (5 ml) and the solution kept at 0°. The mannitol monoglucoside crystallised slowly, and only after several weeks was the separation complete. Yield 0.7 g (24 %), m.p. 132—134°. One further recrystallization from methanol raised the m.p. to 138—139°, undepressed on admixture with the mannitol monoglucoside from *Fucus vesiculosus*.

SUMMARY

1-D-Mannitol β-D-glucopyranoside, identical to the mannitol monoglucoside isolated from *Fucus vesiculosus*, has been synthesized by unambiguous methods.

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Short Communications

A Steroidal Glycol, 22-Hydroxy-cholesterol from *Narthecium ossifragum* Huds.

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In the course of an investigation of the pigments of the flowering parts of *Narthecium ossifragum*, — a tiny lily supposed to be the cause of a sheep disease of photodynamic nature, — there was, during the chromatographic separation of the crude hypophasic carotenoids also isolated a colourless compound (I), which, from the following summary of its reactions, will be seen to be one of the two stereomeric forms of the hitherto unknown 22-hydroxy-cholesterols, m.p. 186°(corr.), $[\alpha]_D^{20}$ −39, $CHCl_3$ (*Anal.* Calc. for $C_{27}H_{46}O_2$: C 80.5; H 11.52. Found: C 80.0; H 11.83). The glycolic nature is demonstrated by the formation of a diacetate, m.p. 101°, $[\alpha]_D^{20}$ −33 (*Anal.* Calc. for $C_{31}H_{50}O_4$: C 76.5; H 10.35. Found: C 76.5; H 10.50), and a dibenzoate, m.p. 256°, $[\alpha]_D^{20}$ −9 (*Anal.* Calc. for $C_{41}H_{54}O_4$: C 80.6; H 8.91. Found: C 80.6; H 8.83). Catalytic hydrogenation furnished a dihydroglycol (II), m.p. 176.5°, $[\alpha]_D^{20}$ + 18; diacetate, m.p. 76°, $[\alpha]_D^{20}$ + 14; dibenzoate, m.p. 215—17°, $[\alpha]_D^{20}$ + 16.

Careful oxidation of (I) with chromium trioxide gave a compound (III) of m.p. 142.5—43.5°, $[\alpha]_D^{20}$ −58. The I.R. spectrum of this oxydation product indicated an unsaturated hydroxy-ketone. The found data were close to those given by Cole and Julian [1] for 22-keto-cholesterol, (m.p. 142—43°, $[\alpha]_D^{20}$ −55). Dr. Cole had the kindness to furnish a sample of this substance with m.p. 142—43°, $[\alpha]_D^{25}$ −60, and supposed by Dr. Cole to be a purer one. Mixed m.p. confirmed the identity of the two substances. Mixed m.p. of the acetate of (III) with the acetate of 22-keto-cholesterol, also kindly provided by Dr. Cole, furnished additional evidence for the identity.

The dihydroglycol (II) was oxidised with chromium trioxide to a diketone, m.p. 148°, $[\alpha]_D^{20}$ + 16, which must be the previously unknown cholestane-3,22-dione. Reduction by the Clemmensen method afforded cholestane, m.p. 80°, undepressed on admixture with cholestane prepared from cholesterol.

The saponins of *Narthecium ossifragum* are presently being investigated. They seem to contain no 22-hydroxy-cholesterol. Their sapogenins have an ordinary number of oxygen atoms.

I want to express my sincere thanks to Professor N. A. Sörensen for valuable advice throughout the work and for determining the I.R. spectra. My thanks are also due to Dr. W. Cole, The Glidden Company, Soya Products Division, Chicago, for supplying samples of 22-keto-cholesterol and its acetate.

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Rotational Isomers of Pentathionic Compounds in Crystals

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Recent work on the crystal structure of pentathionic compounds has shown that these are, in the cases studied so far, built up of unbranched and non-planar chains. This applies to sulphur, selenium and tellurium dimethanethiosulphonate [1,2], $S(S_2O_2CH_3)_2$, $Se(S_2O_2CH_3)_2$ and $Te(S_2O_2CH_3)_2$, barium pentathionate and selenopentathionate dihydrate [3], $BaS(S_2O_3)_2 \cdot 2\ H_2O$ and $BaSe(S_2O_3)_2 \cdot 2\ H_2O$, and ammonium telluropentathionate [4,5], $(NH_4)_2Te(S_2O_3)_2$.

The non-planarity of sulphur chains is principally due to the mutual repulsion of the unshared $p\pi$ electron pairs of neighbouring sulphur atoms [6]. The barrier restricting rotation about a sulphur-sulphur bond may be estimated [6] as roughly 5 kcal/mole.

In pentathionic compounds, with five-membered, unbranched chains, *cis* and *trans* rotational isomers are possible. The terminal sulphur atoms are rotated an angle of about 90° out of the plane of the three middle atoms, either to the same side of the plane — *cis* — or to opposite sides — *trans*. There are two enantiomorphous *trans* forms.

The *cis* form arises from the eight-membered rings of orthorhombic sulphur [7] and monoclinic selenium [8,9] by removal of three neighbouring sulphur or selenium atoms, whereas the *trans* forms are contained in the spiral chains of fibrous sulphur [6] and hexagonal selenium and tellurium [10], and in cesium hexasulphide [11].

A *cis* form of a pentathionic compound possesses a mirror plane of symmetry, provided that bond distances and angles are the same in both halves of the molecule. With the same limitations, a *trans* form possesses a twofold axis of molecular symmetry. The mirror plane and the twofold axis pass through the middle atom and not through any other atoms of the chains.

In solutions, *cis* and *trans* forms probably exist in equilibrium mixtures. In crystals, with fixed atomic positions, the occurrence of isomers is determined in part by lattice energy relations. The nature of the cation, in the case of salts, appears to play a role. The findings are as follows.

Sulphur, selenium and tellurium dimethanethiosulphonate, the crystals of which are isomorphous, occur in *trans* forms [1,2].

The space group of barium pentathionate dihydrate, crystallized from aqueous methanol, is D_{2h}^{16} $-Pnma$ with $Z = 4$. The pentathionate ion has a *cis* form and, by space group requirements, a mirror plane of symmetry [3]. The crystals of barium selenopentathionate dihydrate are orthorhombic and isomorphous with those of barium pentathionate.

The telluropentathionate ion in ammonium telluropentathionate [4,5] has, as different from the pentathionate and selenopentathionate ions in the barium salts, a *trans* form like the methanethiosulphonates.

Now, a series of isomorphous salts of pentathionic, selenopentathionic and telluropentathionic acids exists [4], comprising potassium pentathionate, ammonium selenopentathionate, cesium telluropentathionate, and the three rubidium salts. In the isomorphous salts, the three anions have evidently the same structure. This shows that the pentathionate and selenopentathionate ions, or the telluropentathionate ion, occur in different forms in different salts.

Unit cell and space group data are available for a series of aromatic pentathionic compounds, derived from the methanethiosulphonate series by substitution of phenyl and *p*-tolyl for methyl. These are sulphur dibenzenethiosulphonate and di-*p*-toluenethiosulphonate [12], and selenium and tellurium dibenzenethiosulphonate and di-*p*-toluenethiosulphonate [13]. A twofold axis is crystallographically required as molecular symmetry. Therefore, provided the chains are unbranched, as is strongly indicated from chemical evidence [13] and analogy with the methanethiosulphonates, the molecules occur in *trans* forms. The crystals, except those of tellurium dibenzenethiosulphonate, are based on the enantiomorphous space group $D_4^4 - P4_12_12$ with $Z = 4$. A unit cell thus contains only one of the two enantiomorphous *trans* forms.

This is true also for triselenium di-*p*-toluenesulphinate [13], which is isomorphous with the *p*-toluenethiosulphonates.

The above stereochemical considerations regarding pentathionic compounds are generally valid for molecules X-S-S-S-X where X may be any atom or group and

the three sulphur atoms may be partly or completely substituted by selenium or tellurium. Thus, the arguments apply to compounds of the trisulphide and triselenide type, as has been discussed by other authors [14-16] on the basis of dipole moments in solutions, and with reference to Raman spectra in the case of hydrogen trisulphide [17]. The configurations of the following compounds are known in crystals.

Bis(2-iodoethyl) trisulphide [18,19] has a *trans* form and a twofold axis of symmetry. The space group [12] is the enantiomorphous one, $D_4^4-P4_12_12$ with $Z = 4$. Selenium diselenocyanate [20], $Se(SeCN)_2$, has the space group $D_{2h}^{16}-Pnma$ with $Z = 4$. The molecule occurs in a *cis* form, and possesses a mirror plane of symmetry [21].

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On the Technique of Preparative Electrophoresis of Proteins in Supporting Medium (Starch)

ESKO NIKKILÄ, EERO HAAHTI and RAINER PESOLA

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In efforts to adapt the principle of electrophoresis to preparative purposes, particularly in the field of protein chemistry, several methods have been suggested [1-3]. In their practical application, however, many unexpected factors often prevent a clear-cut and reproducible separation and/or satisfactory recovery of individual fractions. Starch as a supporting medium was recently introduced by Kunkel and Slater [4] and it seems to have many advantages compared with other media. Nevertheless this method also is rather sensitive to various influences and, when performed as presented by them, gives reproducible and otherwise ideal results only accidentally. The following reports briefly on some modifications of the technique which seem to have improved the usefullness of preparative zone electrophoresis.

The experiments were made with human blood serum using a rectangular perspex vessel packed with buffer-moistened potato starch. A point of primary importance is completely to prevent evaporation from the surface of the medium. To this end melted paraffin was poured on the starch block and allowed to solidify, forming quite a tight cover. Serum could then be applied to the starch through a narrow window cut into this cover. The contact from the ends of the block to the buffer vessels was mediated by strips of heavy filter paper.

It appeared that the apparently poor separation of fractions was often due to gravity causing an accumulation of serum in the lower starch layers when the run was made in a horizontal position, and this led to an uneven migration velocity at different levels of the block. This effect was avoided by making the run in the

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vertical direction. To minimize the effect of electroosmotic flow the surface of the buffer solution on the cathodic side was held about 20 cm above that on the anodic side.

The run can be followed by mixing with serum a quantity of stain which is bound to albumin (*e. g.* bilirubin). As coloured fractions are often undesirable, however, inspection of the block in ultraviolet light is to be preferred. Here a green fluorescence shows the exact position of the advancing albumin band (and often also of β-globulin). It seems possible that also other proteins can be made visible by adding some fluorescent substance.

After the separation of the fractions has been effected (usually about 10 hours under the conditions used by us), and provided no exact data on their quantitative distribution are desired, the localization of each protein fraction can be carried out simply and rapidly by pressing the edge of a narrow strip of filter paper (*e. g.* Munktell 20) lengthwise against the still moist surface of the medium so that the liquid (containing the proteins) is absorbed into it. When this strip is then stained with bromophenol blue (for 3—4 minutes) and washed in running tap water the exact position of the fractions becomes visible just as in a paper electrophoresis strip.

The eluation of the proteins from the starch portions, each containing one major fraction, is best done with a slightly alcaline buffer which contains glycine (0.5 *M*). The yield naturally increases with the buffer/starch ratio. The eluates can be concentrated by dialyzing them against concentrated dextran solution or by lyophilization. The purity of serum protein fractions obtained by this method has been controlled by free electrophoresis with an optical device.

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Inhibition by Nitrous Oxide of Biological Nitrogen Fixation and the Uptake of Combined Nitrogen

ARTTURI I. VIRTANEN and SINIKKA LUNDBOM

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

Molnar, Burris and Wilson [1], Repaske and Wilson [2] have found that N_2O inhibits N_2-fixation with *Azotobacter*, but not the uptake of combined nitrogen. N_2O thus seemed to influence nitrogen fixation specifically. These authors used only ammonium nitrogen as the source of combined nitrogen.

Virtanen [3] has suggested that in N_2-fixation may be formed as an intermediate a nitrogenoxygen compound which would be identical with an intermediate formed in nitrate reduction too. Both processes would thus join on this stage. If this suggestion is correct, N_2O may inhibit not only N_2-fixation but also the utilization of nitrate. It was therefore interesting to compare the influence of N_2O on N_2-fixation, and on the utilization of NO_3-N and NH_4-N with *Azotobacter*. Anaerobic *Clostridium butyricum* was also included in the experiments, as there is no previous information on the influence of N_2O on anaerobic nitrogen fixation.

In experiments both with *Azotobacter* and *Clostridium* the atmosphere of the test-flask contained 0, 5, 25 and 50 % N_2O. Primary atmosphere in *Azotobacter*-cultures was air, and in *Clostridium*-cultures N_2. Also *Clostridium butyricum* could be made to grow in synthetic nutrient solution by adding different vitamins to the solution.

The results of our experiments will appear from Figs. 1 and 2. From these can be seen that:

1) N_2O inhibits both N_2-fixation and utilization of nitrate in about the same concentrations.

2) N_2O does not influence the utilization of ammonium-N even in the maximal concentration used (50 % N_2O).

3) N_2O has the same influence both on aerobic *Azotobacter* and anaerobic *Clostridium*. N_2O inhibits N_2-fixation and nitrate

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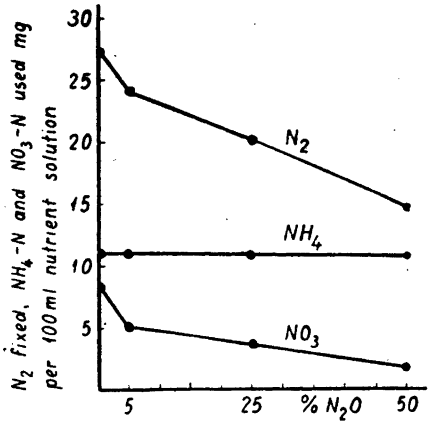

Fig. 1. Influence of N_2O *on* N_2*-fixation and on the use of* NH_4-N *and* NO_3-N *with A z o t o b a c t e r v i n e l a n d i i.*

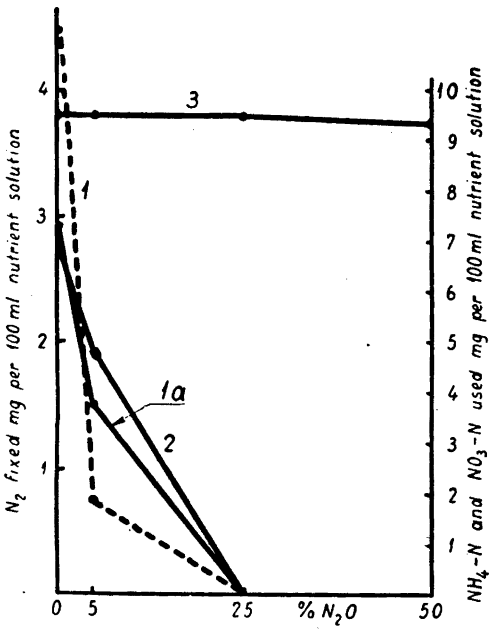

Fig. 2. Influence of N_2O *on* N_2*-fixation and on the use of* NH_4-N *and* NO_3-N *with C l o s t r i d i u m b u t y r i c u m.*
1 and 1a: N_2*-fixation, 2: use of* NO_3-N, *3: use of* NH_4-N.

uptake also with *Clostridium butyricum.* The anaerobic N_2-fixation is more sensible to N_2O than the aerobic one.

The results may be regarded as a corroboration of the conception that N_2-fixation leads to an intermediate which appears also as an intermediate in nitrate reduction. As to the mechanism of aerobic and anaerobic N_2-fixation the results may corroborate the assumption of a similar mechanism in both cases. It would, however, be an exaggeration to state that the results prove these conclusions, because it is possible that N_2O competes with N_2 for the enzyme which causes the activation of N_2, as well as for the enzyme which acts in some phase of nitrate reduction. The possible similarity of the arrangement of electrons in the nitrogen molecule, and in the intermediate of nitrate reduction would thus be the reason for the similar action of N_2O in N_2-fixation and in nitrate reduction. Concerning the mechanism of N_2-fixation, results in this case give no indications.

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A Self-Recording Strip Photometer for Paper Electrophoresis and Paper Chromatography

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Paper electrophoresis was first developed by Wieland and Fischer in 1948 [1] for amino acid analysis, but was quickly and almost simultaneously modified in several laboratories for the purpose of serum protein analysis [2–5]. In these first investigations the separated proteins were analysed quantitatively by retentiometry [2], or, by dying the strips, eluting the dyed zones and measuring the eluates photometrically [3–4]. Grassmann and Hannig [6] first described a quantitative method for paper electrophoresis in which use was made of a manually operated paper strip photometer. In this method pherogram strip was moved, mm by mm, over the slit of a photometer, the density values read and plotted om mm paper against distance from the starting line. The areas of the peaks thus obtained were then measured by a planimeter, these areas being directly proportional to the protein concentrations. Relative concentrations of the serum components were obtained by expressing as percentages the areas of their corresponding peaks compared with the area of all peaks on the densitogram.

The manually operated strip photometer was first developed for paper chromatography by Block [7], and Bull, Hahn and Baptist [8]. Block has used values of optical density, plotted on mm paper, and Bull *et al.* have used percentual transmission values plotted on semilogarithmic paper. Block [9] has also published another, "maximum colour density" method, where optical density is measured only at the centre of the spot. As absolute rather than relative concentrations are usually required in paper chromatography, and as the colour intensity of the reaction product of each amino acid with ninhydrin is different and dependant upon handling conditions, reference standards are usually necessary for the measurement of amino acid paper chromatograms.

We have developed a self-recording photometer for the measurement of paper electropherograms, as well as paper chromatograms. The method has been described briefly in an earlier publication [10]. The new instrument is a combination of a strip photometer of the usual type, and the self-recording galvanometer of the Heyrovsky-polarograph. The movements of the phero-

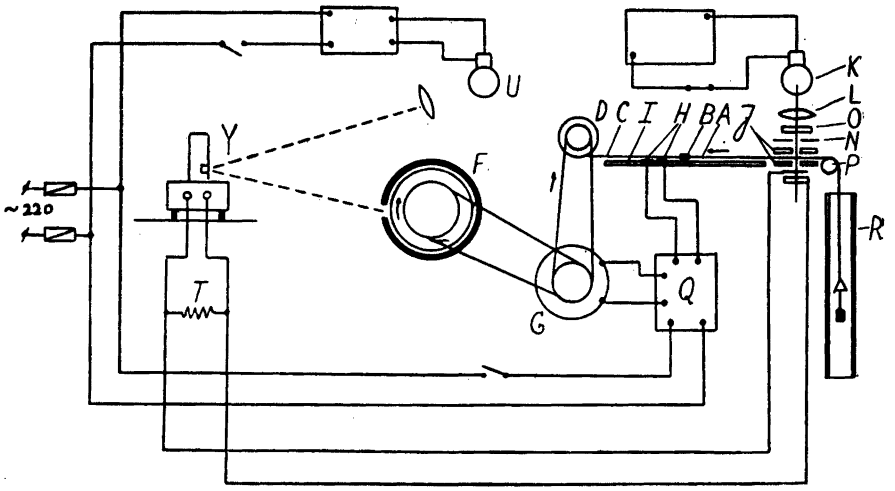

Fig. 1. Scheme of the self-recording strip photometer.

gram strip and the recording drum are synchronized by the polarograph motor. Densitograms are made upon photographic paper containing an extinction scale, divided into narrow columns, and the area of a densitogram peak is obtained as the sum of the extinction values of the columns under that peak. These areas, which can be termed the "extinction areas" of the peaks, are in direct relationship to the amount of dye in the corresponding zone, in contrast to the planimetered areas or "absorption areas" of the peaks.

Logarithmic recording of density values, required by Beer's law, is obtained by a method differing in principal from the above, using an instrument independently developed by Dr. A. Wagner *. This instrument is provided with a "logarithmic galvanometer", which gives directly the "extinction areas", which are measured by planimetring.

The present paper describes in detail our instrument and its use for the measurement of serum electropherograms and one-dimensional amino acid chromatograms.

DESCRIPTION OF THE APPARATUS.

The apparatus (Fig. 1) is a combination of a photometer and a self-recording galvanometer. A galvanometer of the Heyrovsky polarograph Model 1939 has been used in our apparatus, as it happened to be at our disposal, but other corresponding recording galvanometers can evidently be used. Parts G, F and Y originate in our arrangement from the polarograph, which is described in detail in Heyrovsky's monograph [11] and elsewhere in polarographic literature.

* Personal communication. It is a pleasure to acknowledge here a discussion with Dr. Wagner on some theoretical aspects of strip photometry.

The chromatogram- or pherogram-strip A and the recording drum F of the self-recording galvanometer Y have their movements synchronised by a constant speed motor G. A is fastened by a metal clip B to a thin string C which is wound around a reel D. To prevent sagging, A, B and C are sliding upon a glass strip I. H is a pair of thin copper foils. Its position on the glass strip I can be changed to correspond to different chromatogram lengths. When clip B slides over H thus making contact, relay Q switches current from motor G and galvanometer lamp U.

The photometer lamp K (fed from an accumulator) emits an even illumination upon opening M, having first passed through a condensor lens L, an adjustable slit N and a filter O. The glass cell J consists of two microscope slides (see Fig. 2). The chromatogram is cut to be 10.2 mm in width and the opening in J through which it slides is made 10.3 mm in width. J is covered with black paper in which an opening M, 10.0 × 2.0 mm, is cut; thus there is no possibility of extraneous light passing the strip at its edges.

To the other end of chromatogram A a small weight is fastened. This weight which keeps the chromatogram strip taut, moves up and down in a tube R to avoid acting as a pendulum. P is a roller.

The diameter of the axis of reel D is 0.95 cm. The string is wound from side to side upon it. Thus, 20 rotations are needed to draw a 60 cm long chromatogram through the apparatus. Half of this speed (*i.e.* 30 cm by 20 rotations) is obtained by fixing to the metal clip B a roller, around which the string C is drawn back to the side of the reel D, where it is fixed. (The Heyrovsky polarograph has a 1 : 20 reduction gearing which makes the drum F rotate once while the reel D rotates 20 times.) The photographic paper is selected so that its sensitivity is just high enough to give a distinct curve even when the

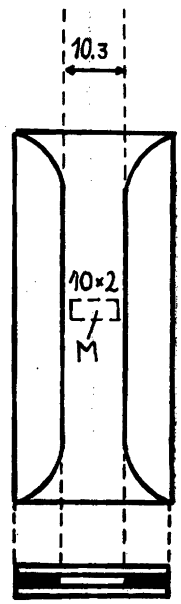

Fig. 2. Glass cell for the sliding chromatogram strip.

galvanometer deflection is rapid. The apparatus can then be used in an ordinarily illuminated work-room. Kodak "Bromars" soft paper and "Labaphot" photostat paper have been ideal for this purpose under our conditions.

We have used a barrier layer photocell, 22 × 40 mm (made by Evans Electroselenium Limited, Harlow, England). The sensitive area of this cell is 5.1 cm^2. The working characteristics of a cell improve as the ratio of sensitive area in use versus total sensitive area approaches unity, and so although this cell has given good results, a smaller size might be preferable, as our slit width has to be kept below 2 mm. However, even with this photocell complete linearity of the current output is obtained under all working conditions because of the high sensitivity and low inner resistance of the galvanometer at our disposal. It has an inner resistance of *ca.* 400 Ω and a critical resistance of *ca.* 7 000 Ω. Damping is effected by the use of a parallel shunt T of 7 000 Ω. Its high sensitivity (1 μA causes a deflection of 30 cm at 1 m distance) enables the use of low light intensities and narrow slit widths.

Slit width has varied in our conditions from 0.5 to 1.5 mm. Calibration of the slit width and control of evenness of illumination is easily made by exposing to different slit widths strips of photographic paper in the place of the chromatogram strip. The photometer part of the apparatus is seen in Fig. 3.

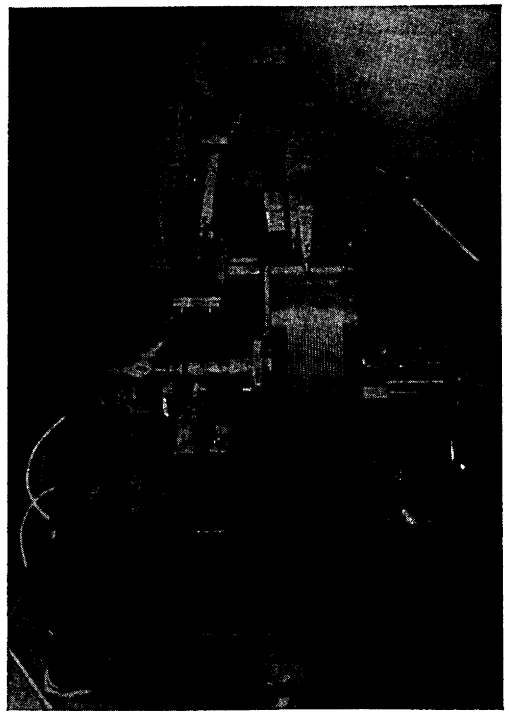

Fig. 3. Photometer part of the apparatus with a chromatogram strip in drawing position.

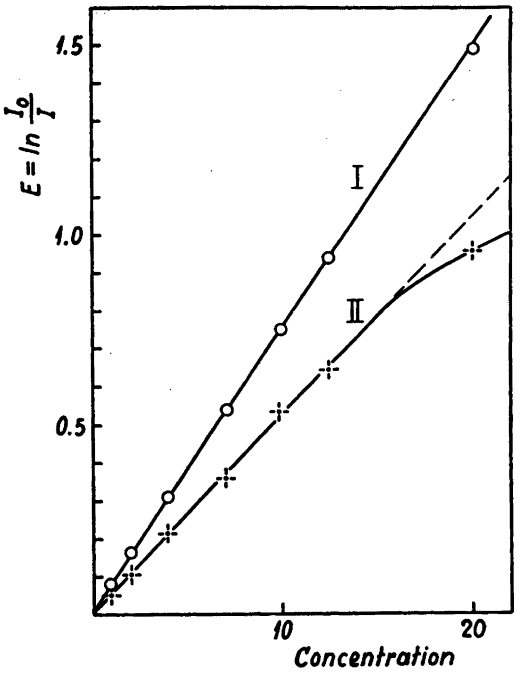

Fig. 4. I. A series of dilutions of amidoblack-10B, measured by Beckman Quartz Spectrophotometer at 620 mμ. II. The same by the strip photometer using Ilford filter N:o 626.

METHOD

Calibration of the apparatus

Deflection of the Heyrovsky galvanometer is directly proportional to the electric current. As current output from the photocell is in linear relationship to illumination as mentioned above, it depends solely on the filter, whether linearity between extinction values and concentration is obtained with substances which follow Beer's law. Linearity of the strip photometer was controlled by measuring a dilution series of amidoblack-10 B in 1 cm cells with the instrument. For this purpose glass cell J was removed, an adequate boring for Beckman cells made and the photometer turned through 90°. The same solutions were measured by the Beckman spectrophotometer. Of the filters at our disposal best results were obtained with the Ilford filter No. 626, No. 607 being inferior. As can be seen from Fig. 4, curve II, complete linearity is obtained with this filter over the finely divided part of the extinction scale, up to $E = 0.8$. The transmittance of this filter and the absorbance of amidoblack-10B can be seen from Fig. 5. Amidoblack-10B follows Beer's law in solution (Fig. 4, curve I).

Strict linearity between density values and dye concentration is necessary in those cases where no reference standards are used, as in the case of paper electropherograms where different peaks, low and high, are directly compared with each other. When standard curve technique is used, as in most cases in paper chromatography, complete linearity is not so essential although the form of the standard curves improves with linearity. For convenient measurement of peak areas in extinction units, areas which are directly

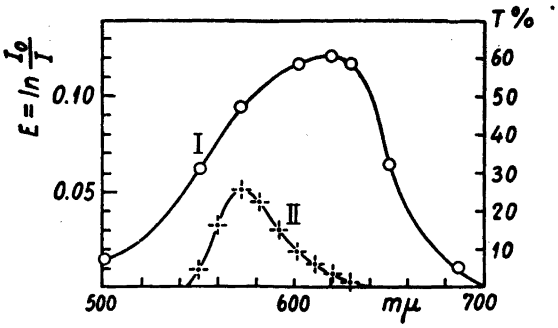

Fig. 5. I. Optical density of amidoblack-10B (left scale). II. Transmittance of Ilford filter No. 626 (right scale).

proportional to the amount of dye in the corresponding zones, the scale given in Figs. 7—9 was designed. It was calculated according to the equation $L = A\,(1-e^{-kc})$, drawn in large size and reduced photographically. This scale is exposed on the recording papers prior to their use by the help of a 10 × 20 cm negative (also commercially available).

Measurement procedure

The measurement procedure is similar for paper electropherograms and paper chromatograms, except when the standard curves for the latter are constructed by using merely heights of the peaks (Block's "maximum intensity"). In this case it is not necessary to expose the scale on the registering paper and the higher drawing speed can be used (Fig. 6 B).

When "extinction areas" are to be measured, the scale, given in Fig. 7—9 is first contact-copied on to a 10.5 × 24 cm piece of suitable photographic paper with the aid of a 10 × 20 cm negative. One edge of the paper is placed in red light exactly along the ∞-line of the negative. This edge is marked with pencil after exposure and, when placing the paper on the recording drum, this edge is placed below the "10 cm" mark.

A 10.2 cm broad strip is cut from the most suitable place of the pherogram or chromatogram with the aid of a straight-edged glass rule and a heavy razor blade. The strip is rendered transparent by keeping it for 10—15 mins. in a trough filled with Grassmann and Hannig's transparancy-oil, and the surplus of the oil is absorbed by drying the strip 2—3 times between sheets of filter paper.

One end of the strip is pointed and pushed through the glass cell J. The light beam of the galvanometer is adjusted with the galvanometer's adjustment screw exactly upon the 10 cm mark of the drum (below which is the ∞-line of the scale) with closed slit. The slit is then opened just enough to move the light beam upon the O-line of the scale when the most transparent place on the strip is below the slit. The clip B and the weight are then fastened to the ends of the strip, the motor started and the drum lock opened. When the contact H is adjusted for electropherograms and "half speed" is used, the current is automatically cut off after 5 minutes, when 15 cm of the pherogram has been drawn through. The recording drum has then turned half way around. A second pherogram can still be recorded upon the same paper, after which the paper is changed in the photographic darkroom and the numbers of the strips just measured marked with pencil behind the recording paper.

'Half speed' is always recommended when the area of the peaks is to be measured, as with pherograms, as it gives peaks of twice the breadth (10 cm densitogram length corresponding to 15 cm strip length). Ordinary speed is recommended when only "maximum intensity" is measured. The relationship densitogram : strip length is then 1 : 3.

After development of the densitogram and scale and drying of the paper, the areas or heights of the peaks are measured. For the measurement of areas the densitogram is

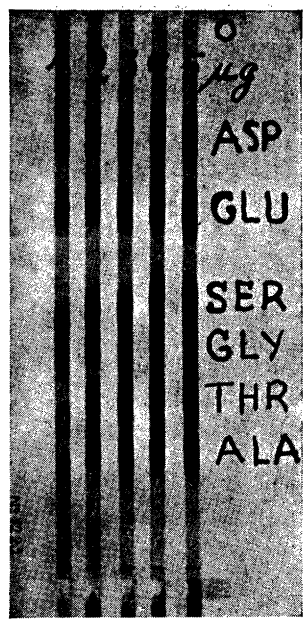

Fig. 6A. Strips for standard curves with 1—5 µg spots. Solvent: water-saturated phenol in NH_3-atmosphere. Paper: Whatman No. 1. 40 hrs, 20° C. Strips cut after ninhydrin reaction.

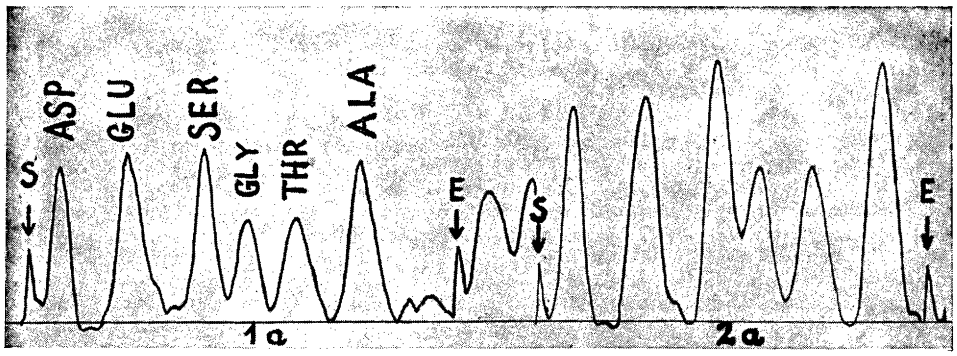

Fig. 6B. Two first strips of Fig. 6A (1 and 2 µg spots) drawn with ordinary speed for measurement by peak heights. S and E = pencil marks showing start and end.

first divided into peaks, after which the density values of the 1 mm broad columns under each peak are read. This is conveniently done with the help of a sharp needle, which is kept in the right hand. To avoid double readings the columns can be marked with needle pricks as they are read. The sum of the columns under each peak, the so-called "extinction area" of the peak, is calculated either from memory or by a computer operated by the left hand. The measurement of one densitogram in this way takes 3—4 minutes. The "extinction area" of a peak is directly proportional to the amount of dye in the corresponding zone, provided that the peak is not higher than $E = 0.8$. If any peak is

Fig. 7A. Two paper electropherograms of normal human serum. Two 10.2 mm wide strips have been cut for measurement from the middle of both pherograms. Pherogram 26 made on "Elphor"-paper, 27 on Whatman No 1 paper.

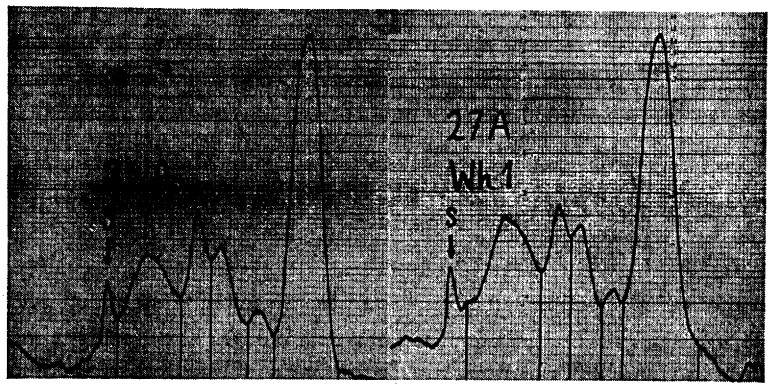

Fig. 7B. Densitograms corresponding to strips 26A and 27A in Fig. 7A, recorded on "Labaphot" photostat paper.

higher than that, it is best to make a new pherogram with a smaller sample. However, if only a few columns exceed $E = 0.8$, their values can be corrected with the help of curve II in Fig. 4, the corrected values being obtained from the dotted continuation.

The accuracy of this method of calculation was determined by allowing several persons to calculate two peaks of medium size without making needle pricks. Standard deviation was found to be 0.2 %. Although the column heights were estimated only by the accuracy of whole scale intervals, it is possible to reduce the error to a minimum by taking the overlap from one column in consideration when estimating the next column.

In the case of serum electropherograms the relative concentration of each component is calculated as a percentage of the peak area from the sum area of all peaks of that densitogram.

In the case of amino acid chromatograms either the area or the height of the peaks is measured. The absolute concentration of an amino acid is arrived at by comparing the value obtained to a standard curve made in exactly the same way with solutions of known concentrations of that amino acid.

Preparation of the amino acid chromatograms

Techniques for preparation of one-dimensional amino acid chromatograms have been published by Block [7,9,12], Bull [8] and others [13-16]. As we have in general followed the same lines as the earlier workers only some aspects of this question are treated here.

Precut strips. To make the chromatograms on narrow strips, already cut to final width [8], is theoretically simplest, since the spreading of the spots out from the strip is

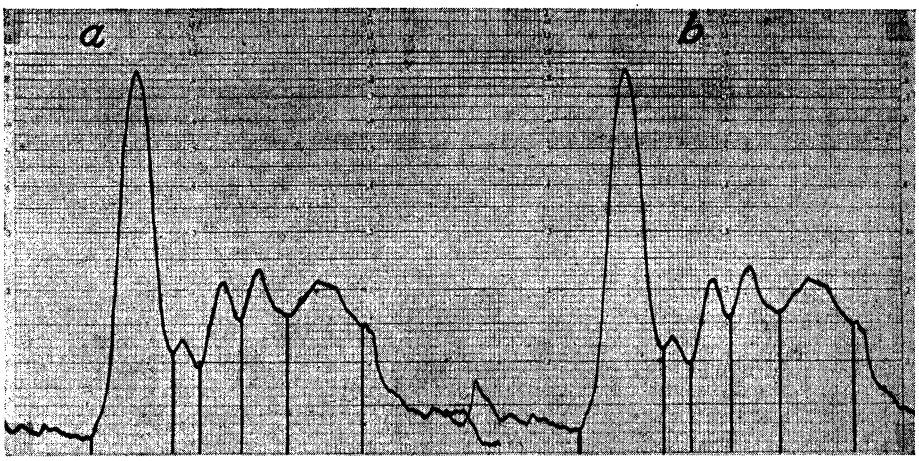

Fig. 8. Reproducibility of the recording. The same strip recorded twice on Kodak "Bromars" paper. Normal human serum.

then impossible and the total colour can thus be measured. In this case the small error caused by variations in paper thickness can also be eliminated by measuring the strips before and after ninhydrin reaction [17]. We have found that the optical density of the paper strips does not change in the ninhydrin treatment except in the region of the amino acid spots. In precut strips, however, *edge effects* are very marked. The spot length is often irregular and the highest colour density is found at the edges. This always reduces accuracy, as it makes a great difference, whether the same amount of dye is spread evenly over the strip width, or concentrated at the edges. In addition, 2 % of the strip width remains unmeasured in our apparatus. With precut strips best results are obtained by using "extinction areas", as fluctuations in spot length have no effect upon these values. On "absorption areas" they have a great effect, since these are not in direct relationship

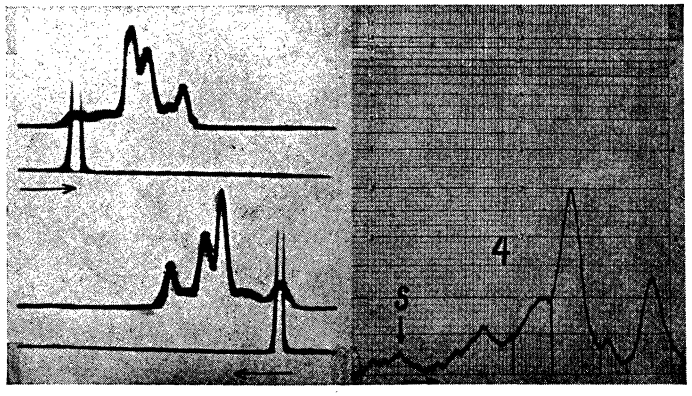

Fig. 9. A nephrosis serum analysed by Tiselius-electrophoresis (A) and paper electrophoresis (B). For quantitative results see Table 1.

to concentration but are a complicated function of diffusion (spot length) and absorption. "Maximum intensity" is of course also sensitive to all irregularities.

Strips cut afterwards. The other possibility is to make chromatograms on wide sheets [12]. Because of partition and diffusion effects spots then spread in such a way that their surface is directly proportionate to the logarithm of concentration (provided the initial area and chromatographing length remain the same) as has been shown by Fischer *et al.* [18,19]. On wide strips the spot form remains much more regular than on narrow strips. After ninhydrin reaction strips are cut to contain the central parts of the spots. Paper correction cannot be done, as it is not possible to cut the strips in the right way before ninhydrin reaction. In comparing measurements the peak heights (Block's "maximum intensities") have given practically the same accuracy as the peak areas. We have therefore mostly used these values for the wide strips as measurement with them is rapid.

None of the techniques for preparation of chromatograms so far used, have been finally approved, as the accuracy of all chromatogram series made has been much inferior to that of the self-recording apparatus. When known solutions of pure amino acids have been analysed using wide strips and peak heights, the error for most amino acids has been 5—10 %, and for alanine, serine and aspartic acid 2—5 %. Two spots of each amino acid at the optimum concentration region, 1—10 mM/l (Block [12]), and a standard curve with 5 points (1, 2, 3, 4 and 5 μg/spot) has been used (Fig. 6A and B). Ninhydrin reaction has been carried out according to Block [12].

Preparation of the electropherograms.

The electropherograms have been prepared essentially according to Grassman and Hannig [6] in a self-constructed apparatus [10], later in the commercial "Elphor"-apparatus (Bender & Hobein, München). Filter paper obtained from the last-mentioned firm and Whatman papers N:o 1, 7 and 11 have given rather similar results.

We have followed Grassmann and Hannig's technique except in two points, these being the application of the sample and decolorization of the strip.

Application is made as follows. The strip is drawn through the buffer, put on some sheets of filter paper, and the place of starting line is heavily dried [22] by pressing it with a narrow "drier", made by winding filter paper two or three times around a glass strip. The pherogram strip is immediately fixed to the moist frame and 10—20 μl of appropriately diluted serum (1 : 2 or 1 : 3) rapidly applied to the middle of the approx. 5 mm wide dried zone. Buffer, siphoning from both sides, diminishes the starting zone, also wetting it and preventing any denaturation and irreversible absorption on dry fibres.

Decolorization procedure has been modified as we have not been able to achieve complete decolorization by dry methanol, containing 10 % acetic acid, and any surplus colour in the strip causes too low albumin and too high γ-globulin values (mean values: alb. = 53, α_1 = 5, α_2 = 10, β = 10, γ = 22 instead of those in Table 1 [10]). Of solvents tried (methanol, ethanol, butanol, acetone and dioxane, containing different amounts of acetic acid and water) "Partridge-mixture"[20], well known from paper chromatography, made according to Campbell *et al* [21]. (630 ml n-butanol + 270 ml water + 100 ml acetic acid; 48 h standing; upper phase used) has given the best results. Corresponding methanol and ethanol mixtures have been too effective causing also decolorization in protein zones. Accurate time control is also necessary with butanol-mixture.

The decolorization procedure finally approved contains 7 5—20 min. washings (5, 10, 15 and 4 × 20 min.) in methanol containing 10 % acetic acid and 5 5 min. washings in the Partridge-mixture. After decolorization the strips have to be completely white except in the protein zones.

Decolorization is the most critical phase in preparation of the electropherograms and comparable results are obtained only if it is always carried out in exactly the same way.

RESULTS

Serum analysis. For comparison, determinations were made from the same samples by Tiselius-electrophoresis and the new method. The determinations

Table 1. Protein components of normal human serum and a pathological serum, determined by optical electrophoresis and paper electrophoresis.

	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Total
By optical electrophoresis:	%	%	%	%	%	%
Normal serum, mean values	59.1	3.9	8.3	14.3	14.4	100
Nephrosis serum	15	6	27	41	11	100
By paper electrophoresis:	Σ* %	Σ %	Σ %	Σ %	Σ %	Σ %
Normal serum, strip 23A	645 54.1	39 3.27	118 9.89	137 11.5	254 21.3	1 193 100.06
» » » 23B	1 014 58.1	49 2.81	149 8.54	237 13.3	300 17.2	1 744 99.95
» » » $24A_1$	816 57.5	59 4.15	116 8.17	192 13.5	237 16.7	1 420 100.02
» » » $24A_2$**	830 58.0	58 4.05	117 8.18	191 13.3	235 16.4	1 431 99.93
» » » 24B	828 57.4	47 3.26	118 8.18	181 12.6	268 18.6	1 442 100.04
» » » 25A	696 55.9	46 3.70	112 9.00	137 11.0	253 20.3	1 244 99.90
» » » 25B	1 038 58.7	71 4.02	148 8.38	211 11.9	299 16.9	1 767 99.90
» » » 26A	836 60.0	51 3.66	132 9.48	146 10.5	228 16.4	1 393 100.04
» » » 26B	708 58.2	42 3.45	118 9.70	149 12.2	200 16.4	1 217 99.95
» » mean values ($\overline{X}$)	57.5	3.54	8.92	12.1	18.0	100.0
» » standard deviation ***	1.7	0.41	0.65	0.99	1.8	
Nephrosis serum, mean values	18.8	4.4	41.9	16.2	18.8	100.1

* Σ = Sum of the columns under the peak in extinction units × 100..

** Duplicate determination to $24A_1$, not included in mean values and standard deviation.

*** Calculated acc. to $\sigma = \sqrt{\frac{\Sigma(X_\nu - \overline{X})^2}{N}}$ (X_ν = individual determinations).

by Tiselius-electrophoresis were kindly made for us by Mr. A. Louhivuori at the State Serum Institute, Helsinki.

Results with two samples of human serum, normal serum (a mixture of 10 sera), and a pathological one from a nephrosis patient are presented in Table 1 (see also Fig. 7—9). A number of determinations were made from both sera by paper electrophoresis and one strip was recorded twice by the photometer to give a picture of the reproducibility of the measurements ($24A_1$ and $24A_2$). All pherograms and densitograms were made in a routine manner, without any special precautions, and give a true picture of variations. In addition to 8 individual determinations by paper electrophoresis, mean values and standard deviations calculated from them are given for normal serum. As can be seen from Table 1, the mean values for paper electrophoresis correspond rather well to those of Tiselius-electrophoresis in the case of normal serum, except that γ-globulins give slightly higher and albumin and β-globulins slightly lower values. This may be due to differences in affinities of these components to the dye used.

In the case of the pathological serum reported in Table 1 (see also Fig. 9) the values of other components also correspond fairly well to each other except those of α_2- and β-globulins. The β-component is a lipoprotein which gives a very high value in Tiselius-electrophoresis, but only slightly higher than normal in the paper electrophoresis. On the other hand, α_2-component gives very high values in paper electrophoresis.

DISCUSSION

The accuracy of paper electrophoresis, using the method described above, is of the same degree as that of the classical Tiselius-electrophoresis and both methods give rather similar results with normal serum. Several authors [22–24] have also reported good conformity between values of Tiselius-electrophoresis and paper electrophoresis in the case of pathological sera. Although this may be true in most cases, it is certainly not always true as is shown by the one case reported here and has also been pointed out by Köiw *et al.*[25]. This is quite understandable as both methods are based on different qualities of protein components: one upon refractivity, the other upon affinity to amidoblack-10 B. Pathological sera may therefore give rather different results by the two methods especially in cases when lipoproteins are present. The methods therefore supplement rather than replace each other. The great advantage of paper electrophoresis is its simplicity and rapidity. If the analyses are made in series and the technique well standardised, one laboratory technician can easily make 5—10 complete determinations in a working day of 7 hours. The method may therefore become clinically valuable.

The error of the measurement of peak areas as sum of the columns is approx. 0.3 % and the accuracy of the apparatus is of the same degree. The accuracy of the method as a whole depends therefore entirely upon the quality of the pherograms and chromatograms. The present accuracy of *ca.* 5—10 % for both electropherograms and amino acid paper chromatograms can evidently be increased. Irregularities of the filter papers available are one of the main sources of error. A more even and thin paper quality would have two advantages: more even baseline for the densitograms and more regular zone and spot form.

For amino acid analysis the regularity of spots obtained with pure samples does not yet, of course, determine the practical value of the method. Tissue extracts and other natural samples usually contain disturbing impurities which have to be removed first. Mixtures of amino acids also have to be rather simple to be analyzable by one-dimensional technique only. Block [12] has developed a technique for measurement of two-dimensional chromatograms, where maximum colour density, an internal standard and a large number of replicate determinations are used. This method is not practical with a self-recording apparatus of this type. Strips, containing central parts of the spots can be measured, but because of the great irregularity of spots in the two-dimensional chromatograms accuracy becomes too low to make the rather laborious procedure practical.

The best way to obtain paper chromatograms from natural amino acid samples for analysis by a self-recording instrument of the present type would probably be through simultaneous purification and quantitative prefractionation of amino acid mixtures into groups by a suitable ion-exchange technique. The groups obtained would then be easier to fractionate by one-dimensional runs.

The high reproducibility of measurements by our method proves that it is not necessary to clamp the paper strip, rendered transparent by oil-immersion, between two glassplates as in the corresponding manual instruments. The

simple filter photometer used has also proved completely satisfactory, even though a filter with higher transmittancy would be desirable. Replacement of the filter with a monochromator would of course greatly enhance the versatility of the apparatus. Strip adapters for Beckman Spectrophotometer [26] and Cary Recording Spectrophotometer [27] have been published. As has been pointed out by Parke and Davis this makes possible the measurement of compounds, having a selective absorption in the ultraviolet, without any colour reaction.

On the practical side of the method development in the quality of chromatograms is of much higher importance, however, than development of the apparatus.

SUMMARY

A self-recording strip photometer has been constructed by combining a filter photometer with the self-recording galvanometer of the Heyrovsky polarograph. An extinction scale, divided into narrow columns, has been designed, this scale being copied photographically upon the recording paper of the galvanometer prior to its use. The scale and curve are developed simultaneously. Peak area is obtained as the sum of the column heights under that peak. For paper electropherograms the relative concentrations of protein components are calculated as percentages of the peak area as compared with the total area under the densitogram. For amino acid chromatograms either peak areas or peak heights and standard curve technique are used.

Replicate determinations of the same strip differ by approx. 0.5 %. Determinations of replicate chromatograms or electropherograms from the same sample differ 5—10 %. Normal serum gives with this method approx. similar results as with Tiselius-electrophoresis, but pathological sera may give quantitatively rather different results, possibly due to different affinity of the components to the dye used, amidoblack-10B.

The practical value and development possibilities of paper strip photometry are discussed.

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A Paperelectrophoretic Investigation on Milk Serum Proteins

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Investigations of milk serum proteins by the classical Tiselius-electrophoresis [1-3] have revealed 5—6 electrophoretically separable components in normal milk serum. The present paper describes a study of milk serum proteins by the newly developed paperelectrophoretic method and a preliminary investigation of the effects of pasteurization and sterilization, as made in the dairy industry, on these proteins.

EXPERIMENTAL

Preparation of the milk samples. Fresh cowmilk was divided into three parts: one part was used as such, one part was pasteurized in a plate pasteurizer for 15 sec. at 72° C **, one part was sterilized for 25 min. at 119° C. These three milk samples were further treated in exactly the same way.

By addition of normal hydrochloric acid the pH was brought to 4.6 under control of the glass electrode. Casein, together with some other (denatured) proteins, was separated by centrifugation and the resulting whey filtered. After an overnight dialysis in cellophane tubes against three times renewed distilled water the clear wheys were lyophilized.

From the dry powders thus obtained samples were analyzed for their moisture and protein contents. The protein content was calculated from the results of micro-Kjeldahl determinations using the factor 6.35.

Preparation of the paper electropherograms. From the lyophilized powders three solutions were made each having a protein concentration of 6 %. These were analyzed by paper electrophoresis using a veronal buffer of pH 8.6 and ionic strength 0.1. The paper electropherograms were made essentially according to Grassmann and Hannig [4], using 110 V potential during 13 hrs. The application of the sample and the decolorization of the amidoblack-dyed strips were, however, made by a slightly modified procedure [5]. The paper strips (Fig. 1) were measured by a selfrecording strip-photometer according to Miettinen and Moisio [5].

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** Kindly arranged by Dr. M. Kreula from this institute, at the dairy of Valio in Helsinki.

RESULTS

The results of protein determinations for 100 mg of powders, dried during 3 hrs at 105° C, are given in Table 1.

Table 1. Protein contents of wheys from normal and heat-treated milks.

Whey-powder of	mg protein per 100 mg dry powder
Natural milk	25.7
Pasteurized milk	25.2
Sterilized milk	13.3

It can be easily seen, that in the case of sterilized milk about half of the whey proteins have been co-precipitated with casein. The brown colour, developed by sterilization of whole milk also separated in the casein precipitate.

The results of paper electrophoresis are presented in Table 2 and Figs. 1 and 2.

5 components are clearly separated in the electropherograms of *normal whey*, as can be seen from Fig. 1A and 2A. They have been tentatively identified as components a, b, c, d + e, and f of Smith [1]. The location of Smith's component "d" in the paper electropherograms is not quite certain yet, but in a number of densitograms the β-lactoglobulin-peak has been unsymmetrical in a way suggesting the presence of a second component.

The mean relative concentrations of the separated components, calculated by the above mentioned method [5], as well as the distances of the peaks on the densitogram from the starting line (a kind of "mobility"-value) are compared in Table 2 with the relative concentrations and mobilities obtained by Smith with the classical electrophoresis [1]. As electroendosmosis was not measured in our experiments, we have made no effort to calculate the real mobility values.

Table 2. Mobilities and relative concentrations of proteins of normal whey, determined by E. L. Smith [1] with the Tiselius-electrophoresis, compared with the relative concentrations and distances of the peaks from the starting line, determined by paper electrophoresis.

	Immunoglobulines a eu-	b pseudo-	c	d	e β-lacto-	f
Classical electrophoresis, mean values, by E. L. Smith [1]	% u *	% u	% u	% u	% u	% u
	6 −1.7	4 −2.5	18 −3.6	12 −4.5	55 −5.1	5 −6.4
Paper electrophoresis, mean values	% mm	% mm	% mm		% mm	% mm
	7 20	13 26	16 37		60 48	4 60

* u = sq. cm per volt per sec. × 10^{-5}.

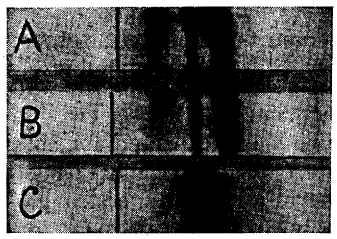

Fig. 1. Whey proteins from normal (A), pasteurized (B) and sterilized (C) milk, separated by paper electrophoresis.

Paper electrophoretic results correspond fairly well to those obtained by Tiselius-electrophoresis, except that values obtained for immunoglobulins by paper electrophoresis are about twice as high. This may be due to a higher affinity of these proteins to amidoblack, as has been suggested by Miettinen and Moisio [5] to be the case with the γ-globulins of human serum. Another explanation would be a real difference between the milk samples analysed by Smith and the present authors.

The patterns obtained with the whey from *pasteurized milk* (Fig. 1B and 2B) do not differ much from those of normal milk. However, the zone of immunoglobulins is usually less sharp, more material being left between this zone and the starting line. The relative concentration of the immunoglobulin-fraction has also a tendency to be slightly lower than in normal whey. It seems therefore, that even the H.T.S.T.-pasteurization used (15 sec. 72° C) has caused some denaturation of this nutritionally important protein fraction.

Sterilization has a very great effect upon the whey proteins. Proteins, which are not already co-precipitated with casein, give in paper-electrophoresis one diffuse zone (Fig. 1C), which migrates with a mobility between that of component c and β-lactoglobulin (Fig. 2C), being evidently denatured β-lactoglobulin. By sterilization almost all of the whey proteins seem thus to be denatured and substantially changed.

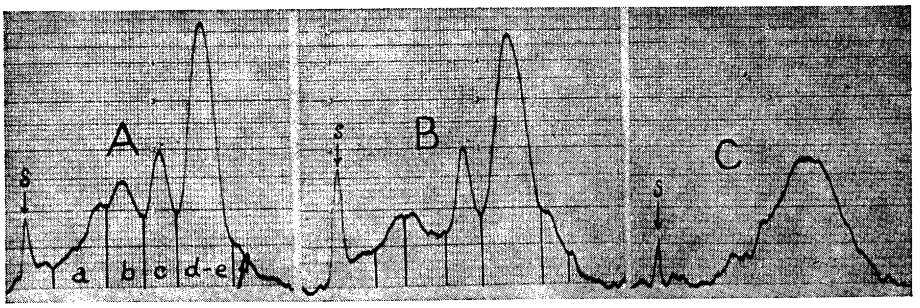

Fig. 2. Densitograms from paper electropherograms, made of whey proteins from normal (A), pasteurized (B) and sterilized (C) milk. S = starting line. Components acc. to Smith [1]: a = euglobulins, b = pseudoglobulins, c = component, d = component, e = β-lactoglobulin, f = component.

SUMMARY

Paper electrophoresis of normal whey, prepared by acid-precipitation of casein at pH 4.6, has given results otherwise comparable to those obtained by earlier workers with the Tiselius-electrophoresis, except that values twice as high have been obtained for immunoglobulins by paper electrophoresis.

Whey from the H.T.S.T.-pasteurized (15 sec. 72° C) milk has given approximately similar results, although the appearance of the electropherograms and the relative concentrations calculated from the densitograms suggest a slight denaturation of the immunoglobulins.

The "whey" from sterilized milk has given only one diffuse zone (β-lactoglobulin with decreased mobility), which shows that milk serum proteins are substantially changed in sterilization.

The authors whish to acknowledge their great indebtedness to Professor A. I. Virtanen for his very kind interest and for the many facilities which have been put at their disposal.

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Nitrogen Metabolism of Pea and Alder

Transamination of γ-Aminobutyric Acid and L(+)-Citrulline with α-Ketoglutaric Acid

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Occurrence of γ-aminobutyric acid and L(+)-citrulline in free form in tissues of alder(*Alnus*) [1] and of γ-aminobutyric acid and β-alanine in pea plant (*Pisum*) [2,3] has arisen interest in the metabolic fate of these non-protein amino acids in plants. Our attention has been specially devoted to citrulline as high concentrations of this amino acid are found in alder tissues but the reactions by which it is connected with the plant metabolism are evidently incompletely known. In a previous paper [4] possible occurrence of arginase and L(+)-glutamic acid decarboxylase in these plants was studied. Arginase activity could not be shown to be present in alder, which means that the urea cycle evidently could not operate. Tissue homogenates of pea effected heavy formation of γ-aminobutyric acid from L(+)-glutamic acid by a soluble decarboxylase, homogenates of alder tissues effected only slight formation by an insoluble decarboxylase.

As an extension of these studies occurrence of transaminases for these amino acids in pea and alder has now been studied. Transamination of γ-aminobutyric acid and β-alanine with α-ketoglutaric acid has been recently shown in animal tissues [5,6], but could not be shown in two plant tissues, ripening avocado and fresh pepper, when studied by Roberts and Bregoff [6]. We have found in pea roots strong transamination of γ-aminobutyric acid and in them and alder leaves slight transamination of L(+)-citrulline with α-ketoglutaric acid, but no reaction could be shown between β-alanine and the keto acid in these tissues.

EXPERIMENTAL

The plant samples studied were harvested at the beginning of August: 1) roots of pea (test plants grown on field, age 6 weeks, at the beginning of flowering, nodules big and red); 2) leaves and 3) root nodules of gray alder *(Alnus incana)*, grown in greenhouse. The experiments with the roots of pea were made as follows:

Homogenate was made by crushing 10 g of fresh, nodulated roots in 5 ml *M*/7.5 phosphate buffer, pH 7.5, to a pipettable suspension. α-ketoglutaric acid solution was made

by dissolving sodium α-ketoglutarate (Hoffman La Roche) in the abovementioned buffer to contain 20 mg of the acid per ml.

Reaction mixtures 1—8 were made by mixing 0.2 ml plant suspension and 0.1 ml ketoglutaric acid solution and adding the other reagents in dry form as follows:

1 20 mg DL-alanine
2 20 mg β-alanine
3 20 mg γ-aminobutyric acid
4 20 mg L(+)-citrulline
5 20 mg γ-aminobutyric acid + 2 mg NH_4Cl
6 20 mg L(+)-citrulline + 2 mg NH_4Cl
7 no addition
8 2 mg NH_4Cl

Reaction mixture No. 9 was made by mixing 0.2 ml plant suspension and 0.1 ml buffer, No. 10 by mixing 0.2 ml *boiled* plant suspension + 0.1 ml buffer.

Reaction mixtures Nos. 11—15 were made as No. 7 and Nos. 1—4 except that prior to mixing the plant homogenate was kept in a test tube in boiling water bath for 5 min. to inactivate all enzymes.

11 no addition
12 20 mg DL-alanine
13 20 mg β-alanine
14 20 mg γ-aminobutyric acid
15 20 mg L(+)-citrulline

The reaction mixtures, with a final phosphate concentration of *ca.* *M*/15 and pH 7.4, were incubated at 38° C for 4 hrs with occasional shaking after which initial spots of 0.01 ml were pipetted for chromatographing on Whatman No. 1 paper. Watersaturated phenol in NH_3-athmosphere (20° C, 40 hrs) was used for irrigation and ninhydrin for colour development.

The experiments with leaves and nodules of alder were made in the same way as those described above, except that the homogenate was made by using for 10 g of fresh plant tissue 20 ml buffer.

With all three tissues two other incubation series were made where the plant homogenate was fortified before mixing with other reagents with 2 mg $MgCl_2 \cdot 6\,H_2O$ and 2 mg pyridoxal phosphate per ml in one series and 2 mg of $MgCl_2 \cdot 6\,H_2O$ + 2 mg pyridoxamine phosphate per ml in the other series. These additions had no detectable effect on the results.

RESULTS

The following results were obtained with *pea roots*. Even addition of α-ketoglutaric acid alone (chromatogram No. 7 in Fig. 1) led to slight formation of glutamic acid from amino acids present in small amounts in the pea plant suspension (No. 9), especially from aspartic acid which reacted quantitatively. A highly significant increase of glutamic acid above this level was obtained with addition of alanine (No. 1) or γ-aminobutyric acid (No. 3). Addition of citrulline also led to a small increase of glutamic acid (No. 4), but addition of β-alanine evidently did not lead to any increase. The amounts of glutamic acid present in the chromatograms (in μg) are, based on visual comparisons with a series of known dilutions: No. 9 = 0.5; Nos. 8, 7, and 2 = 1; Nos. 6 and 4 = 1.5; Nos. 5 and 3 = 10; No. 1 = 20.

A homogenate of *alder leaves* effected with alanine a heavy formation of glutamic acid from α-ketoglutaric acid (ca 30 μg/10 μl), but only a weak although clearly positive formation was obtained with citrulline (1 μg/10 μl). With γ-aminobutyric acid and β-alanine negative results were obtained.

In a homogenate of *alder nodules* negative results were obtained with all abovementioned amino acids and also with aspartic acid.

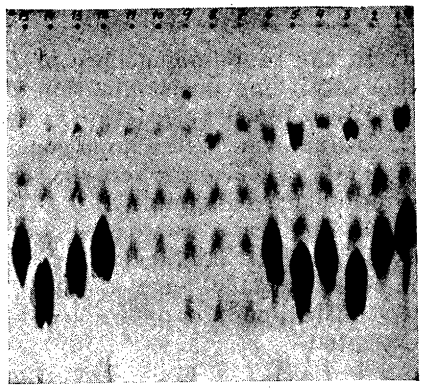

Fig 1. Paperchromatograms prepared from different reaction mixtures (plant suspension + α-ketoglutaric acid + different amino acids etc.) Cf. *composition of the reaction mixtures, and other details in experimental part p. 1244.*

Addition of NH_4Cl (*e.g.* Nos. 5, 6 and 8 in Fig. 1) had in no case any effect upon the amount of glutamic acid formed. The slightly higher mobility and elongation of the glutamic acid spot in these chromatograms is due to a local change of pH, caused by the chloride spot. It is therefore evident that the reactions obtained have not been due to a reductive amination of α-ketoglutaric acid by ammonia liberated from the amino acid added.

Addition of α-ketoglutaric acid only, or this acid and amino acids, to the boiled extract (Nos. 10—15) did not lead to any increase of glutamic acid.

DISCUSSION

The results of the present study show that the roots of the pea plant contain an enzyme system catalyzing transamination between γ-aminobutyric and α-ketoglutaric acids. Whether the reaction in pea is reversible remains to be shown. By this reaction γ-aminobutyric acid may enter the normal metabolic cycles. The only reactions where this acid is known to partake are, so far, its formation from glutamic acid by decarboxylation or from glutamic acid and succinic semi-aldehyde by transamination [5] and its conversion back to the last-mentioned compounds by transamination with α-ketoglutaric acid. Whether it still has some more specific function, is unknown. There seems to be great difference between different plants in respect to this transaminase. The pea plant contains considerable activity, but avocado and fresh pepper [6] nil. As all these plants are rich in L-glutamic acid decarboxylase, there seems to be no correlation between these two enzyme activities either.

The results of the present study suggest also weak transamination of citrulline with α-ketoglutaric acid in pea root and alder leave homogenates. This reaction may play a role in mobilization of citrulline nitrogen in the leaves of alder. The presence of this enzyme system in leaves and its absence from the nodules would be in conformity with the role ascribed to citrulline in alder: transformation of nitrogen, fixed in the nodules, to the other parts of the plant.

SUMMARY

1. In the root homogenate of *Pisum* has been demonstrated an enzyme system catalyzing transamination of γ-aminobutyric acid with α-ketoglutaric acid. The rate of the reaction is about half of that found with alanine.

2. In the same tissue homogenate could be demonstrated weak formation of glutamic acid from α-ketoglutaric acid by L(+)-citrulline, but no reaction was obtained by β-alanine.

3. In the leave homogenate of *Alnus incana* was demonstrated a weak formation of glutamic acid from α-ketoglutaric acid by citrulline, but not by γ-aminobutyric acid or β-alanine.

4. Crushed nodules of *Alnus* did not catalyze any transamination reactions investigated.

The authors are indebted to Miss Vuokko Mickelsson and Miss Sigrid Kari for technical assistance in this investigation.

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An Inverted Microosmometer and Its Use for the Determination of Molecular Weight of some Specimens of Potassium Hyaluronate

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A microosmometer of about 500 μl capacity is described. A semipermeable cap is on the upper end of an open capillary and both are completely immersed in the outer liquid.

The hydrostatic pressure-difference is produced by an air column in the capillary confined between the inner and the outer liquid respectively. The height of the air column is varied by varying the pressure on the outer liquid between 1 and 0.1 atmosphere. At each pressure-difference the flow through the membrane in 10 minutes is determined. The height for zero flow is determined graphically.

One advantage is that the capillary correction is nearly zero, another that pressure-differences of about 1 mm water column can still be measured.

In the course of time many osmometers have been constructed all of which, however, come within one of the two types: 1) the static-elevation cell in which the osmotic pressure of the solution is counterbalanced by the liquid column developed by the influx of solvent into the solution, and 2) the dynamic-equilibrium cell in which the osmotic pressure is counterbalanced by an externally applied pressure of known magnitude. As examples of more recent static-elevation cells we name in chronological order the osmometers of Krogh and Nakazawa [2], Schulz [3], Herzog and Spurlin [4] and Bull [5]. The dynamic-equilibrium principle which underlies the osmometer to be described here, was first used by Tammann [6] and some years later by Berkeley and Hartley [7] in their studies of sucrose solutions, and Sørensen [8] determined the molecular weight of egg albumin by a dynamic-equilibrium osmometer constructed by one of the authors of the present article (described in the paper of Sørensen [8]). Many osmometers based upon the same principle have been described in the literature, a more recent one by Güntelberg and Linderstrøm-Lang [9].

Description of the New Apparatus. The principal parts of the apparatus are outlined in Figs. 1 and 2. The osmometer consists of a testtube-shaped

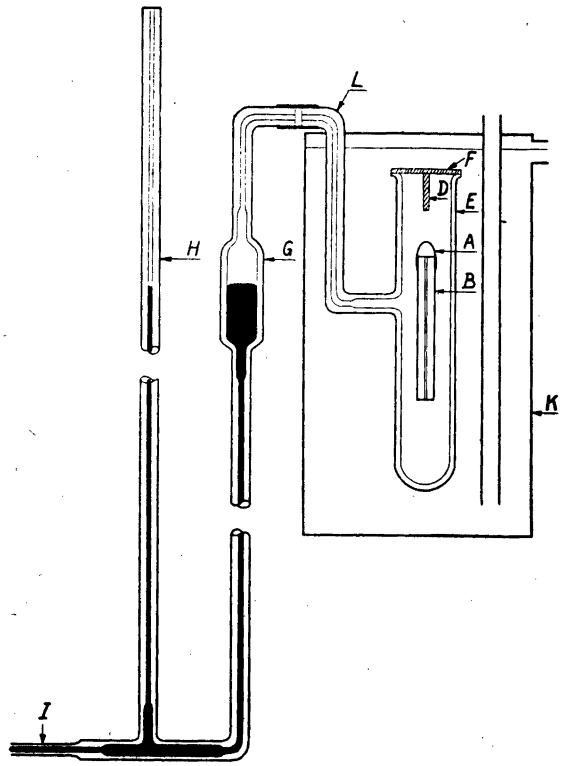

Fig. 1. The principal parts of the apparatus. For explanation see the text.

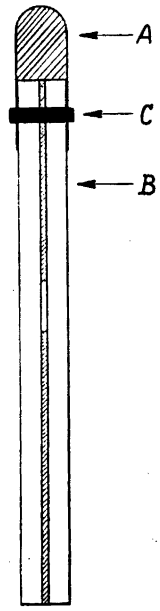

Fig. 2. The osmometer proper showing the air column.

semi-permeable membrane A, tightly connected to a glass capillary B of about 0.7 mm bore, outer diameter approximately 7 mm, length approximately 70 mm. The volume of the cap is about 400 μl. A rubber ring, marked C in Fig. 2, ensures perfect tightness. A support arm D, provided with two clamps, holds the osmometer which is completely immersed in the outer liquid contained in the glass vessel E. D is cemented to a lid F, which covers the vessel E, whose edge is ground plane. The lid, which is also plane, the arm and the clamps are made of perspex. Only the upper part of the arm D is indicated in Fig. 1. By means of a clamping device mounted upon the upper part of E, (not shown in Fig. 1) the apparatus can be made water-tight. E is connected with a pressure device, G H, by means of rubber tubing. Part of the space of the pipette G, (white in Fig. 1) the connecting capillary L and the vessel E are completely filled with the outer liquid. The rest of pipette G, (black in Fig. 1) and the manometer tube H, contain mercury. Tube I is connected to a mercury levelling device. Actually we use a mercury-tight steel-piston pump operated by means of a screw. This device is exceedingly convenient, but we suppose that a mercury levelling device similar to those used in many gas-

analysis apparatuses, *e.g.* in A. Krogh's modification of the Haldane apparatus (described by Lindhardt [10]) may be sufficiently convenient, and it will certainly be much cheaper to manufacture.

The height of the mercury column in *H* is roughly measured on a meter stick divided in millimetres. By means of the arrangement the total pressure on the system can be varied from just above one atmosphere to about one tenth of that amount. Water from a thermostat is circulated through the perspex walled rectangular container *K*, thus keeping the temperature of the container constant at 20° C (Variations about 0.01° C).

The Semi-permeable Caps. For some measurements we used collodion caps prepared by ourselves essentially according to the method elaborated by one of us and described in Sørensen's paper [11] and for others cellophane caps kindly presented to us by the cellophane factory "Kapcello".

As we have modified the procedure for making collodion caps a little it may be useful to describe it in detail. As a mould we use a test tube provided with a small hole in the bottom. The outer diameter of the test tube is about one tenth of a millimeter less than that of the osmometer capillary. Before use it is rinsed thoroughly with soap and water and wiped dry with a towel. It is then attached to a slowly rotating shaft (40—60 r.p.m.) which is so mounted that it can be inclined from zero to at least about 30° relatively to the horizontal position. At first the shaft is inclined and the collodion solution is poured on the bottom end of the testtube. A few seconds after that the rotation is stopped and excess solution is allowed to dribble off. After further rotation for about 2 minutes the application of collodion solution is repeated but so that the desired length of the tube becomes coated. After a momentary stop to remove a drop of excess collodion by means of a spatula the shaft is turned into the horizontal position, and the rotation continued for about 4 minutes, after which time a second coating is applied in the same manner, but concluding with a rotation (shaft horizontal) for 20 minutes. Finally the coated tube is placed in water for at least two hours. Then a ring is cut in the coating with a razor blade so as to produce a cap of suitable length, usually between 10 and 20 mm.

The cap is easily slipped off the test tube by hand and is now ready for use. They should be kept in water saturated with toluene. Under these circumstances they seem to retain their permeability for water at least for months.

The collodion solution is prepared from "Collodion B.D.H." which is dried to constant weight. 15 g of the dry collodion is dissolved in 375 ml anhydrous alcohol and 125 ml anhydrous ether by repeated shaking during some days.

The Kapcello caps are furnished moist from the factory (Kapcello, Mosevej, Copenhagen) and are kept in the laboratory in water saturated with toluene at about 0—3° C. Before use they are placed in running tap-water for a couple of hours and rinsed a few times in distilled water. They are impermeable to the compounds investigated by us and are easily permeable to water and dissolved low-molecular substances. We have therefore omitted the drying procedure used by Rehberg [12]. For further details regarding Kapcello caps see Rehberg [12] and Grandjean [13]. According to our experience they are somewhat less permeable to water than the collodion caps, which follows from Table 1, but both are convenient for practical use. In one case we have determined

the molecular weight of the same specimen both by collodion and Kapcello caps and found mutually agreeing figures, the slopes of the interpolation curve (mentioned later in this paper) being 3.2 and 1.4 respectively.

Anyhow the test-tube shape and the small dimensions tend to facilitate the attainment of partition equilibrium in respect of diffusible particles.

Preparation of the Osmometer. The osmometer capillary is ground in both ends with fine carborundum powder and thoroughly rinsed for grains of abrasive and for grease.

The cap, whose inner diameter must be just a trace smaller than the outer one of the capillary, is placed on one end of the capillary as shown in Fig. 2. To secure complete tightness a narrow rubber ring *C* is placed around the supported part of the cap.

To perform the latter operation we have constructed a special device, Fig. 3. It consists mainly of two pieces of brass tubing one sliding in the outher. The innermost is a little wider inside than the osmometer (the cap included). The relative movement of the tubes is limited by crosspin and slit as indicated. A spring causes the inner tube to protrude about 5 mm relatively to the outer one. In this position the rubber ring is placed on the inner tube. When now the osmometer is held as in Fig. 3 a withdrawal of the inner tube causes the rubber ring to slide onto the cap in the desired position, and the osmometer is now ready for filling.

Filling the Osmometer. To fill the osmometer we have found it necessary or at least very convenient to use a special apparatus. This consists of two parts, a manipulator table provided with a clamp for the osmometer, the table being movable in three directions, and a fixed pipette with a capillary tip which can easily pass through the capillary of the osmometer. The table is shown in Fig. 4, which is drawn to scale and diminished in the ratio (3 : 1).

As base we use the tripod of an ordinary laboratory stand provided with two adjustment screws to secure vertical position of the axis. Just above the base is a cross-table *a* enabling the upper part to be moved by screws in two mutually perpendicular horizontal directions.

The cross-table wears a vertical hollow column *b* which is connected, through a rack-and-pinion device, with a vertical bar carrying a horizontal

Table 1. The figures in the first column give the rate of movement of the upper meniscus of the air column for a pressure-difference of 1 mm water column when the caps quoted in the second column are used.

Slope of interpolation curve	Type of caps
2.3	Kapcello 1
3.0	Collodion 1
3.2	Collodion 1
4.2	Collodion 2
1.2	Kapcello 2
2.6	Kapcello 1
—	—
0.5	Kapcello 3
—	—
0.6	Kapcello 3

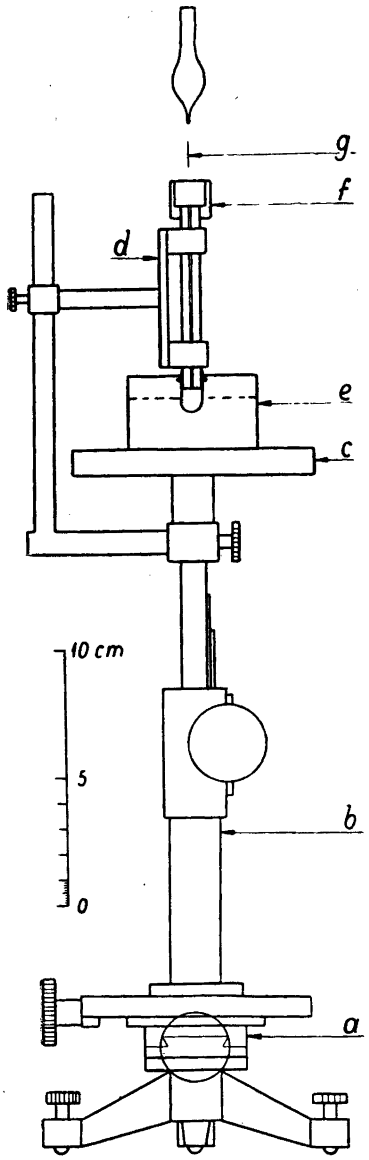

Fig. 4. The apparatus for filling the osmometer. Explanation in the text.

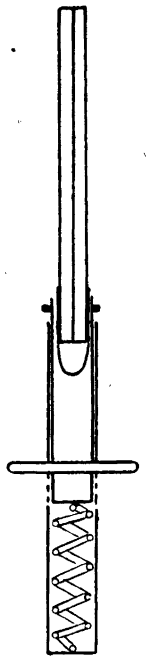

Fig. 3. The device for placing the rubber ring.

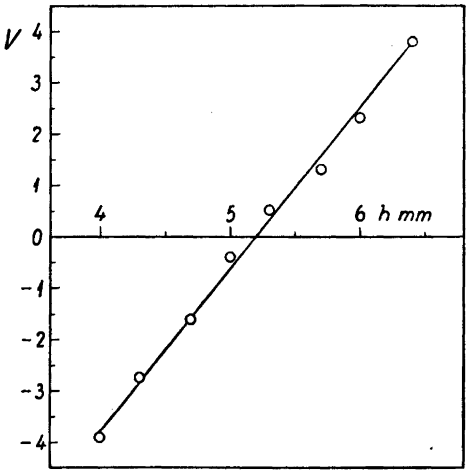

Fig. 5. The graph shows the relation between the height of the air column (abscissae), and the rate of movement of the upper meniscus of the air column (ordinates) in micrometer divisions in ten minutes.

wooden table *c* and a spring-clamp *d* for the osmometer. During the operations the cap of the osmometer can be immersed in a beaker *e* containing the outer liquid to avoid undue drying of the membrane. For filling the osmometer the upper end of the capillary is provided with a removable but tightly fitting perspex cup *f*. Above this is a fixed pipette ending in a capillary *g* so finely drawn that it can easily pass through the whole length of the osmometer capillary. The upper end of the pipette is connected to a syringe the air-tight piston of which can be moved by means of a screw.

The filling is now performed as follows: By means of the manipulator the center of the orifice of the osmometer capillary and that of the capillary tip of the pipette are made to coincide. Next the osmometer is raised vertically so much that the capillary tip is near the bottom of the semi-permeable cap. Now a suitable amount of the inner liquid is filled into the perspex cup. By means of the syringe air is slowly withdrawn from the osmometer which is simultaneously lowered so that the tip is always just above the level of the liquid. The osmometer is thus filled with inner liquid to about somewhat less than half of the capillary. After some practice we have had no difficulties in avoiding air bubbles in the inner liquid, which is essential.

Finally the osmometer is lowered so that the capillary tip is free, and the rest of the capillary is filled with the outer liquid by a similar procedure, care being taken that an air column of a suitable length separates the two liquids. After removal of the perspex cup the osmometer is placed upwards down in the apparatus Fig. 1, which is filled with the outer liquid.

The Microscope. The reading microscope is of the commercial type. It has a vertical stem with two adjustments. By means of the one the microscope is raised to a suitable height and fixed. By means of the other its vertical position can be varied by means of rack-and-pinion. The latter displacements are registered on a vertical scale with vernier (accuracy 0.1 mm). The magnification of the microscope is low and the object distance rather large. The eyepiece micrometer has a scale with 10 × 10 divisions, the whole length of the scale being 5.1 mm.

Calculation of the Pressure-difference. The pressure is calculated from the height of the air column by multiplication with the density of the outer solution and the gravitational constant at the place of the measurements. Strictly speaking three corrections should be applied: The first in regard to the fact that the density of the air column is not zero, the second should take account of the difference in densities of the inner and the outer solutions respectively, and the third should be calculated from a knowledge of the difference in surface tension of the two solutions. All of them are small, however, and we have neglected them in the present investigation, but in precision measurements their magnitude, and especially that arising from the difference in surface tensions must be evaluated.

Mode of Operation of the Osmometer. To begin with the vessel *E*, the connecting capillary *L*, and the bulb *G*, are filled with the outer solution. This can be accomplished by filling *E* and using the mercury levelling device as a pump. In this way all air can be removed. (Very small air bubbles are harmless.) Next the osmometer capillary is placed in the perspex clamp attached to the lid *F*. The latter is placed in its proper place, care being taken that no air

Table 2. Nitrogen and potassium content, viscosity and molecular weight of different preparations of potassium hyaluronate. The nitrogen is determined by a Kjeldahl procedure and the potassium as potassium sulphate. W is the weight in mg of hyaluronate dissolved in 1 ml of the outer solution (0.2 M KCl). h is the height in cm of the air column at equilibrium.

% N	% K	Relative Viscosity Conc. lg/l	*W* mg	*h* cm	Molecular weight × 10^{-3}
3.36	11.36	11.80	13.00	0.62	517
3.35	10.82	11.91	11.00	0.51	530
3.31	8.05	12.19	10.88	0.52	516
3.34	7.12	12.12	10.26	0.48	527
2.90	10.02	5.46	19.35	1.30	367
2.88	11.00	68.46	9.91	0.14	1 750
2.82	13.20	5.29	—	—	—
1.16	17.01	5.75	28.16	2.20	316
5.78	6.56	4.62	—	—	—
2.99	7.86	76.39	27.61	1.23	554

bubbles remain, and fastened by means of the clamping device (not shown in Fig. 1). Finally the container *K*, is filled with water and the circulation of thermostated water is started.

The measurements proper can only begin after the lapse of a certain relaxation time, some 30 minutes or more, during which time diffusion equilibrium is established. To prevent undue dilution of the inner liquid (1 μl corresponds to about 2.5 mm in the capillary), the length of the air column, which must be "too small" at atmosphere pressure, is adjusted as soon as possible by means of the mercury levelling device in such a way that its upper meniscus moves slowly or not at all during the relaxation time.

The measurements proper are performed as follows: The air column is set at a definite length which is measured either by means of the scale on the vertical stem of the microscope or by means of the eyepiece scale. The corresponding displacement of the upper meniscus in 10 minutes is measured on the eyepiece scale. When a number of such measurements have been made, the movements being partly upwards and partly downwards, the results are plotted and the equilibrium length found graphically. Besides this the slopes of the lines in the graphs which, apart from random errors, are straight give a rough measure of the permeability for the outer solution, rough because the surface of the caps are somewhat different in the different experiments.

As an example we give the graph in Fig. 5.

Measurements on Solutions of Potassium Hyaluronate. The outer liquid is in all the experiments reported here a 0.2 molar solution of potassium chloride. To avoid supersaturation with air, which sometimes causes trouble, the solution is boiled and cooled down before use. The inner solutions are prepared by weighing a suitable amount (10—30 mg) of the specimen in question and dissolving in 1.00 ml of the outer solution.

The results are in Table 2. The specimens are those described in a recent paper by one of us [14], and they occur in Table 2 in the same order as in that paper. It must be added that owing to the preliminary nature of the experiments and to the fact that the Donnan effect according to the composition of

the liquids may be expected to be small, no correction for the latter was introduced.

The molecular weight M has been calculated from the expression

$$M = qw/h$$

where w is the amount of substance in grammes dissolved in 1 cm^3, h is the equilibrium height of the air column in cm and q equals RT/gd, where $R = 8.314 \cdot 10^7$ erg/mole degree, $T = 293.16$, $g = 981.6$ cm/sec^2. (latitude of Copenhagen), $d = 1.0078$ gramme/cm^3. Calculation yields $q = 246.8\ 10^5$ cm^4/mole.

The great majority of viscosity determinations of hyaluronic acid vary from 1 to 8 most of them being below 4 as seen in a table by Hadidian and Pirie[15]. On the basis of measurements of double refraction of flow and viscosity Mayer and Palmer[16] and Blix and Snellman[17] have arrived at estimates of the molecular weight of hyaluronic acid ranging from 2×10^5 to 5×10^5. The smaller figures were found for synovial hyaluronic acid, the higher for human umbilical cord hyaluronic acid. As seen in Table 2 some of our preparations show a very high viscosity and the same is true regarding the molecular weights.

Financial support from *Carlsbergfondet* and *Det teknisk-videnskabelige forskningsraad* is gratefully acknowledged. Our thanks are due to J. Nawrocki for excellent mechanical workmanship.

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An Unexpected Formation of 9,10-Dimethylanthracene

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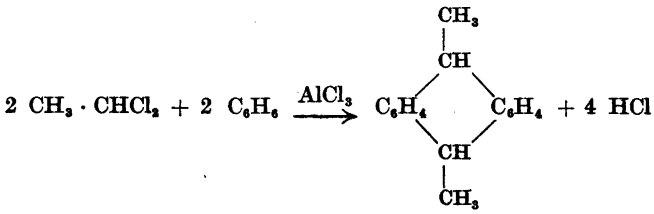
In an attempt to substitute aromatic hydrogen by the acetic acid residue a mixture of benzene and ethyl monochloroacetate was treated with anhydrous aluminium chloride. The reaction product did not, however, contain any phenylacetic acid but consisted of a mixture of hydrocarbons only. Having removed excess of benzene by distillation a yellow crystalline hydrocarbon $C_{16}H_{14}$ separated from the mixture the liquid part of which was found to contain mainly ethyl-substituted benzenes. The crystalline hydrocarbon, purified by sublimation in a vacuum and subsequent crystallization from methanol, had m.p. 181°. In solution it showed the strong blue to violet fluorescence characteristic of the anthracene hydrocarbons. On treatment with bromine two hydrogen atoms were substituted and as well the hydrocarbon itself as the brominated derivative on oxidation gave anthraquinone. From this it was concluded that the crystalline hydrocarbon was 9,10-dimethylanthracene. A preliminary report on this result was given two years ago [1]. Later experiments have shown that it is advantageous by the preparation of the 9,10-dimethylanthracene to use ethyl chloroformate instead of the ethyl monochloroacetate.

On treatment of a mixture of 1,1-dichloroethane and benzene with aluminium chloride Anschütz [2] obtained a yellow crystalline hydrocarbon with m.p. 181° which he regarded as 9,10-dimethyl-9,10-dihydroanthracene formed by the following reaction:



Later Barnett and Matthews [3] showed that the hydrocarbon of Anschütz actually was 9,10-dimethylanthracene. They prepared the dihydroderivative

in another way and found that it was easily dehydrogenated to the hydrocarbon with m.p. 181°.

In our experiments the formation of the dimethylanthracene must evidently be due to the ethyl group in the chloroacetic ester. This would be in accordance with the results of Kunckell and Ulex [4] who found that benzene was alkylated by treatment with chloroformic esters and aluminium chloride. Basing on the modern conception of the Friedel-Crafts reaction we therefore consider the formation of the 9,10-dimethylanthracene to take place through the following reactions.

Firstly the presence of aluminium chloride will give rise to the occurrence of the ethyl carbocation:

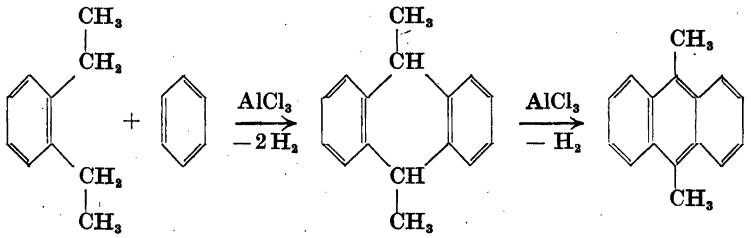
$$ClCH_2 \cdot COOC_2H_5 + AlCl_3 \rightleftarrows C_2H_5^+ + ClCH_2 \cdot COOAlCl_3^-$$

Secondly the electrophilic carbocation adds by a relatively slow process to the benzene nucleus forming a transition complex which rapidly loses a proton:

$$ArH + C_2H_5^+ \xrightarrow{\text{slow}} \left[Ar\begin{matrix} H \\ \\ C_2H_5 \end{matrix} \right]^+ \xrightarrow{\text{fast}} Ar \cdot C_2H_5 + H^+$$

$$H^+ + ClCH_2 \cdot COOAlCl_3^- \rightarrow ClCH_2 \cdot COOH + AlCl_3$$

By continued alkylation a mixture of higher alkylsubstituted benzenes will be formed, in the first hand, the isomeric diethylbenzenes. It is now suggested that the *ortho*-diethylbenzene in the presence of aluminium chloride condenses with benzene to the dihydroderivative of 9,10-dimethylanthracene which is immediately dehydrogenated to the dimethylanthracene itself:



This conception is in accordance with the fact that *ortho*-diethylbenzene (or *ortho*-dimethylbenzene) has never been prepared by the Friedel-Crafts reaction. Kunckell and Ulex [4] and others state explicitly that only the *meta* and *para* derivatives are obtained by this reaction. It would be desirable to test the above scheme by starting with *ortho*-diethylbenzene but as this substance was difficult to obtain a similar reaction was carried out with *ortho*-dimethylbenzene. As expected a small quantity of anthracene could be isolated from the reaction products.

Another explanation of the reaction would be that two molecules of the monoalkylated benzene reacted with each other to give the tricyclic hydrocarbon. However, neither Anschütz [2] nor we have succeeded in isolating

anthracene or 9,10-dimethylanthracene after treating toluene and ethylbenzene respectively with aluminium chloride. On the other hand Anschütz (*l.c.* p. 178) after heating toluene with aluminium chloride isolated another dimethylanthracene evidently having none of the methyl groups in the 9 or 10 positions. This result could easily be explained by a primary formation of higher methylated benzenes by the well-known transmethylation and a secondary condensation of one of these containing two methyl groups in *ortho* position with a toluene molecule according to the above scheme.

It should be added that by the reaction between *n*-propyl monochloroacetate and benzene in the presence of aluminium chloride we isolated a crystalline colourless hydrocarbon $C_{18}H_{20}$ which is evidently the 9,9,10,10-tetramethyl-9,10-dihydroanthracene. The fact that 9,10-diethylanthracene is not formed by this process must be due to a tautomerization of the primarily formed *n*-propyl ion into the *iso*propyl ion.

As mentioned above the dimethylanthracene m.p. 181° was easily substituted by bromine giving a dibromo derivative. Barnett and Matthews [3] found that bromination of 9,10-dimethylanthracene gave ω,ω'-dibromo-9,10-dimethylanthracene. This would be in accordance with our results, *viz.* that the dimethylanthracene m.p. 181° as well as the dibromo derivative were oxidized to anthraquinone and that they gave addition compounds with one molecule of maleic anhydride. It is, however, still an open question whether the addition of the maleic anhydride has taken place in 1,4 or 9,10 positions. On the other hand the colourless dihydro derivative of the 9,10-dimethylanthracene for which we found the m.p. 90° did not react with maleic anhydride. The dihydro derivative reacted with bromine giving the same dibromo compound as obtained from the 9,10-dimethylanthracene which shows that the bromine at the same time acts as a dehydrogenating reagent. A 9,10-dimethyl-9,10-dihydroanthracene has previously been prepared by Badger, Goulder, and Warren [5] from 9-methylanthrone by a Grignard reaction. They found the m.p. to be 101—102° and it is therefore possible that our dihydro compound is a geometric isomer.

Added during proof-reading: A new 9,10-dimethyl-9,10-dihydroanthracene with m. p. 130° has been described by K. Sisido, and T. Isida (*J. Amer. Chem. Soc.* **70** (1948) 1289). The same compound has been prepared also by G. M. Badger, M. L. Jones, and R. S. Pearce (*J. Chem. Soc.* **1950** 1700) who regard it as a geometric isomer of the dihydroderivative with m. p. 101°.

EXPERIMENTAL PART

Preparation of 9,10-dimethylanthracene

Benzene (200 g), ethyl monochloroacetate (20 g), and anhydrous aluminium chloride (60 g) were mixed and refluxed for 3 days. After cooling sufficient water was added and the benzene layer separated in a funnel and dried with sodium sulphate. The excess of benzene was removed by distillation when a dark liquid remained which partly crystallized on standing for some time. The liquid part was absorbed on a porous plate and the crystalline substance (2 g) was purified by sublimation twice in a vacuum and recrystallization from methanol. The hydrocarbon was then obtained as yellow needles with m.p. 181°. Equivalent quantities of the 9,10-dimethylanthracene and picric acid were dissolved

in benzene. On standing the picrate crystallized in dark red needles having m.p. 172–74° (decomp.).

$C_{16}H_{14}$ (206.3) Calc. C 93.16 H 6.84
Found » 93.05, 93.12 » 6.93, 7.12

0.2947 g subst. in 21.83 g benzene, $\Delta = 0.333°$, $M = 207.6$

The liquid part was extracted from the porous plate by means of ether. The material collected from several experiments was fractionated repeatedly in a vacuum, b.p. 78–82° at 15 mm Hg.

$C_{10}H_{14}$ (134.2) Calc. C 89.49 H 10.51
Found » 89.54, 89.28 » 10.38, 10.24

$n_D^{22} = 1.5020$; *m*-diethylbenzene $n_D^{20} = 1.4955$

Oxidation: To a solution of the 9,10-dimethylanthracene (0.32 g) in glacial acetic acid was added CrO_3 (0.94 g) dissolved in the same solvent. After the oxidation had taken place hydrochloric acid and water were added when anthraquinone separated. It was recrystallized from ethanol and obtained as light yellow needles with m.p. 279°, alone and mixed with an authentic sample of anthraquinone. Yield 0.18 g.

$C_{14}H_8O_2$ (208.2) Calc. C 80.76 H 3.87
Found » 80.61, 80.63 » 3.87, 3.95

The oxidation product gave a positive oxanthranol reaction.

Bromination: To a solution of 9,10-dimethylanthracene (0.69 g) in carbon tetrachloride (15 ml) bromine dissolved in carbon tetrachloride was added. On heating very gently hydrogen bromide did evolve and the brominated hydrocarbon (0.98 g) separated. On recrystallization from chloroform it was obtained as yellow needles which had no definite m.p. but decomposed at about 300°.

$C_{16}H_{12}Br_2$ (364.1) Calc. C 52.78 H 3.32 Br 43.90
Found » 53.05 » 3.40 » 43.72

The brominated product (170 mg) was oxidized in the same way as the hydrocarbon. The recrystallized oxidation product (81 mg) formed light yellow needles with m.p. 279°, alone and mixed with anthraquinone.

Preparation of 9,10-dimethyl-9,10-dihydroanthracene

9,10-Dimethylanthracene (1 g) was dissolved in 96 % ethanol and sodium amalgam added in portions. The solution was kept boiling gently and from time to time neutralized with concentrated hydrochloric acid. After 10 hours the solution was poured into water (500 ml) when the 9,10-dimethyl-9,10-dihydroanthracene (0.93 g) separated. It was sublimated in a vacuum and recrystallized from dilute methanol and formed colourless needles, m.p. 90°. Solutions of the dihydro-compound did not show fluorescence.

$C_{16}H_{16}$ (208.3) Calc. C 92.26 H 7.74
Found » 92.29, 92.15 » 7.91, 7.76

Oxidation: The dihydro-compound, oxidized in the usual way, gave anthraquinone, m.p. 279°.

Bromination: To a solution of the dihydro-compound (205 mg) in carbon tetrachloride bromine was added and the solution refluxed for 10 min. Hydrogen bromide evolved and a crystalline substance (272 mg) separated which was recrystallized from chloroform as yellow needles. The substance was decomposed on heating to about 300°.

$C_{16}H_{12}Br_2$ (364.1) Calc. C 52.78 H 3.32
Found » 52.66, 52.74 » 3.56, 3.42

Preparation of 9,9,10,10-tetramethyl-9,10-dihydroanthracene

A solution of *n*-propyl monochloroacetate (30 g) in benzene (300 g) to which anhydrous aluminium chloride (90 g) had been added was refluxed for 3 days. After adding ice-water and shaking the benzene layer was dried with sodium sulphate and the benzene distilled off. The remaining brown syrup did not crystallize and it was therefore subjected to fractional distillation in a vacuum. The first fraction formed a yellow mobile liquid

which obviously contained a di-*iso*propylbenzene (see below). The second fraction was a red viscous liquid. On keeping it in a refrigerator for some time it crystallized partly. The crystals were pressed on a porous plate and then recrystallized three times from ethanol. The new hydrocarbon (0.31 g) formed small white needles, m.p. 159°. Dissolved in ethanol it showed a weak fluorescence.

$C_{18}H_{20}$ (236.3)	Calc.	C 91.47	H 8.53
	Found	» 91.44, 91.40	» 8.14, 8.30

The first fraction was refractionated several times in a vacuum when a nearly colourless fraction 115—125° at 15 mm Hg was obtained.

$C_6H_4(C_3H_7)_2$ (162.3)	Calc.	C 88.82	H 11.18
	Found	» 87.65	» 10.73

Ortho-xylene and benzene with aluminium chloride

o-Xylene (10 g) and benzene (200 g) to which had been added anhydrous aluminium chloride (60 g) were refluxed for 15 hours. The reaction products isolated as described above were fractionated in a vacuum. The fraction collected between 150 and 200° was kept in a cool place when a small amount of crystalline material separated and was filtered off. After recrystallization several times from glacial acetic acid the colourless substance (20 mg) had m.p. 212—213°, alone and mixed with an authentic sample of anthracene. A small quantity of the hydrocarbon was oxidized with chromic acid. The light yellow oxidation product sublimated above 250° and gave a positive oxanthranol reaction when treated with zinc dust in alkaline solution.

Reactions with maleic anhydride

9,10-Dimethylanthracene: To the hydrocarbon (0.27 g) dissolved in benzene (40 ml) maleic anhydride (0.60 g) was added. The solution at first turned strongly yellow but the colour disappeared after boiling for 15 min. Letting the boiled solution stand for some time at room temperature the adduct (0.32 g) separated. On recrystallization from benzene it was obtained as white needles, m.p. 333° (in a closed tube).

$C_{20}H_{16}O_3$ (304.3)	Calc.	C 78.93	H 5.30
	Found	» 78.85, 78.79	» 5.08, 5.12

The corresponding free acid was obtained by dissolving the adduct in alkali and precipitating with hydrochloric acid.

$C_{20}H_{18}O_4$ (322.3)	Calc.	C 74.52	H 5.63
	Found	» 74.64, 74.52	» 5.69, 5.75

30.68 mg required 1.905 ml N/10 NaOH, $M = 322$

ω, ω'-Dibromo-9,10-dimethylanthracene: A mixture of the dibromo-product (0.38 g) and maleic anhydride (1.2 g) was left at 80° for 4 hours when the reaction was finished. In order to remove excess of maleic anhydride the reaction product was spread on a porous plate and again placed at 80°. The adduct was then obtained as a crystalline colourless substance insoluble in organic solvents. It was purified by washing with ether. The yield was quantitative.

$C_{20}H_{14}O_3Br_2$ (462.1)	Calc.	C 51.97	H 3.05
	Found	» 51.62, 51.70	» 3.06, 3.18

SUMMARY

By the reaction of ethyl monochloroacetate or chloroformate with benzene in the presence of aluminium chloride 9,10-dimethylanthracene was obtained besides a mixture of more and less ethylsubstituted benzenes. On using the *n*-propyl ester of monochloroacetic acid a tetramethyldihydroanthracene was obtained which must have all four methyl groups in the middle ring. A small

quantity of anthracene was isolated when *o*-xylene had reacted with benzene in the presence of aluminium chloride. The reduction of 9,10-dimethylanthracene led to a dihydro-derivative with m.p. 90° being obviously different from the 9,10-dihydro-derivative described in the literature.

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Potentiometric and Spectrophotometric Studies on 8-Quinolinol and Its Derivatives. VIII. Calcium Chelates of 7-Iodo-8-Quinolinol-5-Sulphonic Acid

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The 7-iodo-8-quinolinol-5-sulphonic acid (ferron, loretin, yatren, chiniofon) forms two calcium compounds [1] which are both sparingly soluble in water. If we formally write its formula as H_2A, the formulae of these calcium compounds are $Ca(HA)_2$ and CaA without water of hydration. The aim of this study was to determine the solubility products of these compounds. In addition it was necessary to investigate the ionization of this acid and the stability of the chelates in solution.

The ionization of the reagent was investigated previously by us [2], and the results obtained were generally confirmed in the present study, except for the first ionization constant in high ionic strength ($I > 1$) for which we have now obtained somewhat greater values. This was due to a glass electrode used at that time which gave distinctly wrong values at high ionic strengths, as was later observed. The corrected values may be obtained from

$$pK_1' = 2.514 - \frac{0.509\sqrt{I}}{1 + 1.44\sqrt{I}} + 0.13\ I \qquad (1)$$

The chelates in solution were of the type CaA and $CaA_2^{=}$. We were not able to establish the existence of complexes such as $CaHA^+$ and $Ca(HA)_2$. Their absence is also proved spectrophotometrically, as is seen in Fig. 1, where the effect of calcium ions on the ultraviolet spectrum of the reagent at different pH's is presented. No effect can be observed at pH's where the HA^- ion concentration is high but the concentration of $A^{=}$ still very small. With increasing $[A^{=}]$ the effect is gradually heightened. The second curve from the top is obviously the spectrum of CaA. This spectrum very much resembles that of the ligand $A^{=}$, as in the case of oxine [3].

The determination of the stability constants

$$k_1 = [MA] / [M^{++}][A^{=}] \qquad (2)$$

and

$$k_2 = [MA_2^{=}] / [M]\ [A^{=}]^2 \qquad (3)$$

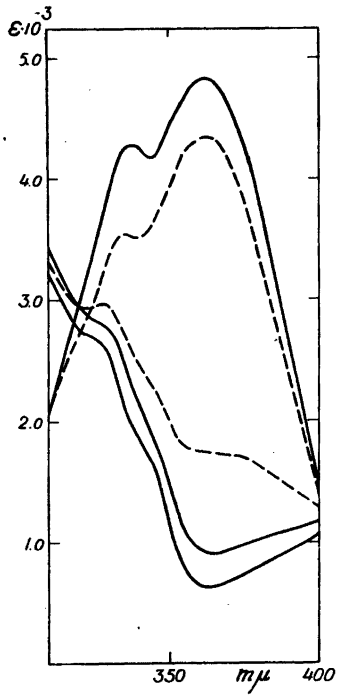

Fig. 1. Effect of calcium ions on the ultraviolet spectrum of 7-iodo-8-quinolinol-5-sulphonic acid at several pH's. From the highest curve downwards: $c = 10^{-4}$, $c_{Ca} = 0$, $pH = 9$. $c = 10^{-4}$, $c_{Ca} = 0.91$, $pH = 9$. $c = 10^{-4}$, $c_{Ca} = 0.51$, $pH = 6$. $c = 10^{-4}$, $c_{Ca} = 0$, $pH = 6$. $c = 10^{-4}$, $c_{Ca} = 0$, $pH = 4$ (the lowest curve) and $c = 10^{-4}$, $c_{Ca} = 0.91$, $pH = 4$ (the lowest curve).

was carried out by the usual titration method [3]. The equations used are as follows:

$$[HA^-] = (2c - c_B - [H^+]) / (1 + 2(H^+) / K_1') \quad (4)$$

$$c' = c - [HA^-] \left\{1 + (H^+) / K_1' + K_2' / (H^+)\right\} \quad (5)$$

$$k_1' + k_2' \frac{x(2c_M - c)}{c_M - c'} - \frac{c'}{x(c_M - c')} = 0 \quad (6)$$

where

$$x = [HA^-] / (H^+) \quad (7)$$

$$k_1' = k_1 K_2' \quad (8)$$

$$k_2' = k_2 K_2'^2 \quad (9)$$

$$K_1' = (H^+)\ [HA^-] / [H_2A] \quad (10)$$

$$K_2' = (H^+)\ [A^{=}] / [HA^-] \quad (11)$$

The symbols are those used previously [2,3].

The results concerning complex equilibria are recorded in Table 1. The data of the table are mean values of several measurements. The thermodynamic constant $k_{1,0}$ was calculated by means of

Table 1. First stability constant of calcium chelate in solutions of potassium chloride at 25° C.

$\sqrt{I}$	pk_1'	pK_2'	log k_1
0.173	4.65	7.22	2.57
0.269	4.78	7.14	2.36
0.524	4.89	7.00	2.11
0.935	4.90	6.90	2.00
1.370	4.70	6.87	2.17

$$\log k_1 = \log k_{1,0} - \frac{4.027\sqrt{I}}{1 + a\sqrt{I}} + BI \qquad (12)$$

The calculation gave

$$\log k_{1,0} = 3.07,\ a = 2.0 \text{ and } B = 0.28$$

For the second constant we obtained $k_2 \sim 10^4$.

The solubility equilibria were studied by the same method as before [3], but the case is now more complicated on account of the two solid components. In the "point titration" method [3,4] used, the calcium concentration was always kept greater than or equal to the concentration of the chelating agent. Equilibrium was reached after two days but the pH was generally measured after one week at the earliest. From Fig. 2 we see the effect of calcium ions on the titration curve when precipitation occurs. By means of the pH values belonging to the lower part, the solubility product

$$[M^{++}]\ [HA^-]^2 = S_1 \qquad (13)$$

may be calculated. From the higher part analogously, the solubility product

$$[M^{++}]\ [A^{=}] = S_2 \qquad (14)$$

is obtained. The equations needed in the first case are:

$$[H_2A] = (c - c_B - [H^+]) \,/\, \left\{1 - K_1'K_2' \,/\, (H^+)^2 - K_1'k_1'[M^{++}] \,/\, (H^+)^3\right\} \qquad (15)$$

$$c' = c - [H_2A]\ (1 + K_1' \,/\, (H^+) + K_1'K_2' \,/\, (H^+)^2) \qquad (16)$$

$$[M^{++}] = (c_M - 0.5c') \,/\, (1 + 0.5\ k_1'K_1'[H_2A] \,/\, (H^+)^2) \qquad (17)$$

$$[HA^-] = [H_2A]\ K_1' \,/\, (H^+) \qquad (18)$$

In the second case, analogously:

$$[HA^-] = (2c - c_B - [H^+]) \,/\, (1 + 2(H^+) \,/\, K_1') \qquad (19)$$

$$c = c - [HA^-]\ (1 + (H^+) \,/\, K_1' + K_2' \,/\, (H^+)) \qquad (20)$$

$$[M^{++}] = (c_M - c') \,/\, (1 - k_2'\ [HA^-]^2 \,/\, (H^+)^2) \qquad (21)$$

$$[A^{=}] = K_2'[HA^-] \,/\, (H^+) \qquad (22)$$

The point where the precipitation of $M(HA)_2$ begins may be calculated from equations (13) and from the equations for complex equilibria. The point where $M(HA)_2$ begins to change to MA is calculated from equations (13), (14), (15), (16), (17), and (18). The point where all $M(HA)_2$ is changed to MA is obtained from (13), (14), (19), (20), (21), and (22). When two solid phases exist, we obtain from (13) and (14)

$$[HA^-] = S_1K_2 \,/\, S_2(H^+) \qquad (23)$$

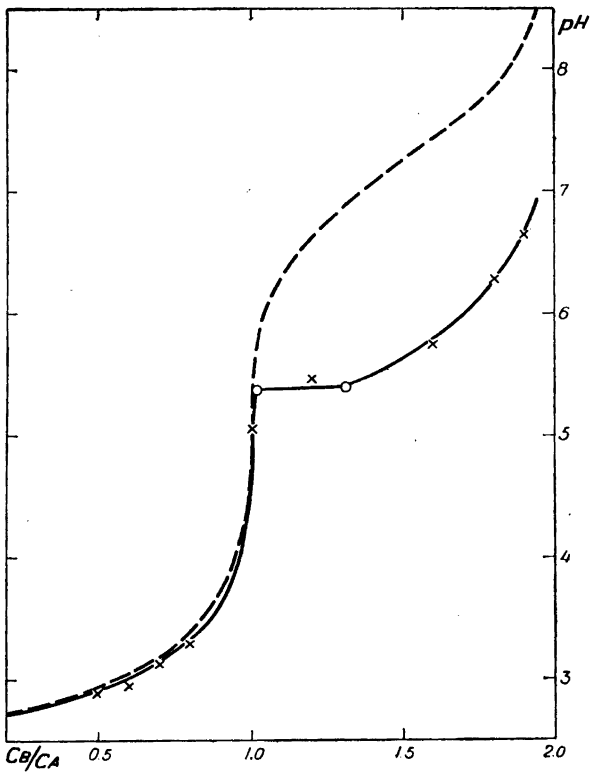

Fig. 2. Titration curve when precipitation of $Ca(HA)_2$ and CaA occurs. $c = 5 \cdot 10^{-3}$, $c_{Ca} = 6 \cdot 10^{-3}$. Between the points o both the sparingly soluble compounds exist. The dotted line represents the titration curve of the reagent. Experimental points: ×.

and

$$[M^{++}] = S_2^2 \, (H^+)^2 / S_1 K_2'^2 \tag{24}$$

The principle of electroneutrality gives

$$c_B = 2c_M - 2[M^{++}] - [H^+] + [HA^-] + 2[A^{=}] + 2[MA_2^{=}] \tag{25}$$

Thus this portion of the curve may be easily constructed. The amounts of $M(HA)_2$ (y_1) and MA (y_2) precipitated may be calculated from

$$c_M = [M^{++}] + [MA] + [MA_2^{=}] + y_1 + y_2 \tag{26}$$

and

$$c = [H_2A] + [HA^-] + [A^{=}] + [MA] + 2[MA_2^{=}] + y_1 + 2y_2 \tag{27}$$

The results relating to the solubility equilibria are recorded in Tables 2 and 3. The thermodynamic constants were calculated according to

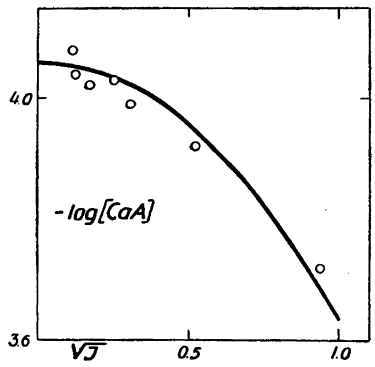

Fig. 3. Solubility of CaA in water at 25° C.

Table 2. Solubility product of $Ca(HA)_2$ *in potassium chloride solutions at 25° C.*

$c \cdot 10^3$	$c_B \cdot 10^3$	$c_{Ca} \cdot 10^3$	c_{KCl}	pH	$\sqrt{I}$	pS_1
4.57	3.20	5.56	—	3.107	0.121	7.35
4.59	2.75	5.51	—	2.949	0.126	7.31
4.55	3.64	5.54	—	3.294	0.127	7.34
4.61	2.30	5.61	—	2.887	0.127	7.30
4.55	3.64	5.54	0.0732	3.347	0.302	7.04
4.27	3.42	10.41	0.2292	3.396	0.513	6.81
3.35	2.68	27.21	0.7192	3.518	0.897	6.58

Table 3. Solubility product of CaA *in potassium chloride solutions at 25° C.*

$c \cdot 10^3$	$c_B \cdot 10^3$	$c_{Ca} \cdot 10^3$	c_{KCl}	pH	$\sqrt{I}$	pS_2
4.33	8.23	5.27	—	6.641	0.113	6.83
4.35	7.83	5.30	—	6.291	0.117	6.76
4.41	6.61	5.37	—	5.720	0.128	6.69
4.27	6.41	5.21	0.0688	5.952	0.307	6.32
4.26	6.39	5.19	0.228	6.119	0.503	5.91
4.17	6.26	5.08	0.894	6.251	0.955	5.79

$$pS_1 = pS_{1,0} - \frac{3.054\ \sqrt{I}}{1 + a\sqrt{I}} + BI \qquad (28)$$

and

$$pS_2 = pS_{2,0} - \frac{4.072\ \sqrt{I}}{1 + a\sqrt{I}} + BI \qquad (29)$$

The following values were obtained:

	pS_0	a	B	
$Ca(HA)_2$	7.64	1.68	0.03	(25° C)
CaA	7.13	1.81	—	(25° C)

From equations (2) and (9) we obtain

$$[MA] = k_1 S_2 \qquad (30)$$

valid in a saturated CaA solution. The dependence on the ionic strength may be presented by

$$-\log [MA] = 4.06 - 0.43\ I \qquad (31)$$

In Fig. 3 this quantity is represented as a function of ionic strength. This result was confirmed spectrophotometrically in a solution with high pH and high calcium concentration. In such a saturated solution nearly all the reagent is in the form of CaA.

7-Iodo-8-quinolinol-5-sulphonic acid has been used for the gravimetric determination of calcium as $Ca(HA)_2$[5]. We see from Fig. 2 that the best pH for precipitation is about 4—5. The relatively high value of the solubility product necessitates the use of an excess of reagent.

These solubility and complex equilibria seem not have been studied previously.

SUMMARY

The complex and solubility equilibria of the calcium chelates of 7-iodo-8-quinolinol-5-sulphonic acid (H_2A) have been investigated. The complexes in the solution are of the type CaA and $CaA_2^{=}$. The first stability constant has been determined and the second roughly estimated. The solubility products of the two sparingly soluble compounds $Ca(HA)_2$ and CaA have been determined. The ultraviolet spectrum of CaA in solution is given. No complexes of the type $CaHA^+$ or $Ca(HA)_2$ have been detected in the solution.

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ACTA CHEMICA SCANDINAVICA 7 (1953) 1267—1270

*iso*Thiocyanates II*. Volatile *iso*Thiocyanates in Seeds and Roots of Various *Brassicae*

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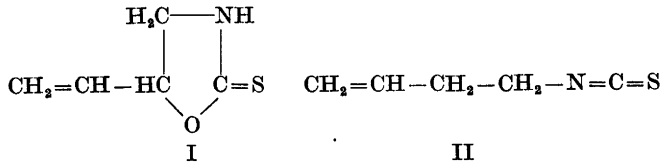
Within the family *Cruciferae* the genus *Brassica* embraces a number of plants of extensive application in human and animal nutrition. Therefore, much interest has been devoted to studies of chemical factors of possible biological importance in these plants. Foremost among those, the goitrogenic substances in vegetables and particularly various seeds of *Brassicae* (see *e. g.* Ref. [1]) have been a subject of much interest, culminating in the isolation from such sources of the antithyroid substance, L-5-vinyl-2-thioöxazolidone, (I) [2].



I

II

The structure, in conjunction with the fact that the active principle seems to be present in the intact biological material as a glycosidic precursor, raised the question as to its possible relationship to the naturally occurring *iso*thiocyanates. Not much is known with certainty regarding the *iso*thiocyanates in *Brassica* species.

The paperchromatographic technique, reported in a previous paper [3], has now been applied to a number of seeds of *Brassicae* in order to determine the chemical nature of the *iso*thiocyanates contained therein. The results, together with the spectrophotometrically estimated contents, are presented in Table 1. Similar investigations of roots and other fresh parts of some common species of the same genus, gave the results summarised in Table 2. Experimental details of the procedure employed are described in a following communication [4]. The results of a closer investigation of rape seeds, from which 3-butenyl *iso*thiocyanate (II) has been isolated, will be found in the following paper.

* The paper: *Unsaturated Five-Carbon Isothiocyanates*, which appeared in this Journal (7 (1953) 518), should be considered as number one in this series.

Table 1. Contents of volatile isothiocyanates *in seeds of various* Brassicae, *determined by paper chromatography.*

Species	% Fat	R_{Ph}	*iso*Thiocyanate(s)	mg %
Brassica nigra Koch (Black mustard)	32	0.27	Allyl	518
Brassica juncea Czern.et Coss. (Indian mustard)	37	0.27	Allyl	353
Brassica pseudojuncea	34	0.26[a] 0.59	Allyl 3-Butenyl	470
Brassica oleracea L. (Cabbage)	35	0.26[a] 0.61	Allyl 3-Butenyl	125
Br. oler.capitata var.*alba* L. (White cabbage)	37	0.27 0.73[b]	Allyl *sec*-Butyl (?)	311
Br.oler.capitata var.*rubra* L. (Red cabbage)	32	0.27 0.91[b]	Allyl Benzyl (?)	221
Br.oler.percrispa L. (Kale)	41	0.27	Allyl	8
Br.oler.botrytis L. (Cauliflower)	34	0.27[a] 0.74	Allyl *sec*-Butyl	270
Br.oler.gemmifera D.C. (Brussels sprouts)	35	0.27[a] 0.73	Allyl *sec*-Butyl	192
Br.oler.sabauda L. (Green savoy)	35	0.27 0.72[b]	Allyl *sec*-Butyl	279
Br.oler.asparagoides D. C. (Broccoli)	42	0.02 0.92[a] 1.28[b]	Methyl (?) Benzyl Unidentified	
Br.napus L. *(rapifera)esculenta* D.C. (Kohlrabi)	40	None	.	
Br.rapa rapifera Metzger (White turnip)	42	1.30	Unidentified	
Br.rapa (rapifera) communis Metzger (Turnip)	45	0.60[a] 1.03[a] 1.33[b]	3-Butenyl Unidentified Unidentified	2—3

[a] predominant; [b] traces only.

DISCUSSION

Upon consideration of the results presented in the tables, two characteristic features are evident. The predominating rôle played by allyl *iso*thiocyanate within the genus *Brassica* is easily perceptible. Second, the appearance of mixtures of two or three different *iso*thiocyanates in a certain species is the rule rather than an exception.

Table 2. Contents of volatile isothiocyanates *in fresh parts of various* Brassicae, *determined by paper chromatography.*

Species	Part	R_{Ph}	*iso*Thiocyanate(s)	mg % in fresh parts
White cabbage	Head	0.27	Allyl	1—2
Red cabbage	Head	0.27[a] 0.60	Allyl 3-Butenyl	5
Cauliflower	Head	0.27[b]	Allyl	
White turnip	Root	0.03[b] 1.01	Methyl (?) Unidentified	

[a] predominant; [b] traces only.

Allyl *iso*thiocyanate, the mustard oil *par excellence*, appears in black mustard seeds, unaccompanied by other volatile derivatives. In this connection it is pertinent to recall the remarkable isolation of β-phenylethyl *iso*thiocyanate from roots of the same species by Stahmann, Link and Walker [5]. Much controversy exists in the literature (see *e. g.* Ref. [6]) concerning the chemical nature of the *iso*thiocyanate(s) in seeds of Indian mustard (*Br. juncea*). Our finding of allyl *iso*thiocyanate as the sole volatile constituent corroborates the results of Schmalfuss and Müller [6]. The additional presence of 3-butenyl *iso*thiocyanate in a "synthetic variety", *Br. pseudojuncea*, is interesting and once again reflects the chemical specificity of even closely related species. The possible utilisation of this fact in heredity studies receives further consideration.

Except for broccoli, all species of *Brassica oleracea* investigated contain allyl *iso*thiocyanate. The minor constituents, however, differ in their chemical nature. A paper by Grimme [7] presents the results of a quantitative estimation of the volatile *iso*thiocyanates in a series of *Brassica* seeds, tacitly assuming, however, that solely the allyl derivative is implied. The only previous establishment of the identity of a mustard oil from cabbage species is furnished by Schneider and Lohmann [8], who isolated allyl *iso*thiocyanate from cauliflower seeds. The absence of this component and the unusual presence of benzyl *iso*thiocyanate plus an unidentified constituent in broccoli seeds urge a closer investigation of this material. Seeds of kohlrabi (rutabaga), known as one of the best sources of the goitrogenic factor (I) [1,2], were found to be remarkably devoid of volatile *iso*thiocyanates. On the other hand, Schultz and Gmelin [9] very recently provided paperchromatographic evidence for the presence in kohlrabi seeds of four glucosides, two of which should give rise to allyl (slight amounts only) and benzyl *iso*thiocyanate on enzymatic cleavage.

The different turnip (*Br. rapa*) species investigated possess a rather complex composition of their volatile *iso*thiocyanate fractions. The identity of the mustard oil, giving rise to the spot with an R_{Ph}-value of 1.03, is still obscure. Work is in progress to isolate this unknown *iso*thiocyanate and reveal its chemical structure.

The deviations between the *iso*thiocyanates in seeds and fresh parts of the same species, as evident upon comparison of the Tables 1 and 2, are indicative of the *iso*thiocyanate contents as a function not only ofthe species but also of the parts of a particular plant (*cf.* Ref. [5]).

Animal tests have indicated practically no antithyroid activity of the naturally occurring *iso*thiocyanates.

SUMMARY

Seeds and fresh parts of various *Brassicae* have been investigated for their contents of volatile *iso*thiocyanates by a paperchromatographic technique.

The tabulated results have been discussed in the light of previous results.

The authors wish to thank Dr. K. J. Frandsen, Øtoftegaard, for the gift of various seed samples.

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ACTA CHEMICA SCANDINAVICA 7 (1953) 1271—1275

*iso*Thiocyanates III. The Volatile *iso*Thiocyanates in Seeds of Rape (*Brassica napus* L.)

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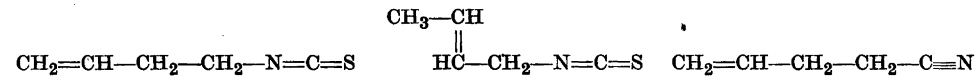
Although the essential mustard oil in seeds of rape (*Brassica napus* L.) has been repeatedly investigated, no clarity exists as to its chemical nature. Considerable practical interest is attached to this problem, because the *iso*-thiocyanate contents have been made responsible for the toxic manifestations occasionally observed upon feeding rape seed cakes in larger quantities to animals.

In 1899 Jørgensen [1] attributed the toxicity of impure rape seed cakes to the presence of a C_5- (or C_6-) *iso*thiocyanate besides the allyl derivative. At about the same time Sjollema [2] reported the isolation of a mustard oil to which the structure (I) was ascribed, mainly on basis of refraction measurements. These, however, seem to be of questionable value as a structure proof.



I II III

Stein [3] obtained from Indian rape seed cakes a C_5-*iso*thiocyanate which he regarded as the *trans*-crotyl derivative (II). Several years later, Schmalfuss *et al.*[4] reported the isolation of "crotonylcyanid" (III), "crotonylsenföl" (I) and a higher-boiling *iso*thiocyanate of unknown structure upon fractionation of the crude rape seed mustard oil. Neither analyses nor other chemical evidence were presented in support of the suggested structures. André and Kogane-Charles [5] described the isolation from rape seeds of an *iso*thiocyanate for which the structure (II) was suggested, though convincing experimental support was missing. André and Delaveau [6] recently presented evidence for the presence of three individual volatile *iso*thiocyanates in rape seeds. Again, no suggestions as to their chemical nature were made.

When defatted rape seeds were submitted to our routine method of investigation, described in the following communication [7], a paper chromatogram resulted (Fig. 1) which left no doubt as to the presence of three individual volatile *iso*thiocyanates. The major component, the thiourea of which gives

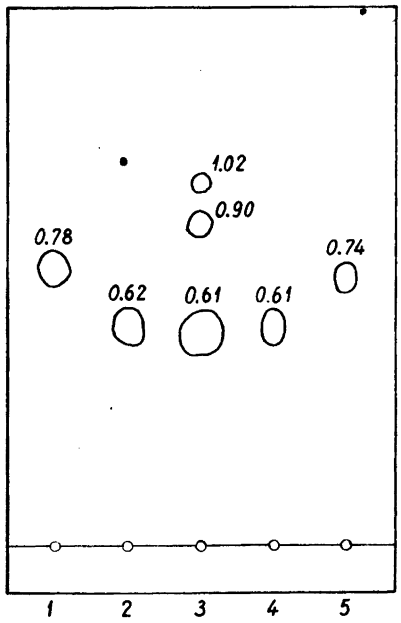


Fig. 1. Paper chromatogram showing the location of: 1. Crotylthiourea; 2. β-Methallylthiourea; 3. The thiourea mixture obtained from rape seed distillates; 4. 3-Butenylthiourea; 5. α-Methallylthiourea. The numbers indicate the R_{Ph}-values (cf. Ref.[9]). Solvent system: chloroform-water.

rise to a spot with an R_{Ph}-value of 0.61, undoubtedly represents the C_5-*iso*-thiocyanate discussed in the literature quoted above. It appears from the paper chromatogram (Fig. 1) that of the four synthetic isomerides previously described [8], the α-methallyl and *trans*-crotyl derivatives can be definitely ruled out as constituents of rape seeds. The chromatographic method does not allow, however, to discern between 3-butenyl- and β-methallyl-thiourea, although the previously reported melting points [2-6] of the thiourea-derivative of the *iso*thiocyanate from rape seeds, point to the latter as being the 3-butenyl derivative. This assumption, however, required confirmation by isolation of the major volatile component, transformation into its thiourea derivative and comparison of the latter with the synthetic isomerides on hand.

Preliminary attempts to provide a homogenous sample upon recrystallization of the crude, semi-crystalline thiourea fraction proved unavailing. Neither adsorption chromatography on activated alumina nor the application of starch and cellulose powder partition columns under varying conditions yielded promising results. Lastly recourse was taken to a counter-current distribution of the crude thiourea fraction between chloroform and water. As the result of a paperchromatographic comparison with a large number of synthetic model thioureas [9], the three components in the rape seed distillates were tentatively regarded as 3-butenyl-, benzyl- and β-phenylethyl thiourea, respectively. On this assumption the distribution coefficients of these compounds in the system chloroform-water were spectrophotometrically determined at 23°. From the values, 0.49, 2.41 and 5.88 respectively, a theoretical distribution pattern (Fig. 2) could be calculated. It appeared that in a twelve-plate procedure the vessels with the serial numbers 0—4 should contain essentially pure

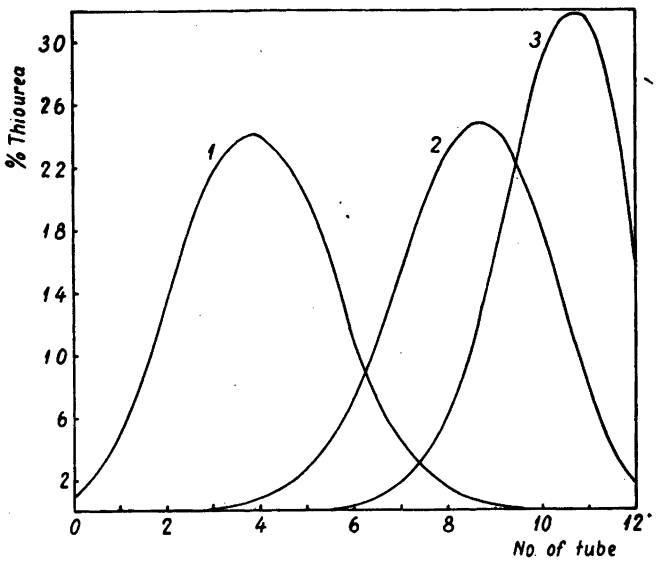


Fig. 2. Hypothetical distribution pattern of: 1. 3-Butenylthiourea; 2. Benzylthiourea; 3. β-Phenylethylthiourea. Solvent pair: chloroform-water.

material of the major component, provided equal amounts of the two phases were employed. The experiment was carried out in twelve separatory funnels and the contents of each funnel checked by paper chromatography. In accord with the theoretical predictions, the first five funnels proved to contain solely the major constituent, whereas no complete separation of the remaining two thioureas could be achieved by this procedure. Upon evaporation of the pooled contents of the five vessels a crystalline product, melting at 65.5—66.0°, separated. Analyses confirmed the composition C_5H_7NS; mixed melting point determination and infrared spectra (Fig. 3) served to establish its identity with an authentic specimen of 3-butenylthiourea, prepared as described in a preceeding paper of this series [8]. Thus it has been definitely proved that 3-butenyl *iso*thiocyanate (I) is the main constituent of the volatile *iso*thiocyanate fraction from rape seeds.

By spectrophotometrical evaluation of the contents of the separatory funnels Nos. 0—4, the total amount of 3-butenyl *iso*thiocyanate was found to be *ca.* 100 mg per 100 g of defatted rape seeds. This amount proved rather independent of the provenience of the seeds; summer and winter seed species were found about equally rich in *iso*thiocyanates. On the other hand, industrially produced rape seed cakes showed strikingly great variations in their contents, apparently dependent on the conditions employed during the manufacture. This problem is being further studied at present and the results will appear in a forthcoming paper.

The occurrence of 3-butenyl *iso*thiocyanate is not confined to rape seeds. Other *Brassica* members [10] as well as seeds of various other *Cruciferae* [7] have

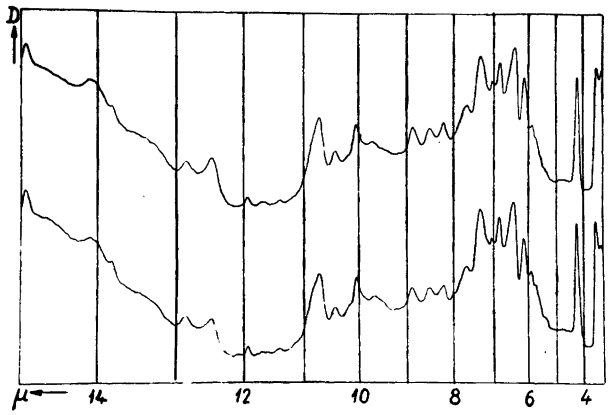


Fig. 3. Infra-red absorption spectra determined in Nujol mulls. Upper curve: synthetic 3-butenylthiourea. Lower curve: thiourea, derived from the major iso*thiocyanate constituent of rape seeds.*

been demonstrated by paper chromatography to contain the 3-butenyl derivative, most often in combination with other *iso*thiocyanates.

The nature of the two minor components of rape seeds is still unknown. Partition chromatography of the middle fractions (Nos. 5—7) from the counter-current distribution on kiselguhr columns afforded a paperchromatographic homogenous thiourea fraction with an R_{Ph}-value of 0.90. The product did not crystallise well and more material is needed in order to reveal its chemical nature. Contrary to expectations, preliminary studies have indicated that the substance is not benzylthiourea, but rather a derivative of a purely aliphatic C_6- or C_7-*iso*thiocyanate. The identification of the latter as well as the third constituent, which is present in rape seeds in only very small amounts, is still being prosecuted.

EXPERIMENTAL

Isolation of a crude thiourea fraction from rape seeds. Industrially produced rape seed cakes, containing less than 1 % of fatty material, were used as a convenient starting material for the isolation and further investigation of the volatile *iso*thiocyanates in rape seeds. In cases, where untreated seeds were investigated, a rapid denaturation of myrosinase by a hot ligroin-ethanol mixture followed by exhaustive extraction with petroleum ether, was the standard pretreatment employed.

Five hundred grams of finely divided rape seed cakes were suspended in 3 l of distilled water. To the mixture were added 26 ml of a cell-free myrosinase preparation, made according to Neuberg and Wagner [11]. After standing at room temperature for 20 hours in a tightly stoppered flask, the liberated *iso*thiocyanates were removed in a stream of steam. The extensive foaming could be somewhat diminished on adding a small amount of ethanol before the distillation. The distillate (10 l) was collected in an ice-cooled receiver containing 4 l of concentrated aqueous ammonia. The clear solution was left overnight at room temperature and then concentrated to dryness *in vacuo* at a bath temperature not exceeding 55°. The semi-crystalline residue (1.80 g) was dissolved in 100 ml of water-saturated chloroform and a drop of the solution used for paper chromatography, the

result of which is shown in Fig. 1. An additional amount of 200 ml of water-saturated chloroform was now added and the solution submitted to the countercurrent separation procedure.

Fractionation of the crude thiourea mixture. In each of 13 separatory funnels (1 liter) were placed 300 ml of water, saturated with chloroform. In the first funnel (No. 0) the chloroform solution of the thiourea mixture was introduced and the contents shaken for about 2 minutes. The chloroform phase was then transferred to the next funnel (No. 1) and fresh chloroform introduced in separatory funnel No. 0. The process was repeated until the first chloroform phase had reached the last vessel (No. 12).

The contents of the separatory funnels were individually evaporated *in vacuo* to a volume of 100 ml at a bath temperature not exceeding 40°. By this procedure the chloroform was completely removed and the individual aqueous solutions were spectrophotometrically evaluated for their thiourea contents, as described in a following paper [7]. After further concentration of the solutions to dryness, methanolic solutions of about 1 % concentration were prepared and utilised for paper chromatography. This revealed that material from the separatory funnels Nos. 0—5 consisted of essentially homogenous material of the predominating thiourea constituent, possessing an R_{PH}-value of 0.61. Therefore, these fractions were combined and carefully recrystallized from aqueous methanol. An additional recrystallization from water yielded 163 mg of beautiful colourless needles, m.p. 65.5—66° (uncorr.), alone or in admixture with an authentic specimen, prepared as previously described [8].

$C_5H_{10}N_2S$ (130.2)	Calc.	C	46.13	H	7.75	N	21.53	S 24.64
	Found	»	46.39	»	7.84	»	21.72	» 24.53

The infrared spectra (Fig. 3) further served to ascertain the identity of the thiourea.

Absorption spectra. The ultra-violet absorption measurements were performed with a Beckman model DU quartz spectrophotometer. The infra-red spectra were determined in Nujol mulls with a Beckman IR 2 instrument.

SUMMARY

By paper chromatography, the presence of three volatile *iso*thiocyanates in enzymatically treated rape seeds has been demonstrated.

The main constituent has been definitely proved to be 3-butenyl *iso*-thiocyanate.

Microanalyses were performed in this laboratory by Mr. W. Egger. The assistance of Dr. K. Rubinstein in the preliminary phases of the present work is highly appreciated.

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*iso*Thiocyanates IV. A Systematic Investigation of the Occurrence and Chemical Nature of Volatile *iso*Thiocyanates in Seeds of Various Plants

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More than a hundred years have elapsed since allyl *iso*thiocyanate was first isolated from seeds of black mustard. Since then, numerous investigations have served to establish the wide-spread occurrence of glycosidically bound *iso*thiocyanates, especially in plants belonging to the family *Cruciferae*. The older results have been previously reviewed [1,2]; here it suffice to mention that only four steam-volatile *iso*thiocyanates with well-established structures were known as constituents of the crucifers when the present studies were initiated, *viz.* allyl-, (+)-*sec*-butyl-, benzyl- and β-phenylethyl *iso*thiocyanate. In a previous paper [3] we added to this series *iso*propyl *iso*thiocyanate while the preceding communication reported the isolation of 3-butenyl *iso*thiocyanate from seeds of various *Brassicae*.

Older investigations have suffered from the disadvantage of requiring large amounts of biological material, thereby rendering only the most common and abundant seeds accessible for detailed studies. Microchemical tests have been reported (*cf.* Ref.[4]) but they do not allow to discern between individual *iso*thiocyanates.

Recently we described a paperchromatographic technique [5] by which minute amounts of thioureas can be separated and identified. This method, permitting the use of as little as one gram of seeds, has now been applied to a systematic scanning of a large number of seeds for their contents of volatile *iso*thiocyanates. In a few cases of special interest the fresh plants or parts thereof have been investigated also. The species studied in the present communication belong to a great extent to the Scandinavian flora, the major part being crucifers. Owing to their special interest the results from similar studies within the important genus *Brassica* have been separately presented. Qualitative and quantitative estimates of the volatile *iso*thiocyanates have been made throughout, while the interesting *iso*thiocyanates, which are not volatile with steam, form the subject of future work.

EXPERIMENTAL

Enzymic liberation and distillation of *iso*thiocyanates

The individual seed sample (*ca.* 2 g) was ground in a small hand mill and than immediately added to a hot mixture of ligroin (b.p. 60—100°) (10 ml) and ethanol (5 ml) and refluxed for half an hour. This treatment denatured the myrosinase present in the seeds and removed much fatty material. In preliminary experiments, where the treatment with hot ligroin-ethanol was substituted with a continuous, cold extraction in a Soxleth apparatus, an undesired and often complete enzymic degradation took place, resulting in removal of the liberated *iso*thiocyanates into the solvent. The partially extracted sample was then ground to a fine powder in the mill and exhaustively defatted by refluxing with a mixture of petroleum ether and ethanol (3 : 1). After filtration and air-drying the total weight loss (fat plus minor amounts of water) was determined. The values found are presented in Table 2.

The finely divided material was then suspended in 20 ml of distilled water; 0.2 ml of a cell-free myrosinase preparation [6] was added and the mixture allowed to stand at room temperature for 18 hours in a glass-stoppered vessel. Next day, the contents were distilled in a rapid stream of steam and the distillate (200—300 ml) collected in a receiver containing 20 ml of concentrated ammonia. After standing overnight the transformation into thiourea was complete and the clear solution was evaporated to dryness *in vacuo* at a bath temperature not exceeding 50°. The often oily residue was dissolved in absolute ethanol and properly diluted before the spectrophotometrical evaluation described below. After being measured, the solution was again taken to dryness *in vacuo* and then, on basis of the found contents, dissolved in enough 96 % ethanol to make a *ca.* 1 % solution. This was applied to a paper strip and chromatographed as previously described [5]. The results obtained are summarised in Table 2.

In a few cases the seeds could not be effectively removed from the siliques in which they were located. Therefore, these samples (Table 2, Nos. 3, 11, 19 and 29) were only qualitatively investigated for their contents of volatile *iso*thiocyanates in the seed-containing siliques.

In cases, where fresh plants or parts thereof were studied, the biological material was homogenised in a Waring blendor, kept for about half an hour at room temperature, distilled and further processed as described above.

Spectrophotometric evaluation of *iso*thiocyanate contents

According to the paperchromatographic results the following volatile *iso*thiocyanates were encountered during the present investigation: methyl-, allyl-, *iso*propyl-, *sec*-butyl-, 3-butenyl-, benzyl- and β-phenylethyl *iso*thiocyanate. The ultraviolet absorption spectra of synthetic samples of the corresponding thioureas were carefully measured in anhydrous ethanol solutions. A characteristic feature of all the spectra is a maximum at 243 mμ (ε 12—13 000). The molecular extinction values at 220 mμ, 240 mμ and 260 mμ are tabulated in Table 1.

Table 1. Absorption data for various thioureas in ethanol solution.

N-Substituted thiourea	ε_{220}	ε_{240}	ε_{260}	K
Methylthiourea	3 750	12 510	1 680	1.28
Allylthiourea	5 100	12 570	2 690	1.45
*iso*Propylthiourea	4 020	12 230	2 270	1.34
sec-Butylthiourea	3 900	11 990	2 180	1.34
3-Butenylthiourea	3 930	12 150	2 160	1.33
Benzylthiourea	10 020	12 140	3 260	2.20
β-Phenylethylthiourea	9 280	12 270	2 730	1.96

It was apparent from the absorption curves of the "natural" thioureas that some nonspecific absorption, due to unknown volatile by-products, was superimposed upon the true thiourea spectra. On the rather likely assumption that the extinction of the impurities increases linearly in the range 220—260 mμ, a corrected extinction value at 240 mμ, e_{240}, could be calculated in the following way.

When E denotes the observed extinctions, k' and k'' the ratios between the extinction values at 260 mμ and 220 mμ, respectively, to those at 240 mμ of the pure substances (calculated from Table 1), the following expressions are obtained:

$$E_{240} = e_{240} + a \quad (1)$$

$$E_{220} = k' e_{240} + a + b \quad (2)$$

$$E_{260} = k'' e_{240} + a - b \quad (3)$$

where $a + b$ and $a - b$ represent the non-specific extinctions at 220 mμ and 260 mμ, respectively. From the equations (1)—(3), the following expression for the corrected extinction at 240 mμ is easily derived:

$$e_{240} = \left(E_{240} - \frac{E_{220} + E_{260}}{2} \right) K$$

where

$$K = \frac{1}{1 - \frac{k' + k''}{2}}$$

The individual correction factors (K) are tabulated in Table 1. From the corrected extinction values the amount of thiourea and consequently of *iso*thiocyanate can readily be calculated. In Table 2 the found contents of volatile *iso*thiocyanates are given in mg per 100 g of untreated (fat-containing) seeds.

All ultraviolet absorption measurements were carried out on a Beckman model DU quartz spectrophotometer in 1 cm cells.

DISCUSSION

The current studies of the distribution of volatile *iso*thiocyanates in seeds and other plant materials were undertaken for several reasons. A general interest in the phytochemical metabolism of sulphur made an extended knowledge of the occurrence and chemical nature of the *iso*thiocyanates desirable. Furthermore, it seemed of interest to attempt to find a possible correlation between the *iso*thiocyanate contents in various species and the traditional use of the latter in a number of drugs and old remedies.

From the experimental results, presented in the tables, some general conclusions may be drawn. First it should be noted that mixtures of two volatile *iso*thiocyanates often are encountered, though no consistency seems to exist with regard to the preferred combinations of the individual *iso*thiocyanates. Surprisingly often, however, *iso*propyl and *sec*-butyl *iso*thiocyanate are found in the same species. It seems rather incomprehensible that the widely distributed *iso*propyl derivative has hitherto escaped notice within the *Cruciferae*. The reason might be found in previous failures to recognise this *iso*thiocyanate in admixture with the closely related *sec*-butyl derivative.

It is noteworthy that quite a few of the seed samples proved to be devoid of volatile *iso*thiocyanates (Table 3). The seeds of *Lepidium perfoliatum* L., *Lepidium Menziesii* DC., *Berteroa incana* (L.) DC., *Eruca sativa* Mill. and *Turritis glabra* L. gave paperchromatographic results which warranted a closer

Table 2. Contents of volatile isothiocyanates *in seeds, determined by paper chromatography.*

No.	Species	% Fat	R_{Ph}	*iso*Thiocyanate(s)	mg %[e]
	Cruciferae				
1.	*Barbarea arcuata* (Opiz.) Reichb.[d]	27	0.02 1.11	Methyl β-Phenethyl [b]	20 70
2.	*Barbarea intermedia* Bor.[d]	29	1.11	β-Phenethyl	43
3.	*Cakile maritima* Scop.[d]	—	0.27	Allyl	—
4.	*Cardamine graeca* L.[d]	15	0.62 0.91	3-Butenyl [b] Benzyl	591
5.	*Cheiranthus cheiri* L.	21	0.02 0.39	Methyl *iso*Propyl [b]	7 11
6.	*Cheiranthus maritimus*[d]	21	0.02 0.71	Methyl *sec*-Butyl [a]	123
7.	*Cochlearia anglica* (L.) Asch. & Grb.[d]	31	0.41 0.73	*iso*Propyl [b] *sec*-Butyl	282 326
8.	*Cochlearia danica* L.	26	0.44 0.76	*iso*Propyl [b] *sec*-Butyl	220 254
9.	*Cochlearia officinalis* L.	35	0.41 0.76	*iso*Propyl *sec*-Butyl [b]	254 292
10.	*Coronopus didymus* (L.) Sm.	23	0.91	Benzyl	154
11.	*Crambe maritima* L.	—	0.26	Allyl	—
12.	*Diplotaxis muralis* (L.) DC.[d]	30	0.27	Allyl	500
13.	*Draba borealis* DC.[d]	23	0.74 0.96	*sec*-Butyl Benzyl (?)	770
14.	*Draba incana* L.	23	0.27 0.59	Allyl 3-Butenyl [a]	690
15.	*Erucastrum gallicum* (Willd.) O. E. Schulz[d]	32	0.27 0.59	Allyl [b] 3-Butenyl	34 36
16.	*Erysimum cheiranthoides* L.[d]	39	0.27	Allyl	8
17.	*Erysimum Perofskianum* Fisch. et Mey.	33	0.02 0.96	Methyl Benzyl (?)	179
18.	*Hutchinsia alpina* R.Br.[d]	20	1.09 1.21	β-Phenethyl (?) [a]	430
19.	*Isatis tinctoria* L.	—	0.59	3-Butenyl	—
20.	*Lepidium densiflorum* Schrad.[d]	25	0.91	Benzyl	203
21.	*Lepidium sativum* L.	27	0.90 1.12	Benzyl β-Phenethyl (?) [a]	942
22.	*Lepidium virginicum* L.[d]	21	0.89	Benzyl	762
23.	*Lunaria annua* L.[d]	27	0.40 0.73	*iso*Propyl [c] *sec*-Butyl [a]	488
24.	*Matthiola annua* R.Br.	25	0.03 0.43	Methyl [a] *iso*Propyl [a]	
25.	*Matthiola fenestralis* (L.) R.Br.[d]		0.03	Methyl [a]	
26.	*Nasturtium officinale* R.Br.	34	1.09	β-Phenethyl	558
27.	*Raphanus sativus* L. var. *alba* D.C.	46	0.02 0.40	Methyl [a] *iso*Propyl	4
28.	*Raphanus sativus* L. var. *radicula* Pers.	41	0.26	Allyl	3
29.	*Rapistrum perenne* (L.) All.[d]	—	0.61	3-Butenyl	—
30.	*Sinapis alba* L.	35	0.39	*iso*Propyl [a]	3
31.	*Sisymbrium sophia* L.[d]	30	0.27	Allyl	30
32.	*Sisymbrium strictissimum* L.	30	0.40 0.73	*iso*Propyl [a] *sec*-Butyl	158
33.	*Thlaspi arvense* L.	35	0.27	Allyl	418

	Resedaceae				
34.	*Reseda Ungnonette odorata*	40	0.03	Methyl	7
	Tropaeolaceae				
35.	*Tropaeolum majus* L.	15	0.90	Benzyl	970
36.	*Tropaeolum majus nanum*[d]	14	0.89	Benzyl	977
37.	*Tropaeolum peregrinum (canariense)*[d]	29	0.41 0.74	*iso*Propyl [b] *sec*-Butyl	37 43
	Capparidaceae				
38.	*Cleome arabica* L.[d]	26	0.03	Methyl	30
39.	*Gynandropsis gynandra* (L.) Briq.[d]	27	0.03	Methyl	251

[a] traces only; [b] predominant; [c] shown by isolation (*cf.* Ref. [3]); [d] not previously investigated for *iso*thiocyanates; [e] calculated as the appropriate *iso*thiocyanate.

investigation of their *iso*thiocyanate contents. The results of these studies now in progress will be presented at a later date.

Many of our results confirm earlier findings whereas other necessitate a revision of statements in the literature. In this connection it should be mentioned that Schultz and Gmelin [7,8] very recently described a paperchromatographic technique for the separation and identification of glycosides in a large number of crucifers. Their method has no limitation as to the nature of the glycosides responding to the separation procedure and therefore provides evidence also of the presence of glycosides containing non-volatile *iso*thiocyanates. However, their R_f-values of the individual glycosides seem to fall within a very narrow range. This fact renders the unambiguous recognition of the spots somewhat difficult. To this circumstance we attribute certain inconsistencies between their results and ours. Space does not permit a detailed discussion of the divergencies which, however, are easily noticed upon comparison of the experimental results in their publication and the present one.

The detection of 3-butenyl and benzyl *iso*thiocyanate in seeds of *Cardamine graeca* (*2*,4) * was surprising in view of the previously established presence of *sec*-butyl *iso*thiocyanate in fresh parts of *C. amara* L.[9] and *C. pratensis* L.[10]. Seeds of the wallflower (*Cheiranthus cheiri: 2*,5) have formerly been known as a source of the non-volatile cheiroline [11], but no previous indication of the presence of other *iso*thiocyanates in this species seems to be on record. From *Cochlearia danica* (fresh plants) Blanksma [10] isolated *sec*-butyl *iso*thiocyanate whereas Urban [12] reported the presence of the same compound in seeds of *C. officinalis*. On this background it was unexpected to find *iso*propyl *iso*thiocyanate to be present in all three species of *Cochlearia* investigated (*2*,7—9), in two of them as the predominant constituent. The detection of benzyl *iso*thiocyanate in seeds of *Coronopus didymus* (*2*,10) confirms recent studies by McDowall *et al.*[13]. In the literature [8] seeds of *Crambe maritima* (*2*,11) are reported to contain a glucoside of unknown structure. Evidence is now provided for the *iso*thiocyanate therein being the allyl derivative. A number of repre-

* In the following, the two figures in brackets refer to the number of the table and species, respectively.

Table 3. Species of seeds found devoid of volatile isothiocyanates *by paper chromatography.*

Cruciferae	10. *Lepidium heterophyllum* (DC,) Benth.[a]
1. *Alyssum alyssoides* L.[a]	*Resedaceae*
2. *Arabis alpina* L.[a]	11. *Reseda lutea* L.
3. *Bunias erucago* L.	*Phytolaccaceae*
4. *Camelina sativa* (L.) Crantz[a]	12. *Petiveria alliacea* L.[a]
5. *Capsella bursa pastoris* (L.) Medic.	*Euphorbiaceae*
6. *Conringia orientalis* (L.) Andrz.[a]	13. *Euphorbia dentata* Michx.[a]
7. *Erophila verna* (L.) Chev.[a]	14. *Euphorbia exigua* L.[a]
8. *Hesperis matronalis* L.	15. *Euphorbia graeca* Boiss. et Sprunn[a]
9. *Iberis amara* L.	

[a] not previously investigated for *iso*thiocyanates.

sentatives of the genus *Draba* were formerly studied [7,8]. In the present work two species (*2*,13—14) were found to be particularly rich with regard to volatile *iso*thiocyanates but with different types predominating.

It is interesting to notice the presence of volatile *iso*thiocyanates in seeds of various *Erysimum* species (*2*,16—17) because those have hitherto been considered as containing only the non-volatile *iso*thiocyanates cheiroline [14] and erysoline [15]. In roots [4,7] and seeds [8] of *Isatis tinctoria* (*2*,19) the presence of allyl and *sec*-butyl *iso*thiocyanate has previously been suggested. We found no indication of the presence of either of these, but repeatedly obtained evidence of 3-butenyl *iso*thiocyanate being present in the seeds. Water-cress seeds (*Nasturtium officinale: 2*,26) have not been previously studied with regard to their contents of volatile *iso*thiocyanates. It was not surprising, however, to find β-phenylethyl *iso*thiocyanate being the sole constituent in view of its well-established occurrence in the fresh plants [16]. The presence of *iso*propyl *iso*-thiocyanate, though only in very small amounts, in seeds of white mustard (*Sinapis alba: 2*,30) was rather unexpected because this material is generally believed to be homogenous as to its glucoside, containing the non-volatile *p*-hydroxybenzyl *iso*thiocyanate. The two species of the genus *Sisymbrium* (*2*,31—32) furnish an illustration to the often encountered phenomenon that closely related species may contain completely different *iso*thiocyanates.

The occurrence of β-phenylethyl *iso*thiocyanate in the essential oil from roots of *Reseda odorata* has been known for many years [17]. Therefore, it was rather surprising to find only slight amounts of presumably methyl *iso*thio-cyanate in the seeds, whereas *R. lutea* (*3*,11) proved completely devoid of volatile mustard oils.

Another striking example of the lacking consistency regarding the *iso*-thiocyanate contents within a certain botanical group is furnished by the seeds of *Tropaeolum peregrinum* (*2*,37) which contain two volatile *iso*thiocyanates. Both of these are, however, different from the long known benzyl *iso*thiocya-nate from *Tr. majus* (*2*,35). The high mustard oil contents in species of the family *Capparidaceae* are interesting and merit further consideration.

The absence of volatile *iso*thiocyanates in seeds of *Alyssum alyssoides* (*3*,1) and *Arabis alpina* (*3*,2) is not in accord with literature reports [8] on the

Table 4. Contents of volatile iso*thiocyanates in fresh plants or parts thereof as determined by paper chromatography.*

Species	Part(s)	*iso*Thiocyanate(s)	mg %
Cochlearia anglica L. [c]	Leaves, stems and flowers	isoPropyl [a]	11
Armoracia rusticana G., M. & Sch. (Horseradish)	Root	Allyl [b] + β-Phenethyl	126
Raphanus sativus L. var. *radicula* Pers. (Radish)	Root	Allyl	~ 1
Capsella bursa pastoris (L.) Medic.	Leaves, stems and flowers	None	
Sisymbrium luteum (Maxim.) O. E. Schulz [c]	Flowers, siliques	None	
» »	Stems, leaves	None	

[a] *cf.* Ref. 3; [b] predominant; [c] not previously investigated for *iso*thiocyanates.

presence of mixtures of glucosides containing different *iso*thiocyanates, mostly non-volatile though, in seeds of related species. We have not been able to confirm older suggestions [18] that *Capsella bursa pastoris* should contain traces of allyl *iso*thiocyanate. Neither seeds (*3*,5) nor fresh plant material (Table 4) gave even the slightest trace of volatile mustard oil. Hopkins [19] isolated from seeds of *Conringia orientalis* the heterocyclic compound 2-mercapto-5,5-dimethyl-oxazoline and suggested α-methallyl *iso*thiocyanate as a possible biogenetic precursor to the heterocyclic substance. We did not, however, obtain any evidence of the presence of α-methallyl or any other volatile *iso*thiocyanate in our paperchromatographic studies.

On account of the isolation by Puntambekar [20] of three volatile *iso*thiocyanates from an Indian plant belonging to the family *Euphorbiaceae*, a series of seeds from plants of this family was investigated (*3*,13—15). None of these contained detectable quantities of mustard oils.

Many problems in connection with the occurrence of *iso*thiocyanates in nature remain to be solved. The distribution of *iso*thiocyanates within the individual plant, the biosynthesis of the mustard oils and their rôle in the sulphur metabolism are only examples of problems to which we hope to return in future communications.

SUMMARY

Paper chromatography has been used for systematic, qualitative studies of the contents of volatile *iso*thiocyanates in numerous seed samples from plants belonging to the *Cruciferae*, *Resedaceae*, *Tropaeolaceae*, *Capparidaceae*, *Phytolaccaceae* and *Euphorbiaceae*.

The method has been combined with a spectrophotometrical evaluation of the amounts of *iso*thiocyanates present.

The results presented in the tables have been discussed in the light of previous findings.

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The Molar Light Absorption of Pyridine Ferroprotoporphyrin (Pyridine Haemochromogen)

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Iron-protoporphyrin proteids are frequently and conveniently assayed spectrophotometrically after conversion to pyridine haemochromogen.

At this institute the determinations have since many years been made in a solution, 2.1 *M* in pyridine and 0.075 *M* in NaOH and with dithionite as reducing agent. A number of determinations of the molar absorption on different specimens of recrystallized haemin [1], made by different operators here during recent years, have given the value $\beta = 7.28—7.32 \times 10^7$ cm^2 × mole^{-1}, based on dry weight of recrystallized haemin, for the α-band at 556.5—557 mμ. de Duve [2] reported a similar value for pyridine haemochromogen obtained directly from haemo- and myoglobin ($\beta = 7.36 \times 10^7$ cm^2 × mole^{-1}).

Drabkin [3], when using 6.15 *M* pyridine and 0.0835 *M* KOH found the value $\beta_{558} = 7.14—7.22 \times 10^7$ cm^2 × mole^{-1} (recalc. from $\varepsilon = 31.05—31.40$ × mM^{-1} × cm^{-1}). Lemberg and Legge [4] found that liver catalase gave a haemochromogen spectrum in 1.25 % NaOH even without the addition of pyridine. Their value was $\beta_{558} = 7.14 \times 10^7$ cm^2 × mole^{-1} (recalc. from $d_{558} = 47.5$ for 1 mg catalase haemin per ml.).

We have occasionally observed, however, that the iron content of haemoproteins, calculated from pyridine haemochromogen determination ($\beta_{557} = 7.3 \times 10^7$) gave higher values than those found by direct determinations by the sulphosalicylic acid method [5,6]. This was especially observed in our recent attempts to prepare myoglobin of high purity. We have therefore redetermined the molar absorption of the α-band of the pyridine ferroprotoporphyrin.

EXPERIMENTS

Haemin with 8.26 % Fe was prepared from cow blood according to Fischer [1] but not recrystallized, since we have found that recrystallized material gives haemochromogen spectra with slightly flattened out minima and maxima.

A sample of recrystallized haemin was kindly put at our disposal by Professor Otto Warburg. Its iron content was 8.42 % according to our determination.

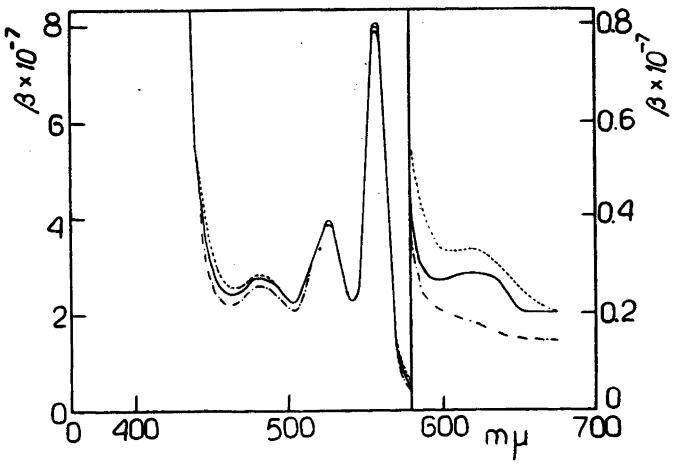

Fig. 1. Absorption spectra of reduced pyridine haemochromogens.
——— *non-recrystallized haemin*
····· *recrystallized haemin*
—.—.—.— *recrystallized myoglobin*

The absorption measurements were carried out and checked in different Beckman spectrophotometers which all were found to give the same values.

Variations in the alkali-concentration between 0.02 and 0.5 *M* NaOH at a constant pyridine concentration of 25 % (v/v) and in the pyridine concentration between 1.2 and 6.2 *M* at a constant alkali concentration of 0.1 *M* did not influence the light absorption of the haemochromogen.

The molar absorption coefficients in Table 1 and in the corresponding absorption curves in Fig. 1 are based on iron determinations [6] on haemin solutions and not on the weights of the dissolved haemin samples. It was thus assumed that the light absorption at 557 mμ of the small amount of impurities which reduced the iron content of the preparations from the theoretical value of 8.57 % to 8.26 resp. 8.42 % was negligible under the experimental condi-

Table 1. Molar absorption coefficients ($\beta = \frac{1}{c} \times \frac{1}{d} \times \ln \frac{I_0}{I}$) *for reduced pyridine haemochromogens.*

	$\beta \times 10^{-7}$ cm² mole⁻¹					
Wave-length in mμ	Max. 557	Min. 540	Max. 526	Min. 503	Max. 480	Min. 460
Non-recrystallized haemin	8.04	2.30	4.03	2.26	2.78	2.44
Recrystallized haemin	7.88	2.28	4.03	2.29	2.83	2.60
Recrystallized myoglobin	7.95	2.29	3.97	2.10	2.55	2.21

tions. The absorption values for the pyridine haemochromogen prepared directly from recrystallized myoglobin were determined and the β calculated on the basis of the iron content. The results are given in Table 1 and Fig. 1.

As seen from the table the data for β_{557} agree with each other, but are considerably higher than those reported earlier in the literature. The true value is $\beta_{557} = 8.0$ rather than 7.3.

As seen from Fig. 1 there is a third band in the visible in addition to the well known α- and β-bands. Its maximum is at 480 mμ. In the regions of low light absorption above 580 mμ and around 460 mμ the recrystallized haemin (8.42 % Fe) gave somewhat higher absorption than the preparation crystallized only once. The opposite was true for the maximum at 557 mμ. Myoglobin gives a definitely better curve than both haemin preparations. This confirms our view that crystallization of Cl-haemin from glacial acetic acid + NaCl leads to some deterioration. The light absorption in the pyridine haemochromogen test is independent of the presence of protein, provided that alkali is present in excess. We determined β_{557} of pyridine haemochromogen for haemin before and after coupling to myoglobin apoprotein and found exactly the same value. It was also found that the addition of crystallized serum albumin to a concentration of 6 mg per ml did not influence the β_{557}.

Keilin and Hartree [7] gave the value 1.61 % haemin for their best horse radish peroxidase preparation, and concluded that they had reached a higher purity than for our crystallized preparations [8]. Their value was based on spectrophotometry of the pyridine haemochromogen. A sample of their preparation, kindly put at our disposal by Dr. E. F. Hartree, was examined for activity in the mesidine test [9] and found to have the same activity per haemin as our preparation. The haemin content of their preparation determined in our pyridine test and calculated on the dry weight value, submitted by Dr. Hartree, was found by us to be 1.36 % ($\beta_{557} = 8.0 \times 10^7$). The haemin content of horse radish peroxidase previously reported from our institute (1.47, 1.39 [8], 1.36 [10], 1.45, 1.38 [11]) had been calculated from the value $\beta_{557} = 7.3 \times 10^7$ cm^2 $\times$ mole^{-1}, and should thus be corrected by the factor 0.915 (=7.3/8). There is thus no reason to assume that the Keilin-Hartree preparation method for peroxidase leads to preparations essentially different from our original one.

SUMMARY

1. The molar absorption of the α-band of the pyridine ferroprotoporphyrin has been re-determined and found to be = 8.0×10^7 cm^2 $\times$ mole^{-1} and thus considerably higher than the values reported earlier in the literature.

2. Variations in alkali and pyridine concentrations within moderate limits have no influence on the spectrum.

3. The presence of myoglobin apoprotein and crystallized serum albumin in the solution does not change the light absorption.

4. The haemin content of Keilins' and Hartrees' horse radish peroxidase preparation was reexamined and found to be lower than reported, and in essential agreement with the values for our preparations.

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Ion Exchange Chromatography of Inosine Phosphates

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A method for the quantitative separation of the different adenosine-5'-phosphates, *viz.* adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) by ion-exchange chromatography has been devised by Cohn and Carter [1]. After adsorption on a column of the strong base anion-exchange resin Dowex-1 in the chloride form, the individual phosphates are removed as separate fractions by successive elution with 1) 0.003 *M* hydrochloric acid (AMP), 2) 0.02 *M* sodium chloride in 0.01 *M* hydrochloric acid (ADP plus any inorganic orthophosphate (P_0)) and 3) 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid (ATP).

For the last two years, extensive use was made of this method in our laboratory both for analytical and preparative purposes. Although the exchange resin Dowex-2 was employed, the eluting solvents of Cohn and Carter could be used unchanged, at variance with the findings of Leuthardt and Bruttin [2]. A drawback of the method as originally described, namely the contamination of the ADP fraction by P_0, could be eliminated by continued elution with 0.003 *M* hydrochloric acid after the removal of AMP; P_0 appears then in the effluent as a separate fraction following AMP. In several large-scale experiments with mixtures of P_0, ADP and ATP, where elution with 0.003 *M* hydrochloric acid was omitted, a sharp separation of P_0 and ADP could nevertheless be obtained. P_0 and ADP appeared either in the 0.02 *M* sodium chloride solvent as separate fractions or else — depending on the volume of the eluents applied — P_0 was obtained in the 0.02 *M* sodium chloride solvent, and ADP in the 0.2 *M* sodium chloride solvent as a separate fraction preceding ATP, without any cross-contamination of the components. Removal of P_0 can also be achieved by elution with 0.025 *M* ammonium chloride in 0.0025 *M* ammonium hydroxide solution, prior to the removal of AMP, according to the method of Khym and Cohn [3] for the separation of sugar phosphates. In some experiments with preparations of high P_0 content, a selective adsorption of the organic phosphates could be obtained by making the solution *ca.* 0.025 *M* with respect to ammonium chloride prior to adsorption.

Inorganic pyrophosphate (PP) — if present — is eluted after ADP and can be collected by elution with 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid solution as a separate fraction preceding ATP.

Application of the method to the separation of inosine-5′-phosphates, *viz.* inosine monophosphate (IMP), inosine diphosphate (IDP) and inosine triphosphate (ITP) gave — as expected — an equally clear separation of all components as in the case of the related adenosine phosphates (Fig. 1). In agreement with the observations of Cohn [4] on the relative elution positions of the isomeric inosinic and adenylic acids, each inosine phosphate is eluted after the corresponding adenosine compound and can be collected in the solvent which elutes the next higher adenosine phosphate. Thus, on chromatography of mixtures, containing both adenosine and inosine phosphates, IMP and ADP on the one hand and IDP and ATP on the other, are removed by the same solvents from the column. But whereas IMP precedes ADP as a separate fraction, no appreciable separation of IDP and ATP occurs. A separation of IDP and ATP can be achieved by a slight modification of the elution system. After removal of ADP by 0.02 *M* sodium chloride in 0.01 *M* hydrochloric acid, elution is continued with 0.05 *M* sodium chloride in 0.01 *M* hydrochloric acid, whereby IDP appears in the effluent as a separate fraction preceded by PP. ATP is eluted thereafter with 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid (Fig. 2). In this way, a mixture of AMP, ADP, ATP, IMP, IDP, ITP, P_0 and PP can be resolved into all its components. Analysis of mixtures, prepared from the chromatographically purified components and containing 1—5 mg total phosphorus, gave recoveries of at least 95 but mostly about 98 % of the individual compounds. Cross-contamination of successive fractions was under 5 %. In preparative runs, the individual compounds could generally be isolated in a yield of about 90 %, without contamination by other components.

EXPERIMENTAL

Test materials. ADP and ATP were prepared as the barium salts from rabbit muscle [5]. AMP was a commercial preparation, free from ultraviolet-absorbing and phosphorus-containing contaminants. IMP, IDP and ITP were prepared as the barium salts from AMP, ADP and ATP by application of the method of Kleinzeller, described for ITP [6]. IMP was purified by two recrystallizations of the barium salt from water and ADP, ATP, IDP and ITP by ion-exchange chromatography. For this purpose, the barium salts were first converted to the free acids. 5—20 ml Dowex-50 resin (250—500 mesh) in the hydrogen form were added to the ice-cooled solution or suspension of 0.5—2 g of the barium salt in 5—20 ml water and after vigorous stirring for 5—10 minutes at 0° C, the resin was filtered and washed with 5—20 ml water of 0° C. The solution of the free acid thus obtained was adjusted to a pH of about 8.5 with ammonia and passed through an ion-exchange column (see below). The appropriate fractions of the effluent were pooled and the barium salt precipitated by addition to the neutralized pooled fractions at 0° C of a small excess of 2 *N* barium acetate solution, followed by 1—2 volumes of ethanol in the case of ADP and IDP. The barium salts were centrifuged, washed with water/ethanol, reconverted to the free acids with Dowex-50 and after neutralization stored at —20° C. Their identity was further established by chemical analysis (purine : pentose : acid labile phosphorus : total phosphorus) and two-dimensional paper chromatography [7].

Ion-exchange chromatography. Columns of the anion-exchange resin Dowex-2 (250—500 mesh) in the chloride form were used throughout. In analytical runs, columns of bed size 0.3 cm^2 × 2.5 cm and 0.8 cm^2 × 5 cm (flow rate 0.5—1 ml/min) were used for the chromatography of material with a total phosphorus content of under 1 mg and 1—5 mg respectively. In preparative runs, as in the purification of the test materials, columns of bed size 4 cm^2 × 15 cm and 8 cm^2 × 30 cm (flow rate 3 ml/min) were used at 0° C for amounts of 50—100 mg and 200—400 mg total phosphorus respectively. The test substances were adsorbed from dilute ammoniacal solution at about pH 8.5 followed by

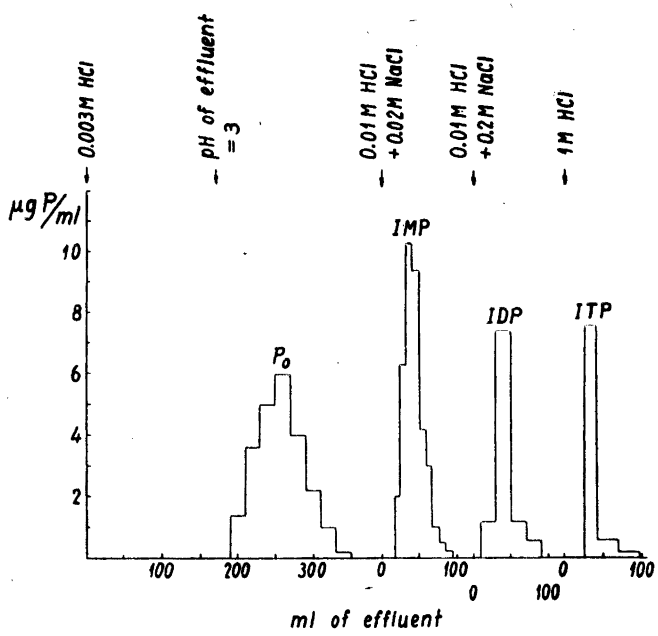

Fig. 1. Ion-exchange separation of inosine phosphates and orthophosphate. Exchanger: Dowex-2, 250—500 mesh, 0.8 cm² × 5 cm, chloride form; flow rate 0.5 ml/min. Test materials: orthophosphate (543 µg), IMP (349 µg), IDP (240 µg), ITP (248 µg) (calculated as total phosphorus present). Recoveries (based on phosphorus analysis): orthophosphate, 98 %; IMP, 98 %; IDP, 99 %; ITP, 97 %.

washing the column with water until the effluent became neutral. The eluting solvents of Cohn and Carter were used except in the separation of mixtures containing both adenosine and inosine phosphates, where elution with 0.05 *M* sodium chloride in 0.01 *M* hydrochloric acid solution was interposed between the eluting agents of next lower and next higher anion content. Fractions of approximately 10 ml in analytical runs and of 50—100 ml in large-scale experiments were taken with an automatic fraction collector adjustable to different time intervals. Each fraction was analysed for total phosphorus and the different fractions were further identified by determination of the acid labile phosphorus, measurement of ultraviolet-absorption and paper chromatography. Individual column runs were carried out with each test substance in order to ascertain the elution position and as an additional test for purity in the final preparations, prior to the analysis of mixtures. Eventual cross-contamination of two successive fractions can be detected by the ratio of total phosphorus : acid labile phosphorus, the ratio of the optical densities at 250 : 260 mµ and by one-dimensional paper chromatography in saturated ammonium sulphate solution : water : *iso*propanol (79 : 19 : 2). This solvent system gives a sharp separation of the adenosine series of phosphates from the inosine series, although no adequate resolution occurs in either series [7]. It is particularly suitable for the present purpose, as cross-contamination by only one compound from each series can be expected.

Analytical procedures. Phosphorus was determined by the method of Allen [8] using a Klett Summerson photoelectric colorimeter with a red filter and was differentiated in separate analyses into total phosphorus, inorganic phosphorus and acid labile phosphorus determined after 10 minutes hydrolysis in *N* hydrochloric acid at 100° C. Pentose was determined by the method of Mejbaum [9] as modified by Albaum and Umbreit [10]. A Klett Summerson photoelectric colorimeter was used with a red filter. Optical densities

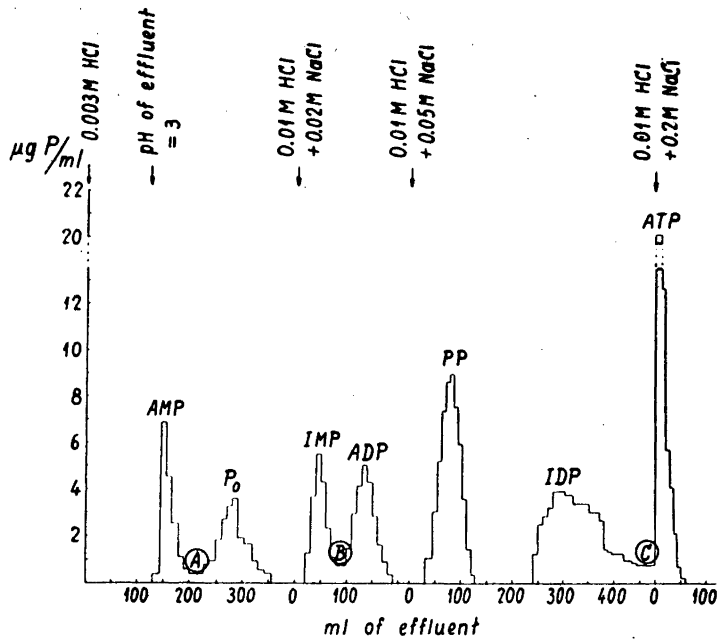

Fig. 2. Ion-exchange separation of inosine phosphates, adenosine phosphates, orthophosphate and pyrophosphate. Exchanger: Dowex-2, 250—500 mesh, 0.8 cm² × 5 cm, chloride form; flow rate 0.5 ml/min. Test materials: orthophosphate (240 µg), pyrophosphate (462 µg), AMP (200 µg), ADP (250 µg), ATP (465 µg), IMP (200 µg), IDP (578 µg) (calculated as total phosphorus present). Recoveries (based on phosphorus analysis): orthophosphate, 102 %; pyrophosphate, 95.5 %; AMP, 97.5 %; ADP, 97.5 %; ATP, 99 %; IMP, 98 %; IDP, 98 %. Cross-contaminated material: 2.3 % at (A), 4.0 % at (B), 2.9 % at (C).

were measured in 0.01 *N* hydrochloric acid solution in a Beckman Universal Spectrophotometer, Model DU, the molecular extinction coefficients of 14 200 at 260 mµ for adenosine phosphates [1] and 13 200 at 250 mµ for inosine phosphates [11] being used.

SUMMARY

By slight modifications of the method of Cohn and Carter for the resolution of adenosine phosphates, a method has been developed for the resolution of a mixture of orthophosphate, pyrophosphate, inosine phosphates and corresponding adenosine phosphates into the individual components.

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Über Pentamethyl-arsen und Pentamethyl-antimon

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Alle Versuche, Pentamethyl-stickstoff und andere Derivate mit fünf am Stickstoff homöopolar gebundenen Kohlenwasserstoffresten darzustellen, waren erfolglos [1]. Wie Wittig und Wetterling [2] nachwiesen, bildet sich bei der Einwirkung von Methyl- oder Phenyl-lithium auf Tetramethyl-ammoniumbromid entsprechend dem Schema:

$$[(CH_3)_3N(CH_3)]\,Br + RLi \rightarrow (I)\ (CH_3)_3\overset{+}{N}-\overset{-}{C}H_2 + RH + LiBr$$

einunddasselbe Trimethyl-ammonium-methylid (I). Dieser Befund entspricht den Forderungen der Oktetttheorie, wonach sich die Achterschale am Zentralatom nicht zu einem Dezett ausweiten kann. Das von Schlenk und Holtz [3] synthetisierte orangefarbene Benzyl-tetramethyl-ammonium: $[(CH_3)_4N]\,CH_2 \cdot C_6H_5$ widerspricht dem nicht, da die Benzylgruppe dem Tetramethyl-ammonium-Rest ionogen zugeordnet ist.

Analog verhält sich das Tetramethyl-phosphonium-jodid gegenüber Methyl-lithium [4], das mit dem Oniumsalz das Trimethyl-phosphonium-methylid (IIa), bezw. Trimethyl-phosphin-methylen (IIb) liefert:

$$[(CH_3)_3P(CH_3)]J + CH_3Li \rightarrow (IIa)\ (CH_3)_3\overset{+}{P}-\overset{-}{C}H_2 \longleftrightarrow (IIb)\ (CH_3)_3P = CH_2$$

Da diese Ausweichreaktionen bei der Umsetzung von Tetraphenyl-oniumsalzen mit Phenyl-lithium nicht möglich sind, entstehen hierbei die Pentaphenyl-verbindungen (III):

$$[(C_6H_5)_4Z]\,Hal + C_6H_5Li \rightarrow (III)\ (C_6H_5)_5Z + LiHal;\ Z = P, As, Sb, Bi,$$

Während Pentaphenyl-stickstoff aus dem oben genannten Grunde nicht existenzfähig sein dürfte, sind Pentaphenyl-phosphor [5], -arsen [6], -antimon [6] und -wismut [7] wohlkristallisierte Verbindungen, die ihren Eigenschaften nach als homöopolare Stoffe anzusehen sind. Bei ihnen muss daher angenommen werden, dass die fünf Liganden am Zentralatom ein Elektronendezett ausbilden. Die bemerkenswert grosse thermische Stabilität der Pentaphenylderivate, die ihr Maximum beim Pentaphenyl-antimon erreicht, lud zu Versuchen ein, das Pentamethyl-arsen und Pentamethyl-antimon darzustellen.

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Bei der Einwirkung von Methyl-lithium auf Tetramethyl-arsonium-jodid in absol. Äther unter Stickstoff beobachtete man eine langsame Gasentwicklung. Das entweichende Gas bestand der Analyse nach aus 93 % Methan und 7 % Äthan. Beim Zusammengeben von äquimolekularen Mengen Methyl-lithium und Tetramethyl-arsonium-jodid blieb noch nicht umgesetztes Arsoniumsalz am Boden zurück, das erst beim Zusatz von Methyl-lithium im Molverhältnis 2 : 1 verschwand. Da bei der nachfolgenden Zugabe von Methyljodid zu dem ätherischen Gemisch Dimethyl-diäthyl-arsonium-jodid gebildet wurde, ist der Verlauf der Reaktion folgendermassen zu deuten:

$$[(CH_3)_3AsCH_3]\ J + CH_3Li \rightarrow \text{(IV)}\ (CH_3)_3As = CH_2 + CH_4 + LiBr \xrightarrow{CH_3Li}$$

$$\text{(V)}\ (CH_3)_2\underset{\displaystyle CH_2Li}{\underset{|}{As}} = CH_2 + CH_4$$

Das intermediär entstehende Trimethyl-arsin-methylen (IV) reagiert also sofort mit Methyl-lithium unter Methanentwicklung weiter und bildet das Metallierungsprodukt (V), das bei der Behandlung mit Methyljodid dann in das isolierte quartäre Arsoniumsalz übergeht. Die Protonenbeweglichkeit im Methyl ist infolge der sich ausbildenden C = As-Bindung so gross, dass weiterer Wasserstoff gegen Lithium glatt ausgetauscht wird; ein Befund, der sich auch bei der Einwirkung lithiumorganischer Verbindungen auf Tetramethyl-ammonium- und phosphoniumsalzen ergab [2,4].

Die in dem entweichenden Gas nachgewiesene geringe Menge an Äthan könnte als ein Indizium darauf betrachtet werden, dass entsprechend:

$$[(CH_3)_4As]\ J + CH_3Li \rightarrow (CH_3)_5As + LiJ$$

Pentamethyl-arsen entstanden ist, das als instabile Verbindung unter den angewandten Bedingungen in Trimethyl-arsin und Äthan zerfällt.

Im Zusammenhang mit diesen Überlegungen wurde das bereits bekannte Trimethyl-arsin-dibromid mit Methyl-lithium umgesetzt, wobei ebenfalls eine Gasentwicklung beobachtet wurde. Der Analyse nach war der prozentuale Anteil an Äthan hier grösser (65 % Methan und 35 % Äthan). Da nach allen Erfahrungen das möglicherweise gebildete Pentamethyl-arsen einen tiefen Siedepunkt haben sollte, wurde die ätherische Lösung nach der Umsetzung destilliert und in der Vorlage auf übergegangene Arsenverbindungen hin untersucht.

Während das bei Zugabe von Brom bezw. Jod ausfallende Trimethyl-arsin-dihalogenid nichts darüber aussagt, ob mit dem Äther Trimethyl-arsin oder Pentamethyl-arsen übergegangen ist, beweist das durch Einwirkung von ätherischem Chlorwasserstoff entstehende Tetramethyl-arsonium-chlorid, das als Doppelsalz * mit Quecksilber-(II)-chlorid isoliert wurde, dass Pentamethyl-arsen — wenn auch in geringem Anteil — im Destillat enthalten ist. Denn das Arsoniumsalz kann nur auf dem folgendem Wege:

$$(CH_3)_5As + HCl \rightarrow [(CH_3)_4As]Cl + CH_4$$

* E. Mannheim, *Ann.* **341** (1905) 197, hatte für das Anlagerungsprodukt die Formel $(CH_3)_4As\ Cl \cdot HgCl_2$ angegeben. Der C-H-Bestimmung nach zeigte es sich doch, dass die Substanz 2 Mol $HgCl_2$ pro Mol enthielt.

entstanden sein. Eine Isolierung des Pentamethyl-arsens gelang nicht, da es bei der Umsetzung des Trimethyl-arsin-dibromides mit Methyl-lithium nur in geringer Menge entstanden sein kann. Wegen seines tiefen Siedepunkts ist es schwer von Äther und Trimethyl-arsin zu trennen.

Die Reaktion von Trimethyl-arsin-dibromid mit Methyl-lithium wurde deshalb in Dimethyläther bei 0° C in einem zugeschmolzenen Schlenkrohr wiederholt. Beim Abdestillieren in eine auf —10° C gekühlte Vorlage bildete sich kein Kondensat. Erst bei Steigerung der Badtemperatur auf 80° und im Vakuum bei 12 Torr ging eine Flüssigkeit über, die offenbar aus Trimethyl-arsin bestand. Denn mit ätherischem Chlorwasserstoff bildete sich nicht das oben beschriebene Tetramethyl-arsonium-chlorid. Dagegen lieferte das Arsin mit Triphenyl-bor ein Addukt, das bei 226—228° schmolz und mit dem aus Trimethyl-arsin und Triphenyl-bor vergleichweise hergestellten Präparat sich als identisch erwies *.

Während also alle Anzeichen dafür sprechen, dass Pentamethyl-arsen bei den genannten Umsetzungen nur nebenher gebildet wird, liess sich Pentamethyl-antimon ohne Schwierigkeit aus Trimethyl-stibin-dibromid und Methyl-lithium gewinnen. Die entsprechend dem Schema:

$$(CH_3)_3SbBr_2 + 2\ CH_3Li \rightarrow (CH_3)_5Sb + 2\ LiBr$$

entstandene Verbindung liess sich bei der fraktionierten Destillation aus dem Reaktionskolben heraus als eine hellgelbe Flüssigkeit isolieren und zeigte bei nochmaliger Rektifizierung den Siedepunkt 126—127° bei 730 Torr.; der Schmelzpunkt lag bei —16 bis —18°. Pentamethyl-antimon ist an der Luft nicht selbstentzündlich, wird aber von Wasser rasch zersetzt. Wärend es sich gegenüber Benzophenon indifferent verhält, reagiert es mit Brom rasch unter Bildung von Tetramethyl-stibonium-bromid, das mit überschüssigem Brom in sein Perbromid übergeht. Triphenyl-bor fällt aus seiner Lösung das ziemlich beständige Tetramethyl-stibonium-triphenylo-methylo-borat:

$$(CH_3)_5Sb + (C_6H_5)_3B \rightarrow [(CH_3)_4Sb]\ [(C_6H_5)_3\ (CH_3)B]$$

Dass bei der Umsetzung von Trimethyl-stibin-dibromid mit Methyl-lithium als Zwischenprodukt das Tetramethyl-stibonium-bromid:

$$(CH_3)_3SbBr_2 + CH_3Li \rightarrow [(CH_3)_4Sb]Br + LiBr$$

anzunehmen ist, wird durch den Befund gestützt, dass dieses Stiboniumsalz bei der Behandlung mit Methyl-lithium ebenfalls in guten Ausbeuten Pentamethyl-antimon bildet.

Da Pentaphenyl-antimon mit Phenyl-lithium den wohldefinierten Komplex Lithium-hexaphenylo-antimonat liefert:

$$(C_6H_5)_5Sb + C_6H_5Li \rightarrow [(C_6H_5)_6Sb]Li,$$

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* Aus der Pentamethyl-arsen enthaltenden Lösung fiel mit Triphenyl-bor das zu erwartende *Tetramethyl-arsonium-triphenylo-methylo-borat* $[(CH_3)_4As][(C_6H_5)_3(CH_3)B]$, aus, das mit dem unscharfen Schmelzpunkt bei 130—140° ganz andere Eigenschaften als das Addukt von Trimethyl-arsin und Triphenyl-bor zeigte.

wurde auch Pentamethyl-antimon auf sein Verhalten gegenüber Methyl-lithium geprüft. Der bei Zusatz von überschüssigem Methyl-lithium zunächst schwach positive, aber dann bald stark positive Gilman-Test weist darauf hin, dass sich die Addenden mit dem Komplex in einem Gleichgewicht befinden:

$$(CH_3)_5Sb + CH_3Li \rightleftharpoons [(CH_3)_6Sb]Li,$$

das im Gegensatz zu dem beim Hexaphenylo-antimonat-Komplex stark nach der Seite der Addenden verlagert ist. Destilliert man die Mischung, geht Pentamethyl-antimon fast vollständig über.

In diesem Zusammenhang wurde auch Tetraphenyl-stibonium-bromid mit Methyl-lithium umgesetzt (Molverhältnis 1 : 2), wobei Pentaphenyl-antimon zu isolieren war. Da bei der nachfolgenden Einwirkung von Benzophenon kein Diphenyl-methyl-carbinol als Reaktionsprodukt des Methyl-lithiums, wohl aber 62 % Tritanol gefasst wurden, ist der Vorgang so zu formulieren:

$$[(C_6H_5)_4Sb]Br + 2\ CH_3Li \rightarrow (VI)\ [(C_6H_5)_4Sb(CH_3)_2]Li \rightleftharpoons$$
$$\rightleftharpoons (C_6H_5)_3Sb(CH_3)_2 + C_6H_5Li;$$
$$[(C_6H_5)_4Sb]Br + C_6H_5Li \rightarrow (C_6H_5)_5Sb + LiBr$$

Also über den Komplex (VI) spaltet sich Phenyl-lithium ab, das mit noch vorhandenem Tetraphenyl-stibonium-bromid Pentaphenyl-antimon liefert und sich an Benzophenon zu Tritanolat addiert.

Ein Rückblick auf die Umsetzung von Tetramethyl-oniumsalzen mit Methyl-lithium führt zu dem Ergebnis, dass das Ammonium- und Phosphonium-halogenid sich in die zugehörigen Ylide umwandeln, wärend das Arsonium-halogenid z.T. und das Stibonium-halogenid ausschliesslich Pentamethyl-metall bilden, die ihren Eigenschaften nach rein homöopolare Verbindungen darstellen. Hieraus folgt, dass die Neigung der Zentralatome, ihre äussere Elektronenschale zu einem Dezett auszuweiten, vom Phosphor über Arsen zum Antimon hin zunimmt.

Die überraschend leichte Bildung von Pentamethyl-antimon legte die Frage nahe, ob unter geeigneten Bedingungen nicht auch Antimonhydrid SbH_5 dargestellt werden könnte. In orientierenden Versuchen wurden Tetraphenyl-stibonium-bromid, Tetramethyl-stibonium-bromid und Trimethyl-stibin-dibromid in ätherischer Suspension mit Lithium-alanat umgesetzt. In allen Fällen beobachtete man bei Zimmertemperatur eine lebhafte Gasentwicklung, die beim Abkühlen auf —70° aufhörte, um beim Auftauen wieder in Gang zu kommen. Dies wies darauf hin, dass die erwarteten Verbindungen:

$$(C_6H_5)_4SbH,\ (CH_3)_4SbH \text{ und } (CH_3)_3SbH_2$$

sehr instabil sein müssen. Wenn überhaupt wird eine Synthese derartiger Verbindungen nur bei sehr viel tieferen Temperaturen möglich sein.

EXPERIMENTELLER TEIL

I. Herstellung einiger Ausgangssubstanzen

a) Tetramethyl-arsonium-jodid. Zu der Methyl-magnesiumbromid-Lösung (aus 90 g Methylbromid und 25 g Magnesium) wurden 50 g Arsentrichlorid in 150 ml Äther zugetropft. Nach beendeter Reaktion destillierte man den Äther und das Trimethyl-arsin [8] ab und zum Destillat gab man 35 g Methyljodid. Nach dreiwöchigem Stehen wurden die gebildeten Kristalle abfiltriert und aus Äthylalkohol (96 %) umkristallisiert. Ausbeute 18 g. Zersp. 315—318°. $C_4H_{12}AsJ$ (262,0) : Ber. J 48,44; Gef. J 48,20.

Zu dem Ätherfiltrat gab man Brom, bis sich kein weiterer Niederschlag bildete. Das ausgefällte Trimethyl-arsin-dibromid wurde 15 Min. mit Aceton gekocht, um den Überschuss von Brom zu entfernen, und nach Abkühlung und Filtrieren aus Eisessig umkristallisiert. Man wusch mit wasserfreiem Petroläther (50–70°) nach. Trimethyl-arsin-dibromid (Nadeln) ist hygroskopisch. Ausbeute 14 g. Zersp. 190–192°. Gesamtausbeute 43 %. $C_3H_9AsBr_2$ (280,0): Ber. Br 57,1; Gef. Br 56,6.

b) Tetramethyl-stibonium-jodid. Setzte man Methyljodid zu einer ätherischen Lösung von Trimethyl-stibin [8] so fiel nach mehrwöchigem Stehen Tetramethyl-stibonium-jodid allmählich aus. Das Salz konnte aus Äthylalkohol (96 %) umkristallisiert werden. Lange weisse Nadeln, die bei Temperaturen oberhalb 250° sublimierten. $C_4H_{12}SbJ$ (308,8): Ber. J 41,10; Gef. J 40,92.

Aus der Ätherlösung konnte durch Zugeben von Brom Trimethyl-stibin-dibromid erhalten werden.

II. Umsetzungen von Arsin- und Arsoniumsalzen mit Methyl-lithium

1) Umsetzung von Trimethyl-arsin-dibromid mit Methyl-lithium

a) Bei Zimmertemperatur. In einem Schlenkrohr wurden zu 5,4 g (19 mMol) getrocknetem und gepulvertem Trimethyl-arsin-dibromid in 25 ml Äther 33 ml 1,25 *N* Methyl-lithium-Lösung (43 mMol) zugetropft. Es setzte sofort Gasentwicklung ohne merkbare Wärmetönung ein. Beim Abkühlen der Mischung hörte sie auf, um beim Auftauen wieder in Gang zu kommen. Nach einigen Stunden war fast alles in Lösung gegangen. Der Äther wurde abdestilliert und das Destillat mit Brom bis zum Eintreten einer Braunfärbung versetzt. Dabei hatte sich ein Niederschlag gebildet, welcher aus Trimethyl-arsin-dibromid bestand. Ausbeute 59 %.

In einem anderen Ansatz wurde das Destillat mit Triphenyl-bor umgesetzt. Es bildete sich sofort ein geringer Niederschlag, der sich beim Stehen über Nacht nicht mehr vermehrte. Rohschmelzpunkt 130–140°. Weisse Nadeln aus Nitromethan. Zersp. etwa 140° unter Gasentw. Mit Trimethyl-arsin bildete Triphenyl-bor in ätherischer Lösung beim mehrstündigen Stehen ebenfalls einen geringen Niederschlag, der aber mit dem oben beschriebenen nicht identisch war. F : 226–228° (aus Nitromethan umkristallisiert). Ein Teil des Destillates wurde mit Jod behandelt, wobei sich Trimethyl-arsin-dijodid bildete.

b) Gasanalyse. Zu 1,37 g Trimethyl-arsin-dibromid in 10 ml Äther wurden 10,0 ml 1,10 *N* Methyl-lithium-Lösung schnell zugegeben. Der eine Schenkel des Schlenkrohres wurde dann über ein kleines Phosphorpentoxyd-Rohr mit einer Gasbürette verbunden. Anschliessend wurde das Gas mit konz. Schwefelsäure gut gewaschen, um den Ätherdampf zu entfernen. Man liess die Reaktion über Nacht gehen. Es wurden 124 ml (reduciert) Gas aufgefangen, berechnet für Methan oder Äthan 110 ml. Die Gasanalyse zeigte, dass es sich um eine Mischung von Methan und Äthan handelte und zwar etwa 65 % Methan und 35 % Äthan. Zu der Lösung wurden 2 g Methyljodid zugesetzt, wobei sich ein Niederschlag bildete. Gleichzeitig trat Gasentwicklung ein (Überschuss von Methyl-lithium). Der Niederschlag wurde als Tetramethyl-arsonium-jodid identifiziert $C_4H_{12}AsJ$ (262,0): Ber. J 48,44; Gef. J 48,55.

Gasanalyse.

Sauerstoff (98,1 %) vorgelegt	69,8 ml
» rein »	68,5 »
Probe	33,9 »
» (Sauerstoff absorbiert)	32,3 »
Volumen nach Verbrennung	44,1 »
» » Kohlendioxydabsorption	21,7 »
» » Sauerstoffabsorption	10,8 »

c) Umsetzung in Dimethyläther. In ein 150 ml-Schlenkrohr wurden unter Stickstoff 40 ml 1,25 *N* Methyl-lithium (50 mMol) eingefüllt und der Äther im Vakuum abgedampft (Badtemperatur nicht höher als 40°). Gleichzeitig hatte man etwa 60 ml Dimethyläther kondensiert und setzte sie unter guter Kühlung zu der im Schlenkrohr zurückgebliebenen

Mischung von Lithiumsalz und Methyl-lithium. Dann wurden 5,6 g (20 mMol) getrocknetes und fein gepulvertes Trimethyl-arsin-dibromid zugesetzt, das Rohr zugeschmolzen und drei Tage geschüttelt. Beim Öffnen (−65°) war der Gasdruck gering. Der Methyläther wurde durch eine auf −10° gekühlte Vorlage destilliert, worin sich jedoch kein Kondensat bildete. Der Gilman-Test im Rückstand war negativ. Erst bei Erhitzen im Vakuum auf 80° (die Vorlage auf −70° gekühlt) erhielt man etwa 2 ml farbloses, leichtflüchtiges Destillat, das zunächst von den Lithiumsalzen festgehalten worden war. Mit Triphenyl-bor wurde nach einer Weile ein geringer Niederschlag erhalten, der mit dem oben unter a) aus Trimethyl-arsin und Triphenyl-bor gewonnenen, identisch war. F : 226−228°. Mit Chlorwasserstoff in Äther erhielt man keinen Niederschlag. Quecksilber-(II)-chlorid gab eine geringe Fällung vom F: 263−264°. $As(CH_3)_3 \cdot 2HgCl_2$ schmilzt bei 265° [9].

d) Umsetzung bei −50° bis −60°. Ein Ansatz aus 6,37 g Trimethyl-arsin-dibromid in 40 ml Äther und 55 ml 1,1 *N* Methyl-lithium-Lösung wurde drei Tage bei −50° bis −60° geschüttelt. Der Äther wurde im Vakuum abdestilliert. Der Rückstand im Kolben wurde in Wasser gelöst und mit einer wässrigen Pikrinsäure-Lösung versetzt. Es bildete sich kein Niederschlag. Beim Zugeben einer Lösung von Jod in Benzol zu einem Teil des Destillats fiel Trimethyl-arsin-dijodid aus. Ein anderer Teil (ein Drittel der Lösung) wurde mit Triphenyl-bor versetzt, wobei sich sofort ein weisser Niederschlag von Tetramethyl-arsonium-triphenylo-methylo-borat bildete. Aus Nitromethan umkristallisiert gab es weisse Nadeln vom Zersp. etwa 140° (unter Gasentwicklung). Die Ausbeute war sehr gering: 0,3 g.

Die Kristalle haben einen widerlichen Geruch und beim Trocknen im Exsikkator verlieren sie an Gewicht.

Im Rest des Destillats (ein Drittel) wurde gasförmiger Chlorwasserstoff eingeleitet. Dabei fiel ein geringer weisser Niederschlag aus. Dieser wurde in Wasser gelöst und mit einer wässrigen Quecksilber-(II)-chlorid-Lösung versetzt. Ein Addukt von Tetramethyl-arsonium-chlorid und zwei $HgCl_2$ bildete sich, das aus Wasser umkristallisiert einen Schmelzpunkt von 176−177° gab. Ausbeute 0,35 g. $C_4H_{12}AsCl \cdot 2HgCl_2$ (713,6): Ber. C 6,73; H 1,70; Gef. C 6,70; H 2,03.

Ein Vergleichspräparat wurde folgendermassen hergestellt. 0,52 g Tetramethyl-arsonium-jodid wurden in das Chlorid durch Behandeln mit Silberoxyd und Ansäuern mit Salzsäure umgewandelt. Zu der wässrigen Lösung des Chlorids wurde eine gesättigte Quecksilber-(II)-chlorid-Lösung gegeben. Weisse Kristalle fielen allmählich aus. Schmelzpunkt und Mischschmelzpunkt 177−178°. Ausbeute 1,1 g.

Ein Versuch über einer Kolonne Pentamethyl-arsen vom Äther zu trennen, war erfolglos. Alles ging beim Siedepunkt des Äthers 34−35° über.

2) Umsetzung von Tetramethyl-arsonium-jodid mit Methyl-lithium.

7,0 ml 1,05 *N* Methyl-lithium-Lösung (7,3 mMol) wurden unter Stickstoff zu 1,00 g (3,8 mMol) Tetramethyl-arsonium-jodid in 15 ml Äther gegeben. Ein Vorversuch hatte erwiesen, dass beim Zusammengeben von äquimolekularen Mengen Methyl-lithium und Tetramethyl-arsonium-jodid noch unumgesetztes Arsoniumsalz am Boden zurückblieb. Eine langsame Gasentwicklung setzte ein. Die Mischung wurde über Nacht geschüttelt. Nach dem Absetzen des gebildeten Niederschlages dekantierte man den Äther ab (der Gilman-Test war positiv) und wusch zweimal mit absol. Äther nach. Zu dem Niederschlag in etwas Äther suspendiert setzte man 3 g Methyljodid, wobei sich unter Aufsieden des Äthers Dimethyl-diäthyl-arsonium-jodid bildete; aus absol. Alkohol umkristallisiert Blätter vom Zersp. 305°. An der Luft wurden sie allmählich gelb. Ausbeute 0,62 g. $C_6H_{16}AsJ$ (290,0): Ber. C 24,84; H 5,56; Gef. C 24,51; H 5,48.

In einem zweiten Ansatz wurde das Gas aufgefangen und analysiert. Es bestand aus fast reinem Methan (93 %) und etwas Äthan (6−7 %).

Analysenwerte

Sauerstoff (98,1 %) vorgelegt	61,2 ml
» rein »	60,0 »
Probe	39,6 »
» (Sauerstoff absorbiert)	36,9 »
Volumen nach Verbrennung	44,7 »
» » Kohlendioxydabsorption	24,4 »
» » Sauerstoffabsorption	14,3 »

III. Umsetzung von Tetramethyl-arsonium-jodid mit Phenyl-lithium

a) 7,6 ml 1,15 *N* Phenyl-lithium-Lösung wurden zu 2,0 g Tetramethyl-arsonium-jodid gegeben. Allmählich bildete sich ein neuer weisser Niederschlag. Nach 20 Stunden war der Gilman-Test schwach positiv. Am Boden konnte noch nicht umgesetztes Arsoniumsalz beobachtet werden, weshalb portionsweise weitere 6 ml Phenyl-lithium-Lösung zugegeben wurden. Der Niederschlag vermehrte sich und nach 12-stündigem Stehen wurde er wieder suspendiert. Zu der Hälfte der Suspension wurden 3 g Methyljodid gegeben, wobei sich unter Wärmeentwicklung Dimethyl-diäthyl-arsonium-jodid bildete. Schmelzpunkt und Mischschmelzpunkt 305°. Ausbeute 0,5 g.

Die andere Hälfte wurde in Eiswasser gegossen und das Hydrolysat mit gesättigter wässriger Pikrinsäure-Lösung versetzt. Gelbe Kristalle von Tetramethyl-arsonium-pikrat fielen aus. Sie wurden aus Wasser umkristallisiert. Gelbe Nadeln von Zersp. 292–294°. Ausbeute 0,3 g. $C_{10}H_{14}O_5N_3As$ (363,2): Ber. C 33,07; H 3,89; Gef. C 33,38; H 3,91.

Zur Herstellung des Vergleichspräparates wurde zu 0,2 g Tetramethyl-arsonium-jodid in 3 ml Wasser ein Überschuss von gesättigter wässriger Pikrinsäure-Lösung gegeben. Gelbe Nadeln fielen aus, 0,2 g. Sie wurden aus Wasser umkristallisiert. Schmelzpunkt und Mischschmelzpunkt 292–294°. $C_{10}H_{14}O_5N_3As$ (363,2): Ber. C 33,07; H 3,89; Gef. C 33,76; H 4,11.

IV. Umsetzungen von Stibin- und Stiboniumsalzen mit Methyl-lithium

1) Umsetzung von Tetraphenyl-stibonium-bromid mit Methyl-lithium.

a) Zu einer Suspension von 2,55 g (5 mMol) getrocknetem und feingepulvertem Tetraphenyl-stibonium-bromid [10] in 15 ml Äther liess man unter Stickstoff 9,0 ml 1,17 *N* Methyl-lithium-Lösung (10,8 mMol) zutropfen, wobei sich ein geringer Niederschlag bildete. Man konnte keine Gasentwicklung beobachten. Der Gilman-Test war erst bei Zusatz von 7–8 ml Methyl-lithium positiv (9 mMol). Die Mischung wurde über Nacht geschüttelt und dann mit Eiswasser zersetzt. Eine merkliche Gasentwicklung war dabei nicht zu beobachten. Eine kleine Menge Niederschlag wurde abfiltriert, die ätherische Schicht von der wässrigen abgetrennt und mit Calciumchlorid getrocknet. Der Niederschlag wurde aus absol. Alkohol umkristallisiert (F: 159–161°) und als Pentaphenyl-antimon [6] identifiziert. Ausbeute 0,2 g. $C_{30}H_{25}Sb$ (507,3): Ber. C 71,03; H 4,97; Gef. C 70,43; H 5,11.

Der Mischschmelzpunkt mit Pentaphenyl-antimon (F: 167–169°) lag zwischen den Schmelzpunkten der beiden Verbindungen. Mit Brom und konz. Bromwasserstoffsäure ergab die Substanz Tetraphenyl-stibonium-bromid zurück. F: 211–214°. Mit überschüssigem Brom wurde das rote Perbromid vom Zersp. 128° erhalten.

Von der ätherischen Schicht wurde der Äther abdestilliert. Im Kolben blieb eine schmierige Masse zurück, die wahrscheinlich aus einer Mischung von Phenyl- und Methylderivaten des fünfwertigen Antimons bestand. Durch Behandeln mit Brom erhielt man nur uneinheitliche Produkte. Auf eine weitere Aufarbeitung der Substanz wurde deshalb verzichtet.

b) Behandlung des Reaktionsprodukts mit Benzophenon. 5,0 g (9,8 mMol) Tetraphenyl-stibonium-bromid in 30 ml Äther wurden mit 23 ml 0,80 *N* Methyl-lithium-Lösung (18,4 mMol; 88 % der zweiten Molstufe) titriert und drei Tage geschüttelt. Dann wurden 1,70 g (0,90 mMol) Benzophenon in 5 ml Äther portionsweise zugegeben, wobei sich ein Niederschlag bildete. Die Mischung wurde noch drei Tage geschüttelt und nach dem Absetzen des Niederschlages wurde der Äther abdekantiert, der Niederschlag einmal mit Äther gewaschen und die Gesamtäthermenge mit Eiswasser zersetzt. Eine Titration mit Säure ergab 6,3 mMol. Der Niederschlag wurde ebenfalls hydrolysiert. Das Hydrolysat verbrauchte 6,5 mMol Säure. Aus dem in Wasser unlöslichen Teil konnte man 1,5 g (62 %) Triphenylcarbinol isolieren. Schmelzpunkt und Mischschmelzpunkt 159–161°. Dagegen konnte kein Diphenyl-methyl-carbinol nachgewiesen werden. Aus der ätherischen Schicht erhielt man wie oben ein uneinheitliches Produkt. 0,4 g Pentaphenyl-antimon wurden isoliert.

2) Umsetzung von Trimethyl-stibin-dibromid mit Methyl-lithium.

a) 40 ml 1,1 *N* Methyl-lithium-Lösung (44 mMol) wurden zu 6,54 g (20 mMol) getrocknetem und feingepulvertem Trimethyl-stibin-dibromid in 40 ml Äther unter Stickstoff und Kühlung mit kaltem Wasser zugetropft. Das Bromid löste sich allmählich auf und man erhielt zum Schluss eine klare Lösung. Eine geringe Gasentwicklung wurde beobachtet. Der Gilman-Test war noch negativ. Der Äther wurde bei gewöhnlichem Druck abdestilliert und die verbliebene Flüssigkeit im Vacuum. Erneuerte Destillation über einer kleinen Kolonne bei 730 Torr unter Stickstoff ergab eine schwachgelbe Flüssigkeit vom Kp 126—127°. Ausbeute 2,5 g oder 63 %. $C_5H_{15}Sb$ (196,9): Ber. C 30,47; H 7,68; Sb 61,85; Gef. C 30,33; H 7,24; Sb 61,90.

Pentamethyl-antimon ist eine gegen Luft und Feuchtigkeit empfindliche Substanz, die aber in Ampullen unter Stickstoff für längere Zeit haltbar ist. Sie erstarrt bei —16 bis —18°. An der Luft entzündet sie sich nicht von selbst.

Umsetzungen von Pentamethyl-antimon

Verhalten gegen Wasser. Pentamethyl-antimon ist in Wasser unlöslich, zersetzt sich aber nach einer Weile und gibt eine stark alkalische Lösung.

Umsetzung mit Brom. Zu einer kleinen Probe von Pentamethyl-antimon in Äther wurde eine Lösung von Brom in Tetrachlorkohlenstoff zugetropft, wobei zuerst ein weisser Niederschlag von Tetramethyl-stibonium-bromid entstand, welches mit einem Überschuss von Brom in ein rotgelbes Perbromid überging. Die weisse Substanz war rein genug für eine Analyse. Die rote wurde zuerst aus absol. Äthylalkohol umkristallisiert. Rotgelbe Nadeln, die sich bei 140° zersetzen. $C_4H_{12}SbBr$ (261,8): Ber. 30,5; Gef. Br 30,2. $C_4H_{12}SbBr_3$ (421,7): Ber. Br 56,98; Gef. Br 56,25.

Umsetzung mit Benzophenon. Zu 0,20 g Pentamethyl-antimon in 5 ml Äther wurden 0,18 g Benzophenon (1 mMol) gegeben. Nach zehntägigem Stehen destillierte man den Äther ab. Im Destillat wurden 30 % Pentamethyl-antimon wiedergefunden. Im Kolben blieb eine feste Substanz zurück, die als Benzophenon identifiziert wurde. Diphenyl-methyl-carbinol konnte nicht nachgewiesen werden.

Umsetzung mit Triphenyl-bor. Zu 0,20 g Pentamethyl-antimon (1 mMol) in 5 ml Äther wurden 0,24 g Triphenyl-bor in 10 ml Äther unter Stickstoff zugegeben, wobei sich sofort ein weisser Niederschlag bildete, der aus Tetramethyl-stibonium-triphenylomethylo-borat bestand. Ausbeute 0,36 g Rohprodukt. Es liess sich aus absol. Alkohol umkristallisieren und gab dabei farblose Nadeln. Die Substanz zeigte keinen definierten Schmelzpunkt. Sie fing bei etwa 180° zu sintern an. Bei rascher Temperatursteigerung schmolz die Substanz um 240° und wurde bald wieder fest. $C_{23}H_{30}SbB$ (439,1): Ber. C 62,88; H 6,87; Gef. C 62,10; H 6,88.

b) Wie oben wurden 1,64 g Trimethyl-stibin-dibromid (5 mMol) in 10 ml Äther mit 16,5 ml 1,0 *N* Methyl-lithium-Lösung versetzt.

mMol Methyl-lithium	Gilman-Test
10,0 mMol	neg.
12,5 mMol	schwach pos.
15,0 mMol	pos.
16,5 mMol	pos.

Die Lösung wurde destilliert und im Destillat konnte man durch Behandeln mit Brom 0,95 g Tetramethyl-stibonium-bromid (72 %) isolieren. Die ursprüngliche Lösung wurde auf —70° abgekühlt. Dabei fielen Kristalle aus, die sich aber als reines Lithiumbromid erwiesen.

3) Umsetzung von Tetramethyl-stibonium-jodid mit Methyl-lithium.

1,05 g feingepulvertes Tetramethyl-stibonium-jodid wurden in 10 ml Äther suspendiert und unter Stickstoff mit 3,5 ml 1,3 *N* Methyl-lithium-Lösung versetzt. Das Jodid löst sich in einigen Minuten auf. Gasentwicklung wurde nicht beobachtet. Nach zweistündigem Stehen wurde der Äther bei Atmosphärendruck abdestilliert. Im Kolben hinterblieb das Pentamethyl-antimon, welches im Vacuum überdestilliert wurde. Durch Behandeln mit Brom wurde es in Tetramethyl-stibonium-bromid übergeführt; 0,25 g oder 28 % Ausbeute. $C_4H_{12}SbBr$ (261,8): Ber. Br 30,5; Gef. Br 30,3.

0,20 g Tetramethyl-stibonium-jodid wurden in wenig siedendem Äthylalkohol (96 %) gelöst und zu der warmen Lösung wurde im Überschuss eine gesättigte Lösung von Jod in Benzol gegeben. Beim Erkalten bildeten sich körnige blauschwarze Kristalle vom Perjodid. Ausbeute 0,30 g. Zersp. 136°. $C_4H_{12}SbJ_3$ (562,7): Ber. J 67,70; Gef. J 67,22.

V. Umsetzungen von Stibin- und Stiboniumsalzen mit Lithium-alanat ($LiAlH_4$)

1) Trimethyl-stibin-dibromid und Lithium-alanat

Zu 1,64 g (5 mMol) getrocknetem und gepulvertem Trimethyl-stibin-dibromid in 10 ml Äther wurden unter Stickstoff 17 ml 0,17 *M* Lithium-alanat-Lösung zugetropft. Eine kräftige Gasentwicklung trat ein, die bei Kühlung auf −70° aufhörte, um bei Steigerung der Temperatur wieder in Gang zu kommen. Als die Reaktion beendet war, wurde der Äther abdestilliert. Im Destillat konnte man durch Zugeben von Brom 1,25 g Trimethyl-stibin-dibromid (76 %) isolieren.

2) Tetramethyl-stibonium-bromid und Lithium-alanat.

In einer mit Stickstoff gefüllten Apparatur nach Zerewitinoff wurden in den einen Schenkel 2 ml 0,17 *M* Lithium-alanat-Lösung gegeben und in dem anderen 0,26 g Tetramethyl-stibonium-bromid in 5 ml Äther vorgelegt. Beim Zusammengeben trat sofort Gasentwicklung ein. Der Niederschlag löste sich fast vollständig auf. Reaktionstemperatur 20°. Gemessene Gasmenge 32,0 ml (reduziert).

3) Tetraphenyl-stibonium-bromid und Lithium-alanat.

In dem Zerewitinoff-Apparat wurden zu 0,51 g Tetraphenyl-stibonium-bromid in 4 ml Äther 3 ml 0,17 *M* Lithium-alanat-Lösung gegeben. Auch hier trat sofort Gasentwicklung ein. Der Niederschlag löste sich unter Wärmeentwicklung fast vollständig auf. Reaktionstemperatur 20°. Gemessene Gasmenge 5,0 ml (reduziert).

ZUSAMMENFASSUNG

Das Verhalten einiger Arsonium- und Stiboniumsalze gegenüber lithiumorganischen Verbindungen wird beschrieben. Bei der Umsetzung von Trimethyl-stibin-dibromid und Tetramethyl-stibonium-bromid mit Methyllithium erhält man in guter Ausbeute das noch unbekannte Pentamethylantimon.

Der eine von uns (K. T.) ist dem *Statens Naturvetenskapliga Forskningsråd*, Stockholm, Schweden, für einen Reisebeitrag zu Dank verpflichtet.

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Furfuryl Mercaptan

An Improved Preparative Method

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It has been observed that 2-furfuryl alcohol reacts exceptionally smoothly with equimolar amounts of thiourea and hydrochloric acid in aqueous solution to give furfuryl *iso*thiourea. This reaction is utilized as a step in a rapid and reliable method for the preparation of 2-furfuryl mercaptan. The yield was 60 %.

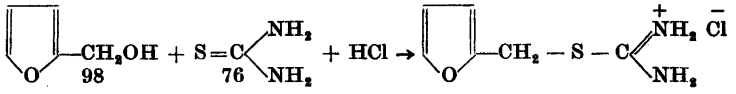
The reaction mechanism is briefly discussed.

Two syntheses of 2-furfuryl mercaptan have previously been reported in the literature. According to one method, which is only briefly described in a patent issued to Staudinger and Reichstein [1], furfural is treated with ammonium hydrosulphide and the resulting *bis*-furfuryl disulphide is subsequently reduced to the mercaptan. Neither physical properties nor yield were stated. Attempts by the present author to reproduce the method gave exceedingly small yields.

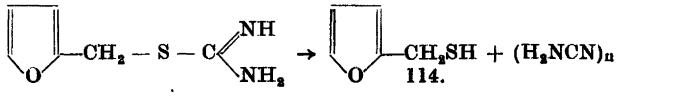
The other method was reported in 1929 by Kirner and Richter [2]. They prepared furfuryl *iso*thiouronium chloride from furfuryl chloride and thiourea. The product was hydrolyzed without being isolated, and the mercaptan was obtained in 33 % yield. Although this is a useful method of quite general applicability to mercaptans, it is, however, less convenient in the present instance. The preparation of furfuryl halides is somewhat laborious, because the furane ring is extremely sensitive to hydrogen halides. Preparation of furfuryl chloride in quantity was in fact only achieved in 1928, when Kirner [3] applied Darzens's method of halogenation [4], *i.e.* treatment of furfuryl alcohol with thionyl chloride in pyridine solution. It is the present author's experience, however, that appreciable resinification always occurs, even when great care is taken; the yield varies, and the pure furfuryl chloride is exceedingly unstable. It decomposes within a few days.

In experiments made during the years 1942—1943, it was observed that the intermediate halide could conveniently be omitted, and the mercaptan obtained in 60 % yield by direct action of furfuryl alcohol on thiouronium

chloride in aqueous medium (I), followed by thermal decomposition of the liberated furfuryl *iso*thiourea (II).



(I)



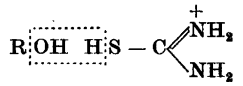
(II)

The direct formation of *iso*thiouronium salts from alcohols was first reported by Stevens [5] in 1902, and it was later employed by Johnson and Sprague [6] as an intermediate step in the synthesis of alkylsulphonyl chlorides. It had not so far been applied to the preparation of mercaptans. The method described in detail in the experimental section has been in frequent use during the last war. It was found convenient and reliable. Furfuryl mercaptan is a major component in roast coffee aroma, and the synthetical product was used in extremely small concentrations for aromatization of artificial coffee.

After the war a paper by Frank and Smith [7] appeared, in which these investigators independently applied essentially the same method to the preparation of a number of acylic and carbocyclic mercaptans. The experimental conditions reported by Frank and Smith were far more rigorous than those described in the present paper. They used a large excess of hydrogen halide (as concentrated hydrochloric or hydrobromic acid), 3 moles per mole thiourea, and refluxed the reaction mixture for 9 hours. The formation of furfuryl *iso*thiouronium halide on the other hand takes place spontaneously in not very concentrated aqueous solution, when equimolar amounts of the reactants in equation (I) are mixed at room temperature. The reaction is strongly exothermic, and it is imperative to control the process by suitable cooling in order to avoid excessive discolouration. It is not quite certain, whether the process goes to completion under these circumstances, but subsequent refluxing, as recommended by Frank and Smith, did not increase the yield, on the contrary it was greatly reduced, and a large amount of tar was obtained. The large excess and much higher concentration of hydrochloric acid used by Frank and Smith is strongly contraindicated because of the sensitivity of the furan ring to mineral acids.

Frank and Smith decomposed the thiouronium compound (reaction II) by refluxing the neutralized reaction mixture for two hours. A more convenient method in the present case was steam destillation of the neutralized mixture. The free furfuryl *iso*thiourea decomposed readily at 100° and the mercaptan, which is practically insoluble in water, separated from the destillate as a colourless heavy oil of high purity. The yield was uniformly 60 % based upon furfuryl alcohol.

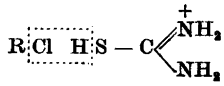
As already pointed out by Frank and Smith [7] two different mechanisms may explain the general process exemplified by reaction (I). Either there is a direct condensation of the alcohol with the *iso*-thiouronium ion



(A)

or an intermediate halide is formed

$$ROH + HCl \rightarrow RCl$$



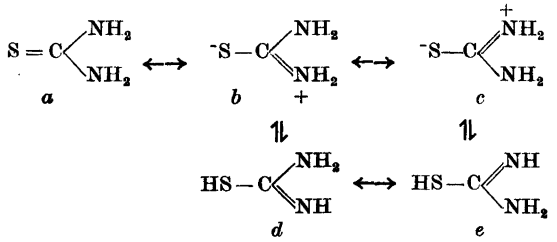
(B)

In mechanism (A) the hydrogen halide acts only as an acid, in mechanism (B) it has the additional function as a halogenating agent.

Frank and Smith obtained some evidence in favour of (B) in that better yields were generally obtained of the various mercaptans, when hydrochloric acid was replaced by hydrobromic acid. This is easily understood on the basis of mechanism (B), since it is well known that alkyl bromides form easier than alkyl chlorides from an alcohol and hydrogen halide. Such difference would not be expected if the hydrogen halides acted only in their capacity as acids as in mechanism (A). On the other hand Frank and Smith found that octyl mercaptan could be prepared, although in minute yield, when using sulphuric acid in stead of hydrogen halides. They considered this observation to be in favour of mechanism (A). It is, however, well known that alkyl sulphates are powerful alkylating agents and may act as intermediates similarly to the alkyl halides, *i.e.* the observation is — *mutatis mutandis* — equally well compatible with mechanism (B).

Regarding the particular process involving furfuryl alcohol it appears at a first view that mechanism (A) must obtain, since the reaction between the alcohol and thiouronium chloride proceeds smoothly without stoichiometric excess of hydrochloric acid as used by Frank and Smith. It must, however, be remembered that thiouronium chloride is almost completely hydrolyzed in aqueous solution [8,9] and consequently there is free hydrochloric acid available for the formation of intermediate furfuryl chloride. From this argument therefore it is impossible to decide about the mechanism.

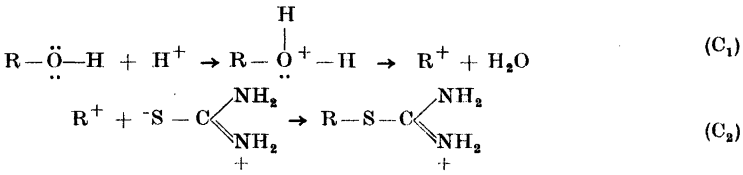
Since very little thiouronium ion exists in aqueous solution, it may be doubtful, whether it is really this ion that reacts, or it is free thiourea. Modern views on the structure of this substance may be summarized in the following scheme



X-ray diffraction methods [10] and Raman spectroscopy [11] have revealed that in the crystalline state thiourea is a planar symmetrical resonance hybrid of the structures $a \leftrightarrow b \leftrightarrow c$, the latter method giving considerable support to the ionic structures. The same formulation may be applied to the substance in solution. In older work on the absorption in ultraviolet it was concluded

that the substance has the *iso*thiourea structure $d \leftrightarrow e$, whereas a was discarded [12]. It is also claimed [13] that the tautomerism between $a \leftrightarrow b \leftrightarrow c$ on one hand and $d \leftrightarrow e$ on the other hand has been confirmed by osmometric measurements.

It seems reasonable to suppose that the reaction (I) is of ionic nature and the following mechanism, which is closely related to mechanism (A), is suggested



(C_1)

(C_2)

Obviously decisive evidence can only be derived from a kinetic examination of the reaction, but the following observations are compatible with the mechanism:

i. The reaction proceeds satisfactorily only in acid solution. The effect of the acid probably is to favour the dissociation of the alcohol (C_1).

ii. It was observed that, contrary to the findings of Frank and Smith [7] for other mercaptans, the course of the reaction and the yields were identical, when using hydrochloric acid and hydrobromic acid respectively. This suggests that only the hydrogen ion participates in the reaction, and that an intermediate halide formation is unlikely in this particular instance.

iii. S-alkyl *iso*thioureas are known to be much stronger bases than thiourea itself. This is manifested in the mechanism. Thiourea appears in its basic form, whereas the product is formulated as an *iso*thiouronium ion (C_2).

Attempts were made to prepare various structurally related primary mercaptans according to the "mild" method described in the experimental section. The alcohols used as starting materials were tetrahydrofurfuryl, allyl, benzyl, *n*-butyl and cinnamic alcohol. There was no spontaneous rise of temperature, and after a time corresponding to at least 60 % reaction of furfuryl alcohol most of the alcohols could be recovered unchanged. It therefore seems true that furfuryl alcohol reacts exceptionally smoothly with thiourea, and that in most other cases either a much longer time of reaction or the rigorous conditions employed by Frank and Smith [7] are required. The slow reaction of tetrahydrofurfuryl alcohol indicates that the double bond is essential. On the other hand the apparent failure of allyl alcohol to react shows that something more than the unsaturation is needed to accelerate the reaction. A possible explanation may be found in the electron distribution in the furan ring. There is a relatively high electron density on the ring carbon atom in position 2, and this negative charge may activate the alcohol group (step C_1).

EXPERIMENTAL

380 g (5 moles) thiourea were dissolved by gently heating in a mixture of 405 ml hydrochloric acid (5 moles) and 500 ml water. The exact concentration of the hydrochloric acid had previously been determined by titration. The solution was cooled to room temperature and 490 g (5 moles) furfuryl alcohol (b.p.$_{760}$ 170°) was quickly added

with stirring. In 1–2 minutes the reaction started. It was accompanied by a considerable evolution of heat and the temperature was held below 60° by cooling the reaction vessel under the water tap. When the reaction slowed down, cooling was discontinued, and the dark green but clear solution was left over night for completion of reaction. Then a solution of 225 g sodium hydroxide, containing 5 moles NaOH, in 250 ml water was added. A heavy brown oil precipitated, consisting of furfuryl *iso*thiourea, which was already partly decomposed to the mercaptan. The flask was equipped with a condenser for steam distillation. The mercaptan separated from the destillate as a colourless heavy oil. The dried yield was 340 g or approximately 60 %. The product distilled uniformly at 84° C at 65 mm Hg and had $n_D^{20} = 1.533$ in accordance with literature [2]. It could be distilled at atmospheric pressure without destruction. The boiling point was 160° at 759 mm Hg.

Grateful acknowledgements are made to the head of the laboratory, professor H. Baggesgaard Rasmussen, for the kind interest, he has shown in this work.

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Short Communications

Guanosine Triphosphate and Uridine Triphosphate from Muscle

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Upon subjecting adenosine triphosphate (ATP) which has been prepared [1] as the barium salt from rabbit muscle to ion exchange chromatography on the strong base anion exchange resin Dowex-2 according to Cohn and Carter [2], the presence is revealed of a contaminating phosphate fraction in ATP preparations, amounting to 2—4 % of the ATP. By application of the ion exchange procedure directly to trichloroacetic acid extracts of muscle (after removal of the bulk of trichloroacetic acid by ether), similar amounts of the contaminating phosphate fraction are obtained. Phosphorus analysis shows the presence of easily hydrolysable (10 minutes in 1 *N* hydrochloric acid at 100° C) and difficultly hydrolysable phosphate in the ratio of 2 : 1, corresponding to that of a triphosphate. The elution position in ion exchange chromatography is that of inosine triphosphate [3], *i.e.* it is eluted by 1 *N* hydrochloric acid following the removal of ATP. Ultraviolet absorption measurements, however, give an absorption curve differing from that of inosine nucleotides. Paper chromatography in the saturated ammonium sulphate solution — water — *iso*propanol (79 : 19 : 2) solvent system [4] — locating the spots both by ultraviolet photography and spraying with the molybdate reagent of Hanes and Isherwood [5] — reveals the presence of two ultraviolet-absorbing and phosphorus-containing components in the unknown phosphate fraction contaminating ATP. Analyses on the eluted spots give the ultraviolet absorption curve of a guanosine derivative for the slower moving spot and that of a uridine derivative for the faster moving one, and an approximate ratio of 1 mole base : 3 atoms phosphorus for each spot. Pentose estimations [6] indicate the presence of 1 mole pentose per mole base in the slower moving spot and the absence of directly estimable pentose in the faster moving one, as expected for guanosine and uridine derivatives respectively. Paper chromatography after hydrolysis for 60 minutes in 1 *N* hydrochloric acid at 100° C gives two spots, the positions of the spots being identical with those of guanine and uridylic acid (four solvent systems). After hydrolysis for 60 minutes in 70 % perchloric acid [7] at 100° C, two spots with positions identical with those of guanine and uracil — chromatographed side by side with the experimental solution — are obtained (four solvent systems). The eluted guanine spots from the experimental solution and the authentic guanine sample gave identical absorption spectra in acid and alkali. The absorption spectra of the eluted spots from the experimental solution were likewise identical with the corresponding spots from authentic uracil and uridylic acid respectively, both in acid and alkali, and after treatment with bromine. After hydrolysis in 1 *N* sulphuric acid (60 minutes, 100° C) guanine could be isolated as the sulphate [8] in the form of colourless needles, indistinguishable from the sulphate prepared under identical conditions from authentic guanine. The ultraviolet absorption curve, identical for both preparations, gave the guanine content calculated for $(C_5H_5ON_5)_2$, H_2SO_4, $2H_2O$.

Summarising, the results of paper chromatography before and after acid hydrolysis indicate the presence of guanosine triphosphate and uridine triphosphate in ATP preparations and in trichloroacetic acid extracts of muscle. Roughly equimolar amounts of the two triphosphates are found, corresponding to approximately 2—4 mg of each phosphate, compared with approximately 200 mg of ATP per 100 g of fresh muscle.

By precipitation of the barium salt in pooled fractions of the effluent from ion

exchange chromatography followed by fractional reprecipitations, we were able to obtain a nearly complete separation of the two components. The less soluble barium salt fraction gave the absorption spectrum of a guanylic acid derivative and a ratio of 1 : 1 : 2 for guanine : difficultly hydrolysable phosphate : acid labile phosphate. Only a trace of the uridylic acid derivative could be detected by paper chromatography before and after hydrolysis. The most soluble barium salt fraction gave the absorption curve of the uridylic acid derivative and a ratio of 1 : 1 : 2 for uracil : difficultly hydrolysable phosphate : acid labile phosphate. Paper chromatography revealed the presence of traces of the guanylic acid derivative. Pentose estimations using the orcinol reagent before and after bromination [9] for the determination of purine- and pyrimidine-bound pentose, gave confirmatory results. Experiments on the periodate oxidation and copper complex formation of guanosine and uridine triphosphates in comparative tests with ATP, and adenosine 5′- and 3′-phosphates showed that the two new triphosphates behaved as if the phosphate groups were in the 5′-position. Although location of the acid labile phosphate groups in the purine or pyrimidine part of the molecule is not excluded, we consider that their rates of acid hydrolysis as compared with ATP render their formulation as 5′-triphosphates most likely. The structural analogy of ATP coupled with the isolation of uridine 5′-diphosphate derivatives [10], from which uridine triphosphate may be formed [11,12] supports this view. In spite of many attempts, no carbohydrate other than pentose and no ninhydrin-reacting material has so far been detected in our triphosphate preparations.

An unequivocal proof of the constitution of guanosine triphosphate and uridine triphosphate must await the results of further investigations at present in progress. Fuller details together with a consideration of possible biochemical implications will be given in a separate communication.

This work has been supported by a grant from the *Swedish Natural Science Research Council.*

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Molecular Structure of Perhydroanthracene, M. P. 90° C

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If the "*cis*"connection between the six-membered rings in the perhydroanthracene is of the same kind as that observed for *cis* decalin [1] the "*trans-trans*", "*trans-cis*" and "*cis-cis*" forms of perhydroanthracene would be represented by the forms I, II and III of Fig. 1. Judging from the physical constants of these substances J. W. Cook *et al.*[2] have suggested that the substance melting at 90° C is the "*trans-trans*" form (I).

Crystals of this substance are usually twinned, but in two cases we succeeded in growing single crystals suited for X-ray measurements. The crystals are triclinic:

$a = 5.46$; $b = 5.60$; $c = 10.20$ (Å)
$\alpha = 93.9°$; $\beta = 94.6°$; $\gamma = 102.0°$

The space group is $P\bar{1}$ and the unit cell contains *one* molecule which must therefore have a centre of symmetry. Suggesting that the strong 008 reflexion corresponds to the 1.26 Å spacing of the "*trans-trans*" form and taking into account the pseudo-symmetry respective to the *a* and *b* axes it proved possible to find approximate carbon atomic coordinates which explained in a

exchange chromatography followed by fractional reprecipitations, we were able to obtain a nearly complete separation of the two components. The less soluble barium salt fraction gave the absorption spectrum of a guanylic acid derivative and a ratio of 1 : 1 : 2 for guanine : difficultly hydrolysable phosphate : acid labile phosphate. Only a trace of the uridylic acid derivative could be detected by paper chromatography before and after hydrolysis. The most soluble barium salt fraction gave the absorption curve of the uridylic acid derivative and a ratio of 1 : 1 : 2 for uracil : difficultly hydrolysable phosphate : acid labile phosphate. Paper chromatography revealed the presence of traces of the guanylic acid derivative. Pentose estimations using the orcinol reagent before and after bromination [9] for the determination of purine- and pyrimidine-bound pentose, gave confirmatory results. Experiments on the periodate oxidation and copper complex formation of guanosine and uridine triphosphates in comparative tests with ATP, and adenosine 5′- and 3′-phosphates showed that the two new triphosphates behaved as if the phosphate groups were in the 5′-position. Although location of the acid labile phosphate groups in the purine or pyrimidine part of the molecule is not excluded, we consider that their rates of acid hydrolysis as compared with ATP render their formulation as 5′-triphosphates most likely. The structural analogy of ATP coupled with the isolation of uridine 5′-diphosphate derivatives [10], from which uridine triphosphate may be formed [11,12] supports this view. In spite of many attempts, no carbohydrate other than pentose and no ninhydrin-reacting material has so far been detected in our triphosphate preparations.

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$\alpha = 93.9°$; $\beta = 94.6°$; $\gamma = 102.0°$

The space group is $P\bar{1}$ and the unit cell contains *one* molecule which must therefore have a centre of symmetry. Suggesting that the strong 008 reflexion corresponds to the 1.26 Å spacing of the "*trans-trans*" form and taking into account the pseudo-symmetry respective to the *a* and *b* axes it proved possible to find approximate carbon atomic coordinates which explained in a

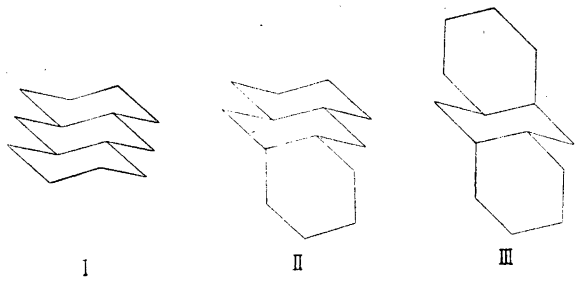

Fig. 1. "trans-trans"-, "trans-cis"- and "cis cis"-Perhydroanthracene.

qualitativ way the intensities of observed reflexions. On the basis of these coordinates Fourier maps along the *b* and *a* axes were worked out (Compare Fig. 2). It follows from these projections that the substance indeed is the "*trans-trans*" isomeride.

The X-ray analysis was carried out using CuK-radiation only. This in combination with the fact that good single crystals are difficult to obtain, makes the experimental material rather limited and we think therefore that a reinvestigation using X-rays of shorter wavelength will be necessary in order to derive exact values of atomic distances and valency angles.

The analysis of the lower melting isomerides is in progress. Unfortunately these crystals are also triclinic and mostly twinned. In both cases there are *two* molecules in the unit cell.

We wish to express our gratitude towards professor J. W. Cook for placing the substances mentioned at our disposal.

1. Bastiansen, O., and Hassel, O. *Tidsskr. Kjemi, Bergvesen, Met.* **6** (1946) 70.
2. Cook, J. W., McGinnis, N. A., and Mitchell, S. *J. Chem. Soc.* **1944** 286.

Received October 5, 1953.

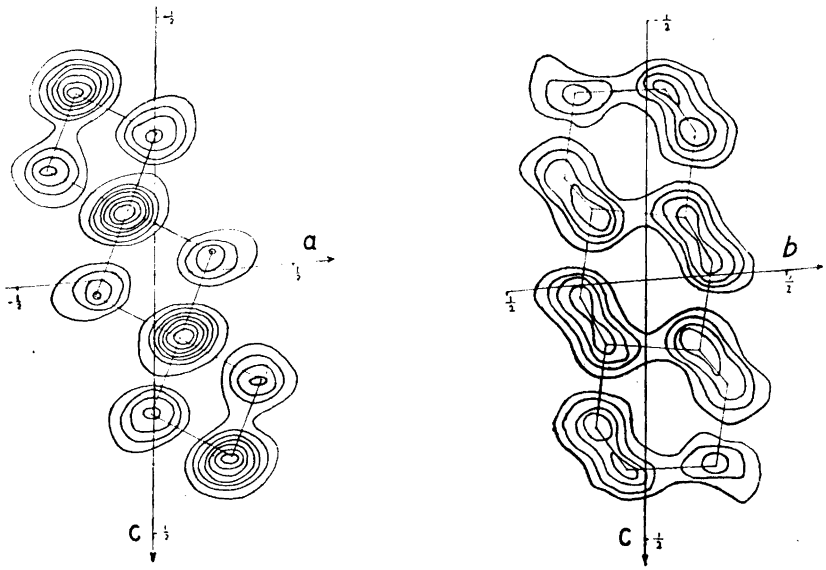

Fig. 2. Electron density maps projected: a) along the b-axis, b) along the a-axis.

Free Sarcosine in Reindeer-Moss (*Cladonia silvatica*)

P. LINKO, M. ALFTHAN, J. K. MIETTINEN, and ARTTURI I. VIRTANEN

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During investigation of free amino acids in plants we have found sarcosine in reindeer-moss (*Cladonia silvatica*). As far as we know this N-methyl-glycine has not earlier been observed in a free state in plants. Haworth *et al.*[1] have, however, found that sarcosine is formed by acid hydrolysis of protein separated from the groundnut. If the protein preparation investigated did not contain other components this would be the first time sarcosine has been found as a constituent of a protein molecule. Plattner and Nager [2] have found that the antibiotic enniatines contain N-methyl-L-isoleucine, N-methyl-L-valine, and N-methyl-L-leucine. Dalgliesh *et al.*[3] have found sarcosine and N-methyl-L-valine in their "Antibiotic X-45". These N-methyl-amino acids are also components of the actinomycin C of Brockmann *et al.*[4] Participation of N-methyl-amino acids in the formation of some antibiotics is thus proved.

The isolation of the free amino acids was accomplished in accordance with earlier methods used in this laboratory. An extract of ethanol, which was made by crushing 500 g of fresh moss in ethanol (alcohol concentration after extraction being about 70 %), and keeping the mixture for 6 days in a refrigerator, had a volume of 2 000 ml. This was passed through a column containing 40 g Amberlite 120. The amino acids remain in the resin, and were displaced by 300 ml of 1 *N* ammonium hydroxide. Using a fraction-collector, and collecting fractions of 10 g, amino acids were mainly found in fractions 12–16. The fractions were of a reddish brown colour.

In fractions 12–16 the following amino acids were found through two dimensional paperchromatographic analysis (butanol-acetic acid and phenol in NH_3-atmosphere): aspartic acid, glutamic acid, serine, glycine, asparagine, glutamine, threonine, alanine, proline, lysine, arginine (?), *sarcosine*, α-amino-*n*-butyric acid, γ-aminobutyric acid, tyrosine, valine, isoleucine, phenylalanine. In addition ethanol-amine was found.

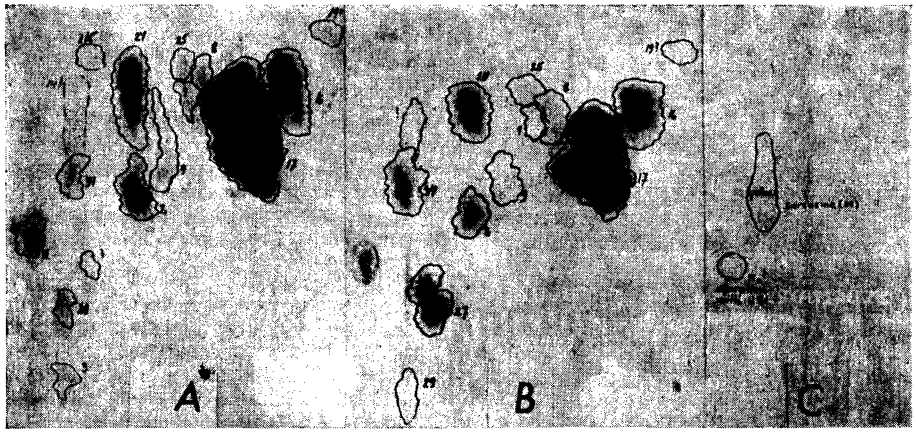

Fig. 1. Free amino acids in Cladonia silvatica. Two-dimensional chromatograms. A = extract, spots with ninhydrin, B = extract + addition of sarcosine and α-aminobutyric acid, spots with ninhydrin, C = extract, spots with p-nitrobenzoylchloride in toluene solution. 1 = gly, 2 = ala, 3 = val, 8 = ser, 9 = thr, 11 = pro, 14 = arg, 15 = lys, 16 = asp, 17 = glu, 24 = glu NH_2, 25 = asp NH_2, 27 = α-amino-n-butyric acid, 29 = γ-aminobutyric acid, 34 = sarcosine.

The identification of sarcosine is founded on the following observations:

1. The colour given by ninhydrin is typical for sarcosine.
2. The spot of added sarcosine coincided with the spot given by the plant extract.
3. With *p*-nitrobenzoylchloride the spot gave a yellow colour [2].
4. When *α*-amino acids were deaminated with nitrous fumes (from $NaNO_2$ and dilute HCl) only spots of sarcosine (characteristic colour) and proline developed when spraying the paperchromatogram with ninhydrin [3].

When the plant residues after ethanol extraction were hydrolyzed with acid, sarcosine was not to be found by the paperchromatographic method. Accordingly, the proteins did not contain sarcosine.

1. Haworth, R. D., Mac Gilliwray, Peacock, D. H. *Nature* **167** (1951) 1068.
2. Plattner, Pl. A., and Nager, U. *Helv. Chim. Acta* **31** (1948) 2192, 2203.
3. Dalgliesh, C. E., Johnson, A. W., Todd, A. R., and Vining, L. C. *J. Chem. Soc.* **1950** 2946.
4. Brockmann, H., and Grubhofer, N. *Naturwissenschaften* **37** (1950) 494.

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Carbethoxylation of 2-Thenyl Cyanide

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In an earlier paper the present author has described the carbethoxylation of 2-thenyl cyanide by a general process [1]. This paper seems to have been overlooked by Leonard and Simet, who about a year ago described a modified procedure [2]. During the last three years a very similar method has been used at this laboratory for the preparation of ethyl 2-thienylcyanoacetate. The differences appear to be of some interest from the practical point of view, however, and are therefore reported here.

Brändström has described a method for the carbethoxylation of ketones, esters and nitriles, using sodium ethylate (prepared from sodium and ethyl carbonate) as condensing agent, and ethyl carbonate as solvent [3]. A slight modification of this method (alcohol being removed *in vacuo*) gives a good yield of ethyl 2-thienylcyanoacetate.

The above-mentioned paper [1] contains a reference to a paper by Wideqvist [4], which perhaps gives the solution of Leonard and Simet's problems concerning the hydrolysis of the alkylated 2-thienylcyanoacetates.

Experimental: 200 ml of dry ethyl carbonate was placed in a 500 ml, three-necked, round-bottomed flask, fitted with a dropping funnel, a glycerol-sealed stirrer and a Widmer column, connected to a distillation condenser. The flask was heated in an oil bath until it boiled gently. The oil bath was removed, and 3.2 g (0.14 moles) of sodium was added to the hot, stirred solution at such a rate that refluxing occurred. When all the sodium was dissolved, the mixture was allowed to cool to about 80°, and the apparatus was evacuated to a pressure of about 300 mm Hg. 16.0 g (0.13 moles) of 2-thenyl cyanide was then added in a rapid stream to the mixture in the flask. The alcohol was removed, and when the temperature at the top of the column attained the expected boiling point of ethyl carbonate, the oil bath was removed and the flask allowed to cool. The contents were poured over a mixture of 20 ml of glacial acetic acid and ice. The organic layer was separated and the water layer extracted with ether. The combined organic layers were washed with water, dried over anhydrous sodium sulphate, and the ether removed. The residue was finally fractionated *in vacuo*. The yield of ethyl 2-thienylcyanoacetate, boiling at 115°—120°/1—2 mm, was 17.7 g (70 %).

1. Pettersson, K. *Acta Chem. Scand.* **4** (1950) 395.
2. Leonard, F., and Simet, L. *J. Am. Chem. Soc.* **74** (1952) 3218.
3. Brändström, A. *Acta Chem. Scand.* **4** (1950) 1315.
4. Wideqvist, S. *Svensk Kem. Tidskr.* **55** (1943) 125; *Arkiv Kemi, Mineral. Geol.* **26A** (1948) No. 16.

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Diffusion of Argon and Air through Polyvinylchloride and Rubber

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In connection with experiments in which argon was circulated in a closed apparatus, a remarkable diffusion of the gas through rubber and polyvinylchloride tubing was observed. The gas was circulated by means of a pump described by Clusius [1], using tubing of rubber or polyvinylchloride (PVC).

The permeability constant P was measured for PVC and argon or air, respectively. P is equal to unity if one cm^3 of gas at NTP per sec. is passing through one cm^2 area of the membrane (thickness 1 mm) when the difference in partial pressure is one cm of mercury. In these units the results at 18 ± 2° C are:

$$P = 0.83 \times 10^{-9}$$ for argon and PVC,

and

$$P = 0.48 \times 10^{-9}$$ for air and PVC.

The diffusion of these gases through rubber tubing was similar. If the Clusius pump was working, the permeability was still higher.

The conclusion is, that in chemical experiments using gases, rubber or PVC containers, connections and especially Clusius pumps should not be used if diffusion out of or into the system has to be avoided.

1. Clusius, K. *Chimia* **4** (1950) 275.

Received October 2, 1953.

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Liquid Structure and Solubility of Sparingly Soluble Salts in Water

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The theoretical formula for the solubility of solids in liquids assuming the solution to be ideal, is applied to aqueous solutions of sparingly soluble salts. The process of dissolution involves chemical reaction between salt and water. The equilibrium constants for a few such reaction equilibria are computed from the coordination numbers of the liquid structure. The results are compared with experimental data on silver halides, lead halides, and Ag_2O.

1. THEORY

When a system is in equilibrium, its free energy is a minimum. For instance, when two phases are in equilibrium with one another, there is no change in the free energy of the entire system, when matter is transferred from one phase to the other. We will apply this principle to the solubility of a sparingly soluble salt in water.

We will consider the process of dissolution as consisting of two steps. In the first step the solid salt is transformed from the solid into the liquid state. In the second step the liquid salt is mixed with the liquid water. We will assume that the mixture is ideal, *i. e.* there is no heat of mixing, and the entropy of mixing is equal to R times the logarithm of the mole fraction X of the salt (Raoult's law). From the fundamental principle

$$\Delta F = 0 \tag{1}$$

we thus obtain

$$\Delta F_m = -T\ \Delta S_m = -\Delta F_f \tag{2}$$

when m denotes mixing and f fusion.

With Raoult's law

$$\Delta S_m = -R \ln X,\ \Delta E_m = 0 \tag{3}$$

we have from (2)

$$X = e^{-\frac{\Delta F_f}{RT}} \tag{4}$$

Applying the principle (1) to the process of melting, we find at the melting point T_f

$$\Delta F_f = \Delta E_f - T_f \Delta S_f = 0 \tag{5}$$

Hence,

$$X = e^{-\frac{\Delta E_f}{R}\left(\frac{1}{T} - \frac{1}{T_f}\right)}. \tag{6}$$

This is the familiar formula for the solubility of a solid in a liquid on the assumption that the solution is ideal.

Few aqueous solutions are ideal. As a rule the water reacts with the salt. This reaction is accompanied by a free energy of reaction ΔF_e which must be included in (1). We will write ΔF_e in the form

$$\Delta F_e = \Delta E_e - T\ \Delta S_e \tag{7}$$

and assume that

$$\Delta S_e = \frac{\Delta E_e}{T_f} + \Delta S_{struct.} \tag{8}$$

Hence,

$$\frac{\Delta F_e}{T} = -\ \Delta S_{struct.} + \Delta E_e\ \left(\frac{1}{T} - \frac{1}{T_f}\right) \tag{9}$$

The entropy change $\Delta S_{struct.}$ derives from the structural change involved in the reaction,

$$\Delta S_{struct.} = R \ln W \tag{10}$$

when W denotes the ratio of the numbers of ways in which the two states, before and after the reaction, may be realized.

Including ΔE_e and ΔE_f in the heat of solution ΔE_s of the solid salt we have thus with (1), (6), (9) and (10)

$$X = W\ e^{-\frac{\Delta E_s}{R}\left(\frac{1}{T} - \frac{1}{T_f}\right)} \tag{11}$$

This formula is valid for a solution such that

$$\Delta S_m = -R \ln X \tag{12}$$

i. e. for a sufficiently dilute solution.

Since there are 55.55 moles of water per litre of pure water, and since the concentration of the water is not appreciably changed by the presence of a sparingly soluble salt in the solution, we may express the solubility in mole/litre instead of mole fraction by rewriting (11) in the form

$$c = 55.55\ W\ e^{-\frac{\Delta E_s}{R}\left(\frac{1}{T} - \frac{1}{T_f}\right)} \tag{13}$$

We will now consider reactions of the types

$$AB + H_2O \rightleftharpoons (AB\ H_2O) \tag{14}$$

$$AB + H_2O \rightleftharpoons AOH + HB \tag{15}$$

The equilibrium is characterized by its equilibrium constant

$$K = e^{-\frac{\Delta F_e}{RT}} \tag{16}$$

or with (9)

$$K = We^{-\frac{\Delta E_e}{R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} \tag{17}$$

We may therefore calculate K by way of calculating W from the structure of the liquid. Instances of such calculations will be given in section 3.

2. EMPIRICAL RELATIONS

In the following we will apply these formulae to a few cases of sparingly soluble salts. However, before we do so we will consider the experimental data and derive corresponding empirical formulae which we will then interpret by comparing them with the theoretical formulae and by considering the reactions involved. The data employed have been taken from Landolt-Börnstein and from the Handbook of Chemistry and Physics. The values of $-\Delta E_s$ have been taken from those tables ("observed"), from a plot of $\log c = f\left(\frac{1}{T}\right)$ "(from curve"), or computed according to the rule of heats of formation in dilute solution derived elsewhere [1] and from the observed heats of formation in the solid state ("computed").

Tables 1 and 2 give data for silver and lead halides, respectively.

Table 1. Solubility of silver halides.

Salt	AgF	AgCl	AgBr	AgI
$-\Delta E_s$ (computed)	+ 4.3	−15.9	−20.2	−26.5
$-\Delta E_s$ (from Fig. 2)	—	-2×7.79	—	—
c mole/litre at 16°	14	$9.70 \cdot 10^{-6}$	$5.60 \cdot 10^{-7}$	$6.48 \cdot 19^{-9}$
$^{10}\log c$	0.146 + 1	0.987−6	0.748−7	0.812−9
T_f (observed)	708	728	703	830
T_f (from Fig. 1) *	1 410	1 410	1 410	1 410

* T_f is regarded as an empirical constant the significance of which will be discussed in section 4.

Table 2. Solubility of lead halides.

Salt	$PbCl_2$	$PbBr_2$	PbI_2
$-\Delta E_s$ (computed)	−6.5	−9.1	−15.2
$-\Delta E_s$ (observed)	−6.55	−10.04	—
$-\Delta E_s$ (from Fig. 3)	-2×3.25	-2×4.58	-3×4.71
T_f (observed)	774	646	675

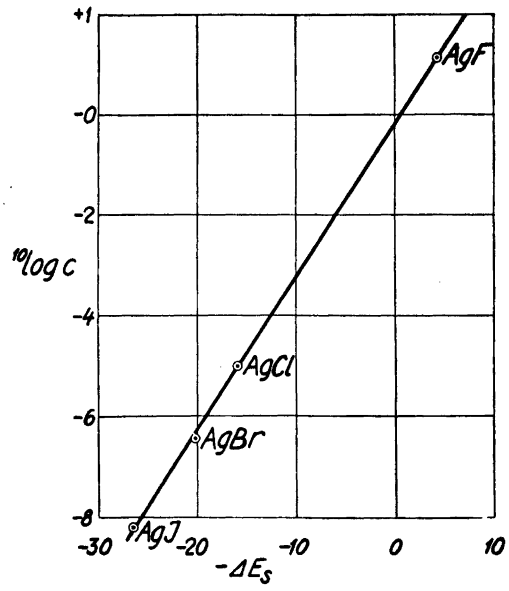

Fig. 1. $^{10}\log c = f(-\Delta E_s)$ *for silver halides at 16°.*

Fig. 1 shows a plot of $^{10}\log c = f(-\Delta E_s)$ on the observed data of c and the computed values of $-\Delta E_s$. Fig. 2 shows a plot of $^{10}\log c = f(\frac{1}{T})$ for AgCl. The two curves satisfy the formula

$$c = 0.67\ e^{-\frac{\Delta E_s}{2R}(\frac{1}{T}-\frac{1}{T_f})} \tag{18}$$

where $T_f = 1\,410°$ K.

Fig. 3 shows plots of $^{10}\log c = f(\frac{1}{T})$ for $PbCl_2$, $PbBr_2$ and PbI_2. Inserting the values of T_f given in Table 2 we obtain the following formulae

$$PbCl_2: \quad c = 1.15\ e^{-\frac{3.25}{R}(\frac{1}{T}-\frac{1}{T_f})} \tag{19}$$

$$PbBr_2: \quad c = 1.70\ e^{-\frac{4.58}{R}(\frac{1}{T}-\frac{1}{T_f})} \tag{20}$$

$$PbI_2: \quad c = 0.158\ e^{-\frac{4.71}{R}(\frac{1}{T}-\frac{1}{T_f})} \tag{21}$$

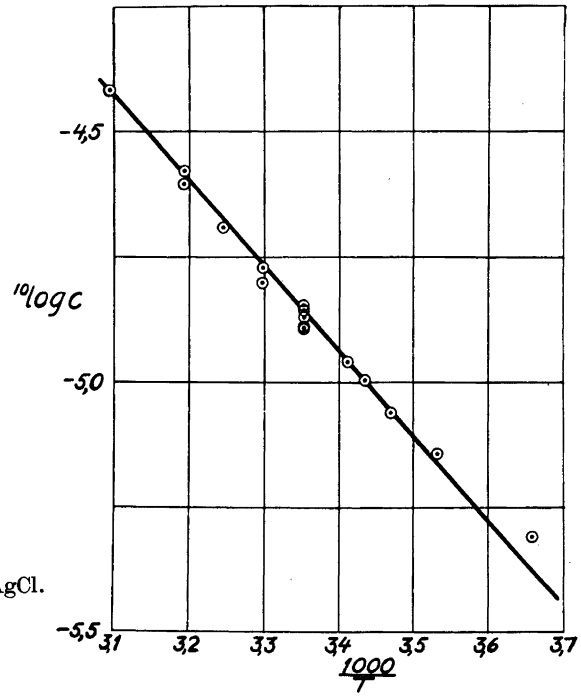

Fig. 2. $^{10}log\ c = f\ (\frac{1\,000}{T})$ *for* AgCl.

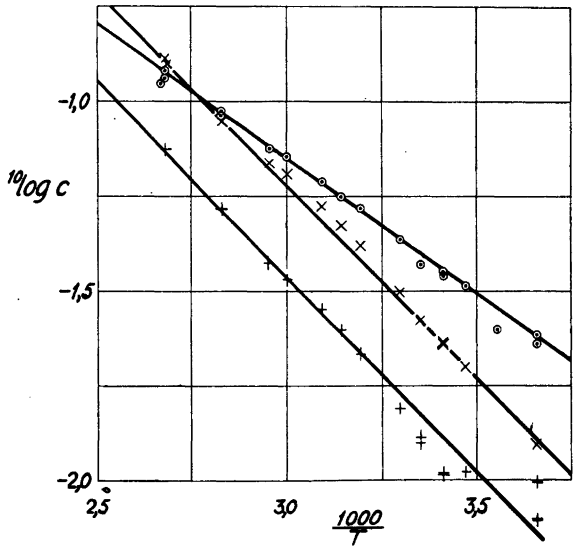

Fig. 3. $^{10}log\ c = f\ (\frac{1\,000}{T})$ *for* $PbCl_2$ (⊙) *and* $PbBr_2$ (×).
$^{10}log\ c + 1.9 = f\ (\frac{1\,000}{T})$ *for* PbI_2 (+).

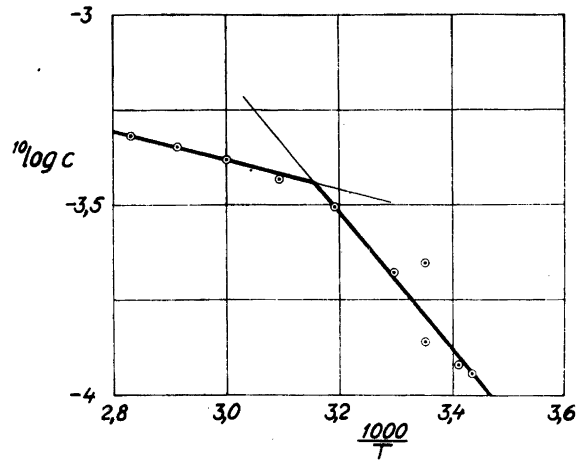

Fig. 4. $^{10}log\ c = f(\frac{1\,000}{T})$ *for* Ag_2O.

Fig. 4 shows $^{10}\log c = f(\frac{1}{T})$ for Ag_2O. The slope of this curve gives $\Delta E_s = -8.2$ for $T < 317°$ K. Inserting $T_f = 1\,410°$ K * we obtain from the curve

$$Ag_2O: \quad c = 8.7\, e^{-\frac{8.2}{R}(\frac{1}{T} - \frac{1}{1\,410})} \tag{22}$$

3. COMPARISON OF THEORETICAL AND EMPIRICAL FORMULAE

A. Solubility of Ag_2O

When a hypothetical liquid Ag_2O is mixed with liquid water, it is built into the structure of the water. Each Ag_2O molecule is surrounded by H_2O molecules and bound to those with O—H—O and Ag—O bonds. Fig. 5 illustrates this structure schematically.

The breaking of the O—H—O bond as indicated in Fig. 5 brings about the reaction

$$Ag_2O + H_2O \rightarrow (AgOH)_2.$$

The re-formation of the same bond leads to the reaction

$$(AgOH)_2 \rightarrow Ag_2O + H_2O.$$

We will therefore consider the equilibrium

$$Ag_2O + H_2O \rightleftharpoons (AgOH)_2. \tag{23}$$

* This choice of T_f will be discussed in section 4.

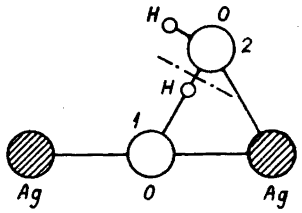

Fig. 5.

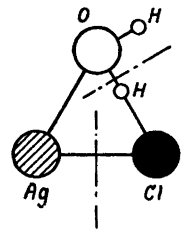

Fig. 6.

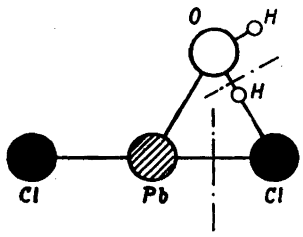

Fig. 7.

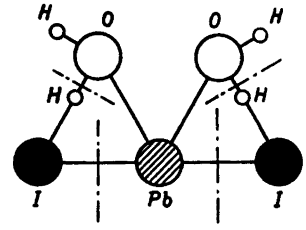

Fig. 8.

When the bond is broken, the two OH groups can form new bonds with surrounding H_2O molecules. In pure water each H_2O molecule or OH group is surrounded by 4 neighbors. We will assume this to be the case with the OH groups of $(AgOH)_2$ as well. The number of ways in which new bonds can be formed is thus, 2 for oxygen atom No. 1 and 3 for oxygen atom No. 2. Hence,

$$\frac{1}{W} = 2 \times 3 = 6, \quad \Delta S_{struct.} = -R \ln 6 \tag{24}$$

and

$$c_{(AgOH)_2} = \frac{55.55}{6} e^{-\frac{\Delta E_s}{R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} e^{-\frac{\Delta E_s}{R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} = 9.3\, e \tag{25}$$

B. Solubility of silver halides

The equilibrium is

$$AgX + H_2O \rightleftharpoons AgOH + HX \tag{26}$$

X denoting, F, Cl, Br, or I. The structure is shown in Fig. 6.

This case is considerably more complicated than the previous case. When the two bonds are broken, the AgOH and HX molecules formed may separate. When those separated molecules join so that the Ag and X atoms occupy adjacent lattice sites, they will recombine to form AgX. We have thus to consider two processes: the formation of AgX from AgOH and HX, and the number of ways in which the broken bonds may be reformed.

From the heat of vaporization of HCl in aqueous solution [2] we know that each HCl molecule is surrounded by 10 H_2O molecules. We will assume that the AgOH molecule occupies two sites and that one AgOH molecule may replace one of those ten H_2O molecules. Around the HCl molecule we may thus consider 11 sites, two of which may be occupied by an AgOH molecule.

The number of pairs of Ag and X atoms per litre of the solution is then[3]

$$\frac{11}{2} \cdot \frac{c_{AgOH} \cdot c_{HX}}{55.55}$$

But this is the number of AgX molecules per litre. Hence,

$$c_{AgX} = \frac{11}{2} \cdot \frac{c_{AgOH} \cdot c_{HX}}{55.55}$$

or

$$c_{AgOH} \cdot c_{HX} = \frac{2}{11} \cdot 55.55 \; c_{AgX} \tag{27}$$

We next consider the number of ways in which new bonds can be formed instead of the two broken bonds. There are 10 H_2O molecules with which the Cl atom may form bonds. The number of ways in which the two broken bonds may be reformed is thus $\binom{10}{2} = 45$. The O atom may, again, form 3 new bonds instead of the one broken. For the Ag atom we will assume that it may be surrounded by 10 H_2O molecules, just as is the Cl atom. This assumption is based upon the similarity of the structures of HCl and NaOH in aqueous solution [4]. Hence, the number of ways in which the broken bond may be reformed is 9. The total number of ways in which the broken bonds can be reformed is thus $45 \times 3 \times 9 = 1\,215$ and

$$\frac{1}{W} = 1\,215 \tag{28}$$

Hence, from (27) and (28),

$$c_{AgOH} = c_{HX} = \sqrt{2 \, \frac{55.55}{11 \times 1\,215} \cdot c_{AgX}} = 0.68 \, e^{-\frac{\Delta E_s}{2R}\left(\frac{1}{T} - \frac{1}{T_f}\right)} \tag{29}$$

in agreement with (18).

C. Solubility of $PbCl_2$

We consider the equilibrium

$$PbCl_2 + H_2O \rightarrow PbClOH + HCl \tag{30}$$

according to Fig. 7.

This case differs from that of AgCl simply by the two Cl atoms and, accordingly, by a factor of 2 in W. Hence,

$$c = 0.68 \times \sqrt{2}\, e^{-\frac{\Delta E_s}{2R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} = 0.96\, e^{-\frac{\Delta E_s}{2R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} \tag{31}$$

The agreement with the empirical formula (19) is not quite so good as in the previous cases. For $PbBr_2$ we should have the same result (31) which differs from (20) by a factor of 1.8, however. The discrepancy may probably be attributed to complex formations or to an inadequate choice of T_f.

D. Solubility of PbI_2

We consider the equilibrium

$$PbI_2 + 2\ H_2O \rightleftharpoons Pb(OH)_2 + 2\ HI \tag{32}$$

according to Fig. 8.

Comparing this case to that of AgCl we find

$$W_{PbI_2} = W_{AgCl}^2 \tag{33}$$

and, thus,

$$c_{Pb(OH)_2} = \sqrt[3]{(2\frac{55.55}{11 \times 215})^2\, c_{PbI_2}} = 0.157\, c^{-\frac{\Delta E_s}{3R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} \tag{34}$$

in agreement with (21).

4. THE SIGNIFICANCE OF T_f

In deriving the formula (6) for an ideal solution we have disregarded chemical reactions, since otherwise the solution could not have been considered as ideal. The significance of T_f is then the melting point of the solid. However, when reactions occur as illustrated in section 3, the significance of T_f is less obvious, since we have several salts in the solution, *e. g.* AgCl, AgOH, and HCl. The significance of T_f may therefore warrant some comments.

In order to bring all the salts into the liquid state we have to raise the temperature to the highest melting point. Thus, for such a mixture, the solute is the salt with the highest melting point, and the solvent is an aqueous solution of the salts of lower melting points. The significance of T_f is then the melting point of this solute, *i. e.* T_f is the highest melting point among those of the individual salts.

It follows from Fig. 1 that T_f is the same for all the silver halides and about twice as high as the melting points of the halides. We may therefore assume that $T_f = 1\,410°$ K is the melting point of the hydroxide, AgOH. There is no reason why the double hydroxide $(AgOH)_2$ should have a different melting

point; in fact, AgOH and $(AgOH)_2$ should be identical in the solid state. Hence, $T_f = 1\,410°$ K in the case of Ag_2O as well.

For the lead salts PbXOH and $Pb(OH)_2$ the melting points are not known, nor is T_f the same for all the lead halides. We have therefore arbitrarily taken for T_f the melting points of the halides. In view of the spread of the data as shown by the plots in Fig. 3, it is difficult to say whether the discrepancy between theoretical and empirical formulae is due to an erroneous choice of T_f. It should be pointed out, though, that in order to bring the empirical data in agreement with the theoretical formulae we have to chose lower values of T_f.

5. CONCLUSIONS

The agreement between theoretical and empirical formulae shows that the formula (6), valid for an ideal solution, is applicable to aqueous solutions of sparingly soluble salts, provided due corrections are made for the reactions involved in the process of dissolution. It further shows that the statistical computation of equilibrium constants is possible, provided the coordination numbers of the liquid structure are known.

There is no reason for applying the simple formula (6) to concentrated solutions, *e. g.* the solubility of alkali halides, since those do not follow Raoult's law. Hence, for concentrated solutions, X should be replaced by the actual value of $e^{-\frac{\Delta F_m}{RT}}$. We will take up this problem in a following publication.

Acknowledgment. The results reported in this paper were obtained in the Laboratory of Avesta Jernverks AB, Sweden. I am indebted to the Management of the Company for permission to publish this report.

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The Structure of Some Alleged 3-Hydroxyflavanones

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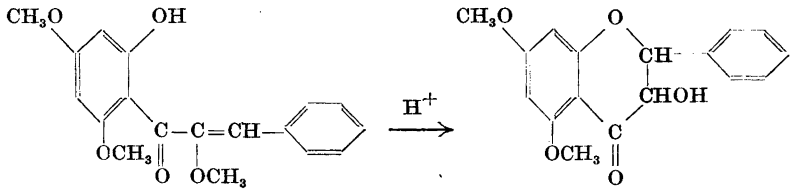
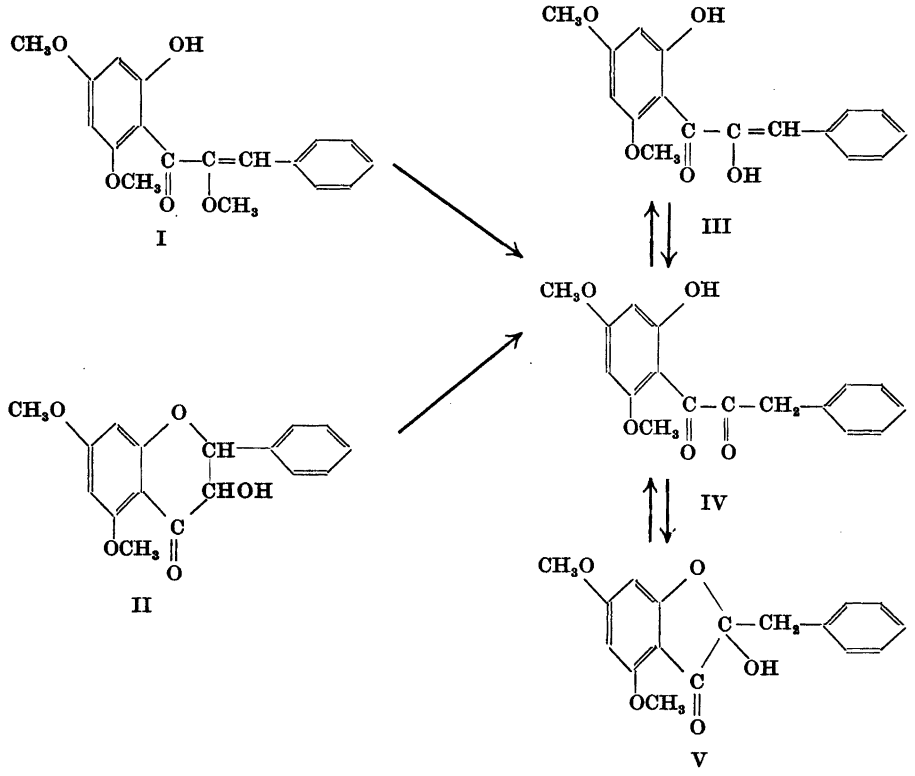
Some years ago Lindstedt [1] showed that the so-called apoalpinonemonomethylether of Kimura could not have the structure assigned to it [2]. Kimura obtained the compound by condensing 2-hydroxy-ω,4,6-trimethoxyacetophenone with benzaldehyde and treating the resulting chalcone (I) with acid; he assumed that hydrolysis of the enolether was followed by ringclosure to a flavanone, giving 3-hydroxy-5,7-dimethoxyflavanone (II)



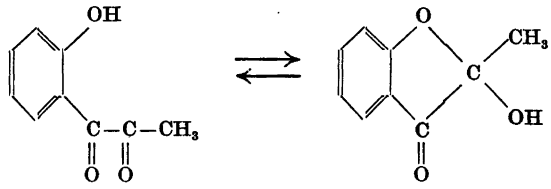
I II

Lindstedt [1], found that "apoalpinonemonomethylether" could also be obtained by treatment of pinobanksindimethylether (3-hydroxy-5,7-dimethoxyflavanone) with alkali, and subsequent acidification, but that it was soluble in alkali and did not give the colour reaction with magnesium and hydrochloric acid, typical of flavanones. It was thus clear that the compound was not a flavanone, but Lindstedt made no attempt to elucidate its true structure.

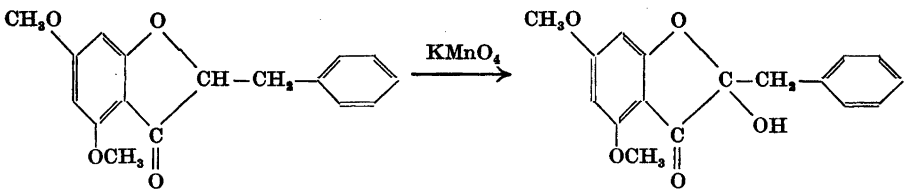
Bearing in mind that the compound is formed both by acid hydrolysis of 2′-hydroxy-ω,4′,6′-trimethoxychalcone (I), and by treatment of pinobanksindimethylether (II) with alkali, it is apparent that the most probable structure is represented by one of the three tautomeric formulae (III), (IV) or (V), the reactions being:



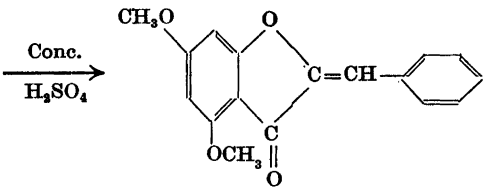
Of these formula (V) is the most probable, for the following reasons: (1) v. Auwers and Müller[3,4] have shown that attempts to prepare compounds of the type (VI) invariable lead to products whose properties were better represented by the tautomeric formula (VII).



(V) is a derivative of (VII). (2) "Apoalpinonemonomethylether" upon treatment with conc. sulphuric acid loses one molecule of water, giving 2-benzal-4,6-dimethoxycoumaranone (IX). (3) It can also be obtained by oxidation of 2-benzyl-4,6-dimethoxycoumaranone (VIII) with potassium permanganate.



VIII V

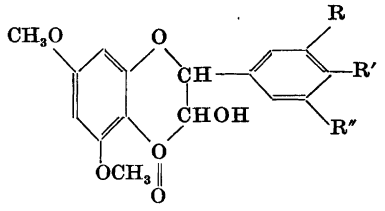


IX

This last mentioned reaction is closely analogous to the oxidation of a degradation product of griseofulvin [5].

Although there is always some uncertainty in deciding between tautomeric structures on the basis of chemical reactions, it is felt that structure (V) best explains the behaviour of the "apoalpinonemonomethylether", which is thus 2-benzyl-2-hydroxy-4,6-dimethoxycoumaranone.

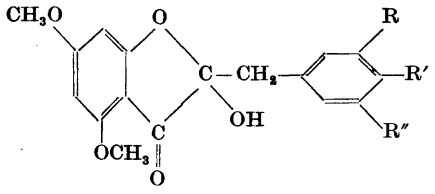
Kimura [6] has used the same method for the synthesis of some other alleged 3-hydroxyflavanones namely: 3-hydroxy-4′,5,7-trimethoxy-(X), 3-hydroxy-3′,4′,5,7-tetramethoxy-(XI), 3-hydroxy-3′,4′,5,5′,7-pentamethoxy-(XII) and 3-hydroxy-3′,4′-methylenedioxy-5,7-dimethoxyflavanone (XIII)



X; R = R″ = H; R′ = OCH_3
XI; R = H; R′ = R″ = OCH_3
XII; R = R′ = R″ = OCH_3
XIII; R = H; R′ + R″ = OCH_2O

Although it has not been proved experimentally, analogy makes it very reasonable to regard these compounds also as substituted 2-benzyl-2-hydroxy-

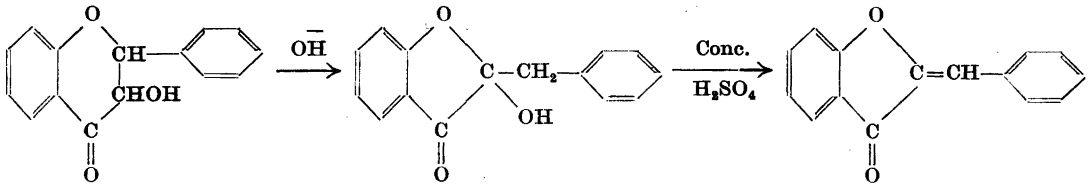
coumaranones, namely: 2-(4-methoxybenzyl)-(XIV), 2-(3,4-dimethoxybenzyl)-(XV) 2-(3,4,5,-trimethoxybenzyl)-(XVI) and 2-(3,4-methylenedioxybenzyl)-2-hydroxy-4,6-dimethoxycoumaranone (XVII) respectively.



XIV; R = R″ = H; R′ = OCH_3
XV; R = H; R′ = R″ = OCH_3
XVI; R = R′ = R″ = OCH_3
XVII; R = H; R′ + R″ = OCH_2O

Of these compounds (XVI) has been synthesised, apparently independently, by Kotake and Kubota [7], using the same method. But these authors obtained it also by treatment of ampelopsinpentamethylether (XII) with alkali, a reaction quite analogous to the formation of (V) from pinobanksindimethylether (II). Kotake and Kubota [7] regarded their product as a stereoisomer of ampelopsinpentamethylether and called it epiampelopsinpentamethylether. This view was supported by the ultraviolet absorption spectrum of "epiampelopsinpentamethylether", which was found to be almost identical with the spectrum of ampelopsinpentamethylether. However the similarity of the spectra is not at all incompatible with the structure (XVI) for "epiampelopsinpentamethylether", which has the same chromophore as the corresponding 3-hydroxyflavanone, and indeed can be taken as a further support for a structure of the type (V) in preference to (III) or (IV). A more detailed study of the ultraviolet absorption of the 2-benzyl-2-hydroxycoumaranones and the corresponding flavanones and chalcones is planned.

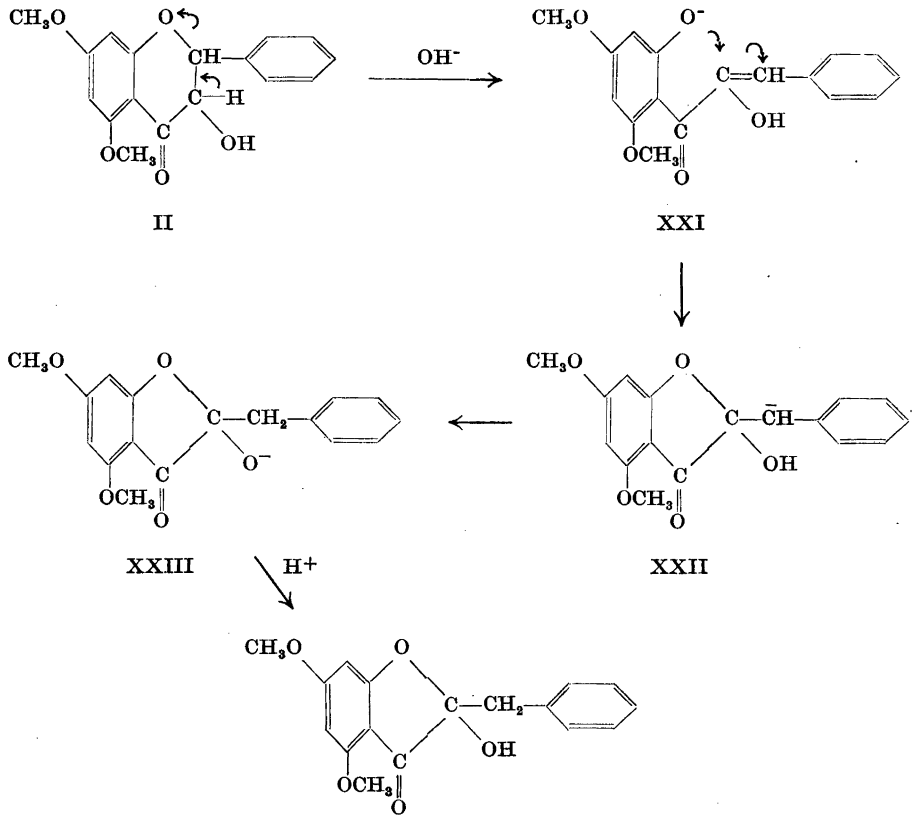
Geissman and Fukushima [8] have described a method of converting suitably substituted chalcones into benzalcoumaranones by means of hydrogen peroxide and alkali. It was demonstrated that there must be a methoxyl group in the 6′-position of the chalcone in order to give a benzalcoumaranone, which will thus have a methoxyl group in its 4-position. In order to find whether the formation of the coumaranone ring from 3-hydroxyflavanones is similarily dependent on the presence of a methoxyl group in the same position, — that is the 5-position in the flavanone —, 3-hydroxyflavanone (XVIII) was treated with alkali unde the same conditions as in the earlier cases. There was obtained, together with considerable amounts of 3-hydroxyflavone, an alkali soluble compound, isomeric with the starting material, which upon treatment with conc. sulphuric acid was converted into 2-benzalcoumaranone (XX). The compound must therefore be regarded as 2-benzyl-2-hydroxycoumaranone (XIX), and is apparently identical with the unidentified solid of m.p. 105—106°, obtained by Marathe, Chandorkar and Limaye [9] under similar conditions.



XVIII XIX XX

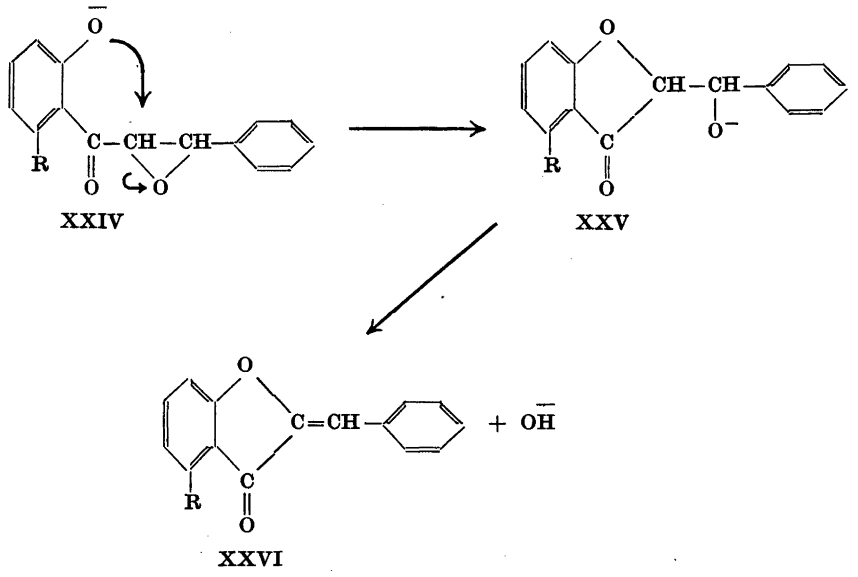
Thus the formation of the coumaranone ring in this case takes place even when there is no methoxyl group whatsoever in the molecule.

The following mechanism is proposed for the formation of a 2-benzyl-2-hydroxycoumaranone from the 3-hydroxyflavanone by treatment with alkali:



II XXI XXII XXIII V

The intermediates (XXI) and (XXII) are, however, those which Geissman and Fukushima [8] have proposed as intermediates in the formation of 2-benzalcoumaranones from chalcones. It is of course very improbable that the intermediates should be the same, as both reactions occur under almost identical conditions. It is therefore proposed that the formation of 2-benzalcoumaranone takes place in the following way:



In agreement with Geissman and Fukushima [8] the first step is considered to be the formation of the chalcone oxide ion (XXIV), the oxide ring of which is then opened by attack of the electron pair of the negatively charged oxygen atom — but opened in the opposite direction to that proposed by Geissman and Fukushima —, leading to (XXV). This is of course the intermediate which must also be assumed to be formed in the preparation of benzalcoumaranone from coumaranone and benzaldehyde under alkaline conditions. The discussion of Geissman and Fukushima regarding the influence of the group R on the course of the reaction holds equally well for this mechanism.

EXPERIMENTAL

2-Benzyl-4,6-dimethoxycoumaranone (VIII). 2-Benzal-4,6-dimethoxycoumaranone [8] (0.63 g) was hydrogenated in alcoholic solution over $Pt(O_2)$-catalyst. The uptake of hydrogen amounted to 56 ml (Calc. for 1 mole 53 ml). The catalyst was removed and the alcohol was evaporated *in vacuo* until crystallisation set in. The crystals (0.43 g) were collected. Addition of water to the mother liquor precipitated a further amount (0.15 g) of slightly less pure compound. The 2-benzyl-4,6-dimethoxycoumaranone after recrystallisation from dilute alcohol had m.p. 109.5 – 110.5° (Found: C 71.6; H 5.75. $C_{17}H_{16}O_4$ requires C 71.8; H 5.7 %).

2-Benzyl-2-hydroxy-4,6-dimethoxycoumaranone (V). 2-Benzyl-4,6-dimethoxycoumaranone (0.1 g) was dissolved in acetone (50 ml); a few drops of 2 *N* sodium carbonate and finely powdered potassium permanganate (0.3 g) were added. After standing for four days at room temperature the mixture was poured into water, acidified, and the manganese dioxide dissolved by introducing sulphur dioxide. The aqueous solution was thoroughly extracted with ether and the ether solution first washed with sodium hydrogen carbonate and then extracted with 2 *N* sodium hydroxide. The sodium hydroxide extract acquired a yellow colour. Acidification caused the 2-benzyl-2-hydroxy-4,6-dimethoxycoumaranone (0.03 g) to be precipitated in an almost pure condition. After crystallisation from dilute alcohol it had m.p. 170—170.5°. No depression was observed when mixed with "apoalpinonemonomethylether" prepared by Lindstedt [1] from pinobanksindimethylether (The author wishes to thank Prof. H. Erdtman for supplying this specimen). (Found: C 67.4; H 5.4. $C_{17}H_{16}O_5$ requires C 68.0; H 5.4 %.)

2-Benzal-4,6-dimethoxycoumaranone (IX) 2-Benzyl-2-hydroxy-4,6-dimethoxycoumaranone (0.02 g) was dissolved in conc. sulphuric acid (2 ml) giving an orange coloured solution. The solution was immediately poured into water (50 ml), and the slightly yellow precipitate was collected and crystallised from dilute alcohol. The m.p., 151.5—152.5° was undepressed, when the compound was mixed with an authentic specimen of 2-benzal-4,6-dimethoxycoumaranone prepared by the method of Geissman and Fukushima [8].

3-Hydroxyflavanone (XVIII) was prepared by the method of Murakami and Irie [10]. Crystallisation from alcohol gave a m.p. of 185—187° (Murakami and Irie [10] report m.p. 174—177°, Geissman and Fukushima [8] 177—180°, whereas Oyamada [11] for a sample prepared in another way reports 183—184°).

2-Benzyl-2-hydroxycoumaranone (XIX). 3-Hydroxyflavanone (1.0 g) was boiled for five minutes with a 5 % alcoholic potassium hydroxide solution (100 ml), in a stream of nitrogen. The solution was then rapidly cooled and poured into 2 *N* hydrochloric acid (200 ml). The precipitate was collected (0.3 g) and crystallised from alcohol to m.p. 165—167°. (A m.p. of 169—170° is reported for 3-hydroxyflavone [12]). The alcohol was removed from the mother liquor which was then extracted with ether. The ether solution was washed with sodium hydrogen carbonate solution and then extracted with 2 *N* sodium hydroxide. The yellow alkaline solution was acidified whereupon an oil, that partly solidified, was precipitated. The solid material was collected (0.1 g) and crystallised from ether — light petroleum to m.p. 102—103° (Found: C 74.85; H 5.05. $C_{15}H_{12}O_3$ requires C 75.0; H 5.0 %).

2-Benzalcoumaranone. 2-Benzyl-2-hydroxycoumaranone was converted into 2-benzalcoumaranone by the method described above for 2-benzyl-2-hydroxy-4,6-dimethoxycoumaranone. The m.p., 110—111°, after crystallisation from dilute alcohol, was undepressed when the compound was mixed with an authentic specimen of benzalcoumaranone.

SUMMARY

It has been shown that the alleged 3-hydroxyflavanones prepared by the method of Kimura are in fact substituted 2-benzyl-2-hydroxycoumaranones. These can also be obtained by rearrangement of the true 3-hydroxyflavanones. The mechanism of this rearrangement and that of the formation of 2-benzalcoumaranones from chalcones, discovered by Geissman and Fukushima, are discussed.

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A Reaction between Certain Quaternary Ammonium Compounds and Sulphinic Acids

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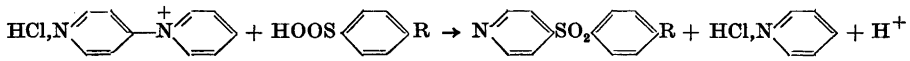
It is known that sulphinic acids can be added to α,β-unsaturated acids, ketones, and aldehydes with formation of sulphones [1]. Thus it was to be expected that Mannich bases easily splitting off the amine group with formation of α,β-unsaturated ketones [2] would react with arylsulphinic acids with formation of β-arylsulphonyl ketones, and this was confirmed. Probably the reaction with the quaternary Mannich bases does not proceed *via* a decomposition to unsaturated ketone, but the quaternary base is likely to act alkylating. The facts that Mannich bases do not decompose under the conditions of synthesis employed, and that pyridylpyridinium chloride reacts correspondingly favour the assumption of this reaction type, an S_2 reaction.



Wieland *et al.*[3] have synthesized indolylmethanesulphonic acid from gramine and sodium hydrogen sulphite, and gramine is expected to react with sulphinic acids with formation of sulphones.

The arylsulphoneketones formed are easily crystallizing substances soluble in ethanol and chloroform but insoluble in water. They form oximes, dinitrophenylhydrazones etc. and can be isolated by Girard reagents.

Aryl-γ-pyridyl sulphones are easily crystallizing substances, slightly basic.

EXPERIMENTAL

All melting points are uncorrected micro melting points. Microanalyses are made by Mr. P. Hansen.

Quaternary Mannich bases. The tertiary Mannich bases were prepared according to the method in Organic Syntheses [4]. 150 ml of ether and 45 ml of 10 % NaOH were added to 0.1 mole of the crude product of the hydrochloride. The ether phase was dried with K_2CO_3, and 40 g of 30 % methyl bromide in methanol were added. The mixture was left in the ice box for 24 hours. The white precipitate was collected, washed with ether and recrystallized from a mixture of 4 parts of methanol and 1 part of acetone (Table 1).

The sulphinic acids were prepared according to the method in Organic Syntheses [5].

2-p-Acetamino-phenylsulphonyl-1-benzoylethane; Compound 1. 6 g of trimethyl-ω-benzoylethylammonium bromide and 4 g of *p*-acetaminobenzenesulphinic acid were dissolved in 40 ml of ethanol and refluxed for 4 hours. The solid was collected and recrystallized from ethanol. Yield 4.5 g.

The other sulphonyl ketones were prepared according to the above method (Table 2).

2-p-Aminophenylsulphonyl-1-benzoylethane; Compound 2. Compound 1 (2g) were refluxed with a mixture of 15 ml of ethanol and 15 ml of 75 % H_2SO_4 for 1 hour. The solution was poured into 100 ml of water. The precipitate was collected and recrystallized from 75 % ethanol with charcoal. Yield 0.9 g.

Oxime of compound 3. Compound 3, (1.2g), 0.5 g of hydroxylamine hydrochloride and 8 ml of pyridine were refluxed for 1 hour, and after cooling poured into 20 ml of water. The mixture was left in the ice box for 12 hours. The solid was collected and recrystallized from dilute ethanol. Yield 0.7 g of white crystals, m.p. 130°.

$C_{15}H_{15}O_3NS$ (289.4)	Calc.	N	4.8
	Found	»	4.8

Oxime of compound 1. Was prepared analogously to oxime of compound 3, m.p. 162°.

$C_{17}H_{18}O_4N_2S$ (346.4)	Calc.	N	8.1
	Found	»	8.0

Dinitrophenylhydrazone of compound 5. Yellow needles, m.p. 206°.

$C_{22}H_{20}O_7N_4S$ (484.5)	Calc.	N	11.6
	Found	»	11.3

Isolation by Girard P reagent. Impure compound 10 (0.5g) and 2 g of Girard P were dissolved in 20 ml of ethanol + 1 ml of acetic acid by heating on a waterbath for 2 hours. After cooling in ice the solution was poured into 30 ml of ice-cold water and immediately extracted with 50 ml of chloroform. On addition of 1 ml of concentrated HCl to the aqueous phase a crystalline precipitate was formed which was collected and washed with cold dilute ethanol. Yield 0.2 g, m.p. 152°.

p-Tolyl-γ-pyridyl sulphone. Compound 13. 4.5 g of pyridylpyridinium chloride hydrochloride [6] and 3.1 g of *p*-toluenesulphinic acid were refluxed with 50 ml of 75 % ethanol

Table 1. R_1—C$_6$H$_4$—$COCH_2 \cdot CH_2\overset{+}{N}(CH_3)_3\overset{-}{Br}$

R^1	m.p.	Formula	Mol.w.	Calc.	Found
H	238°	$C_{12}H_{18}ON\ Br$	272.2	N 5.1 Br 29.3	N 4.7 Br 29.7
CH_3	185°	$C_{13}H_{20}ON\ Br$	286.2	N 4.9 Br 27.9	N 5.2 Br 28.7
OCH_3	180°	$C_{13}H_{20}O_2N\ Br$	302.2	N 4.6 Br 26.5	N 4.7 Br 26.4
Cl	178°	$C_{12}H_{17}ONCl\ Br$	306.7	N 4.6 Br 26.1	N 4.5 Br 27.0

Table 2. R_1—C$_6$H$_4$—CO · CH_2 · CH_2—SO_2—C$_6$H$_4$—R_2

No	R_1	R_2	m.p.	Formula	Mol.w.	Calc.		Found	
1	H	$NHCOCH_3$	182°	$C_{17}H_{17}O_4NS$	331.4	C H N	61.6 5.2 4.2	C H N	61.5 4.9 4.1
2	H	NH_2	160°	$C_{15}H_{15}O_3NS$	289.4	N	4.8	N	4.9
3	H	H	99°	$C_{15}H_{14}O_3S$	274.4	C H S	65.7 5.1 11.7	C H S	65.7 5.2 11.4
4	H	Cl	136°	$C_{15}H_{13}O_3SCl$	308.9	C H Cl	58.4 4.3 11.5	C H Cl	58.0 4.1 11.2
5	H	OCH_3	123°	$C_{16}H_{16}O_4S$	304.5	C H S	63.2 5.3 10.5	C H S	62.9 5.0 10.4
6	H	CH_3	136°	$C_{16}H_{16}O_3S$	288.5	C H	66.6 5.6	C H	66.5 5.4
7	H	NO_2	185°	$C_{15}H_{13}O_5NS$	319.3	C H N	56.4 4.1 4.4	C H N	56.2 4.1 4.3
8	OCH_3	H	106°	$C_{16}H_{16}O_4S$	304.5	C H	63.2 5.3	C H	63.5 5.3
9	CH_3	H	117°	$C_{16}H_{16}O_3S$	288.5	C H	66.6 5.6	C H	67.2 5.8
10	Cl	H	154°	$C_{15}H_{13}O_3SCl$	308.9	C H Cl	58.4 4.3 11.5	C H Cl	58.0 4.0 11.3
11	CH_3	CH_3	105°	$C_{17}H_{18}O_3S$	302.4	C H S	67.5 6.0 10.6	C H S	67.4 6.0 10.3

Table 3. N—C$_5$H$_4$—SO_2—C$_6$H$_4$—R

No.	R	m.p.	Formula	Mol.w.	Calc.		Found	
12	H	125°	$C_{11}H_9O_2NS$	219.3	C H N S	60.3 4.1 6.4 14.6	C H N S	60.2 3.8 6.4 14.5
13	CH_3	134°	$C_{12}H_{11}O_2NS$	233.3	C H N S	61.8 4.8 6.0 13.7	C H N S	61.4 3.9 5.9 13.7
14	NH_2	265°	$C_{11}H_{10}O_2N_2S$	234.3	C H N	56.4 4.3 12.0	C H N	56.5 4.1 11.9

for 2 hours. The solution was filtered while warm, neutralized with Na_2CO_3 and left in the ice box. The crystalline solid was collected and recrystallized from ethanol. Yield 2 g (Table 3).

Compound 14. This compound was prepared analogously to compound 13, *p*-acetaminobenzenesulphinic acid being employed. During the synthesis a deacetylation takes place.

Compound 2 and compound 14 have been tested for antibacterial effect on *Mycobacterium avium*. The effect was negligible. (B.Noer.)

SUMMARY

A procedure is described for the preparation of sulphones from arylsulphinic acids and certain reactive quaternary ammonium compounds.

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Studies of Critical Phenomena in Carbon Dioxide contained in Vertical Tubes

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An accurate knowledge of the isotherms in the critical region is of great importance for problems of equations of state, for studies of liquid-gas phase interaction and many others. For the computation of the isotherms close to the critical point, the determination of the mass distribution of the substance in vertical tubes as a function of distance perpendicular to the original liquid-vapour interface might be useful. Previously, techniques such as the measurement of the position of small glass floats of known density or of a single spring-actuated float [1], and the measurement of radiation intensity from radioisotope loaded samples [2] have been used for the determination of mass distributions in vertical tubes.

It has appeared to me that a technique of greater simplicity and of potentially far greater resolving power could be developed by taking advantage of the relationship between density and refractivity. In this paper the apparatus developed for this work will be briefly described, and preliminary results for carbon dioxide presented.

The assembled equipment is shown in Fig. 1. It consists essentially of a cylindrical glass-jacketed thermostat containing the sample tube made of ordinary Pyrex tubing, and an optical system designed to focus an image of a pair of parallel vertical lines (illuminated by a sodium lamp) as seen through the sample tube and thermostat on a photographic plate. The thermostat and sample tube together may be regarded as a part of the optical system since they form a cylindrical lens. The telescope is focussed a few millimeters beyond the sample tube.

The photographic plate shows the image of two vertically running lines, the horizontal distances between which are determined by the extent of the refraction of the light which passes through the tube at whatever the height of the sample tube, and hence by the density of the substance in that region. One must, of course, establish the relationship between density and the distances between the lines, that is, calibrate the apparatus.

The line distance is found to increase nearly linearly with increased density. Therefore, each of the two lines on the photographic plate represents a mass

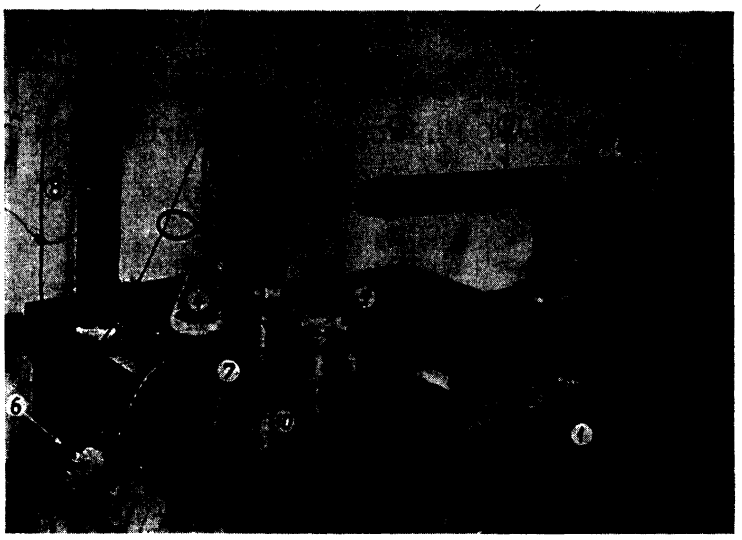

Fig. 1. Apparatus.

1) Steel frame (300 kg) mounted on 3 rubber blocks.
2) 3 thermostats (the one in front not fully mounted).
3) Screw for selecting the temperature of the thermostat. One turn of the screw changes the temperature about 50 millidegrees.
4) Reservoir of thermostat-water. Is used in the case of rough temperature selection.
5) Circulation pump, magnetically driven. The motor is inside the hollow foundation.
6) Electronic relays for switching off and on heating current on one of the thermostats.
7) Coil for additional heating of thermostat.
8) Lamp carrying vertical, illuminated lines (Na-light). The lamp is movable and can be placed in fixed position behind each thermostat.
9) Telescope can face any of the thermostats and can be raised and lowered for measuring at any level of the thermostats. (Screw and wheel not visible.)
10) Planed iron table which allows exact vertical placing of lamp and thermostats.

distribution curve of the carbon dioxide in the sample tube at the temperature of the thermostat. The same principle has been employed previously, but only by making direct visual observations and therefore without significant results [3].

THE THERMOSTAT

The thermostat consists basically of a glass cylinder (390 mm long, 60 mm inner diameter) hermetically sealed at both ends and totally filled with de-aired water. A magnetically-driven pump wheel spinning like a top on the bottom of the thermostat forces the water up through the 5 mm intermediate space created by the wall of the thermostat and a second glass cylinder placed inside and coaxial with it. (About 50 recirculations per minute.)

The spinning of the pump wheel is maintained by a motor below the thermostat bottom, the motor being supported by a rubber ring and placed coaxially inside the hollow foundation of the thermostat. The top of the vertical shaft of the motor carries a permanent horseshoe magnet. The pump wheel inside the thermostat also consists basically of a permanent magnet formed as a cylindrical disc. Naturally, the magnetic

connection between the motor and the pump wheel does not transfer the vibrations of the motor to the thermostat construction. In fact, in spite of the relatively high speed of the motor and the pump wheel — 1 400 rpm — the thermostat is practically free from vibrations — this being of great importance for the measurements close to the critical point.

Heat is supplied by means of a low-voltage alternating current (about 4 watts) conducted by naked resistant wires directly immersed in the water. The heating current is controlled by a relay actuated by a mercury switch, which is constructed in the form of a U-tube with a tungsten wire contact inside one arm. The contact end of the tube is sealed off, and the space above the mercury is filled with hydrogen. The other arm is open and the whole U-tube immersed in the thermostat water, so that the thermostat construction operates as the bulb in a great water contact thermometer.

The intense (not visible) stirring of the water provides for homogeneous temperature in the thermostat. The mercury switch reacts almost immediately by changes in the average temperature of the water. The resistant wires, spun of fine nichrome filaments, allows for an almost immediate heat transfer to the water when the heating current is switched on. Thus the inertia factors of an off-on thermostat are greatly reduced and, in fact, the calculated "hunting" (energy × time/heat capacity) of this thermostat can be as low as less than 0.001° in periods of one second. In cases where the calculated "hunting" even exceeded the last-mentioned value, the actual "hunting" was not registered by the resistant thermometer employed.

A rough temperature selection is obtained by adding or withdrawing water through a valve: withdrawing water causes, for example, the mercury contact to be broken and heat to be supplied to the system. The specific volume of the water will increase and the reduced mass of water will, due to the increased temperature, re-establish the balance of the mercury switch. An accurate temperature selection (0.001° intervals over about 1°) is obtained by screwing a plunger in or out of the thermostat, thus changing the volume. The easy regulation and the small size of the thermostat allow a convenient and rapid selection of desired temperatures. For precise work the thermostat has to be shielded by reflecting aluminium sheets.

The main difficulty which I met when designing this new type of thermostat was to secure a constant interior volume in the thermostat construction, a volume that was to be independent of exterior temperatures and air pressure changes, and to avoid leaks which tend to cause a constant rise or fall in temperature.

Through the top enclosure and almost all the way down the entire length of the thermostat a coaxial glass tubing (closed at the bottom and open at the top) form a "well", inside of which the test-tubes can be conveniently placed. The test-tubes are fully immersed in an oil with almost the same refractive index as the pyrex glass, thus reducing optical imperfections and securing good thermal conduction between the thermostat water and test tube. In the same oil and above the test tube, the thermometer is placed. Above the oil, but still inside the thermostat, a bobbin of wool yarn provides for adequate thermal insulation. FS Precision-Bore Tubing of Pyrex glass is used for the construction of the thermostat. The outside of these tubes is ground cylindrically and polished for optical perfection.

PREPARATION OF CARBON DIOXIDE

Carbon dioxide is prepared by dripping sulfuric acid into a solution of potassium-hydrocarbonate in an apparature described by Reihler [4]. This author described the carbon dioxide to be airfree. The carbon dioxide is dried by passing through 4 U-tubes containing concentrated sulfuric acid and glass beads, while the last of the 4 tubes is cooled by solid carbon dioxide. At this temperature the drying power of the solid sulfuric acid distributed on the surface of the beads is calculated to be about as high as that of phosphorus pentoxide at ordinary temperature.

From the fourth drying tube the carbon dioxide is led through a valve into an all-fused glass tubing system, consisting of the sample tubes to be filled, a trap cooled with solid carbon dioxide and an open mercury manometer. Finally, a glass valve leads to a diffusion pump and a mechanical vacuum pump. The glass tubing system is repeatedly filled with carbon dioxide heated and evacuated below 10^{-4} mm Hg, and, filled with

carbon dioxide, allowed to rest for several days. Then it is evacuated and refilled before the carbon dioxide is finally precipitated by immersing the bottom of the sample tubes in liquid air. The sample tubes are then individually fused off and thereby sealed. The purity of the enclosed carbon dioxide was probably better than 99.999 %. I am indebted to Cand.real. Arne Almenningen, who has done the preparation work of the carbon dioxide.

When using heavy-walled capillary tubing as sample tube, the tubes are deliberately under-filled when fused off. By warming the tubes to a temperature close below the critical, inaccuracies in the filling proportions can be determined. When recooling the bottom of the sample tube in liquid air the carbon dioxide will again precipitate and vacuum be reestablished in the tube. If now the top of the tube is fused, the interspace of the tube will be reduced. By repeating the test and thus shortening the tube the interior volume, corresponding to the mass of the enclosed carbon dioxide and its critical density, can be attained with adequate exactness.

GENERAL DESCRIPTION OF THE MASS DISTRIBUTION PHENOMENA

The mass distribution in tubes filled with CO_2 and heated to temperatures near the critical was studied in 3 series, the first as early as in the autumn of 1951. The purity of the CO_2 enclosed in the tubes has been successively improved and only the tube of the last series was prepared with the accuracy described above. In the first two series the tubes were about 20 cm long and with an inner diameter of 6 mm. In the last series the tube used for the mass distribution measurements was 5 cm long and of 1.8 mm inner diameter.

Qualitatively, the mass distribution found in the tubes in all 3 series was such as is to be expected when determined by isotherms of classical type and if the pressure increase downward in the tube is computed as being due to the weight of the substance itself.

At temperatures below the critical the carbon dioxide is separated into vapour (upper part) and liquid (lower). On approaching the critical temperature pronounced density gradients are built up in the two formerly homogeneous phases, the vapour being denser near the surface than higher up, and the liquid less dense near the surface than lower down. At a certain temperature the brilliant reflecting surface is replaced by a region with a sharp density gradient between the still fairly homogeneous fillings in the extremities of the tube. The density difference between both ends of the tube is then 10—15 % depending on the length of the tube investigated. When the temperature is further increased the densities at the extremities approach each other. More marked, however, is the increase in height of the region with density gradient. In the case of proper filling proportions the density gradient will soon reach both ends of the tube, and the fairly homogeneous filling, formerly present in the extremities of the tube, will have disappeared.

At temperatures just below the critical, the surface in tubes containing too much carbon dioxide will, with increasing temperatures, move upwards, in the opposite case downwards. Above the critical temperature the region with density gradient which has replaced the surface continues the movement of the surface and will, on further heating, in the case of improper filling, soon "move out of" one of the ends of the tube. If the two phases were homogeneous at all temperatures up to the critical point, then the surface would always "move out of" one of the ends of the tube already before the critical tem-

perature had been reached. As stated above, this movement of the surface will always exist but, due to the fact that the density difference between the fillings in the extremities of the tube is greater than the density difference at the surface, the surface will not reach one of the ends of the tube before a region with density gradient has been established. This happens when the filling proportion does not deviate more than about ± 10 % from that corresponding to the critical density. These limits must depend on the height of the tube employed.

In my experiments the method of raising or lowering the temperature in steps was employed, thus allowing the tubes to remain for a considerable time at the selected temperatures before the photographs were made.

In practice the mass transportation which takes place when changing the temperature is slow. When selecting the time in which the tube was kept at the chosen temperature, regard was paid to the changes in mass distribution to be expected. In some cases, the time during which the tube was kept at constant temperature was as long as 8 days. Working carefully enough it must be expected that the distribution actually measured will not be far from an equilibrium distribution.

The new material seems to indicate very strongly that the mass distribution in the tubes in equilibrium is determined by the isotherm of the substance at the temperature selected. If this is so, the mass distribution curves will show parts of the isotherms, and the determination of the mass distribution in the vertical tube will make possible a determination of the isotherms substantially closer to the critical point than any previously known method.

If a simultaneous determination of the pressure inside the tube had been carried out, the position of the isotherm in the *pv*-diagram would have been known. However, in the region in question which is very close to the critical point, the $\frac{\mathrm{d}\,p}{\mathrm{d}\,t}$ of substances at critical density can be regarded as constant. At the critical point $\frac{\mathrm{d}\,p}{\mathrm{d}\,t}$ is known for a series of substances.

The relation between line distances and actual densities

For the calibration of the apparatus the sample tube was heated to the temperatures 29.01°, 29.98° and 30.76° (2.03°, 1.06° and 0.28° below the critical). The horizontal distances between the vertical lines on the photographic plate which recorded the densities of the liquid and vapour phases were at these temperatures 1.745, 1.382; 1.698, 1.414; 1.639 and 1.460 (mm).

Fig. 2 shows the top of the coexistence curve. The densities of the liquid and vapour phase at the temperatures mentioned according to the curve are as follows: 0.623, 0.308; 0.593, 0.338; 0.545 and 0.380 (g/cm³). Using these density data and the corresponding line distances the calibration curve was constructed. It is a slightly curved line which is almost straight at the densities found in the tube near and above the critical point.

For future work a more satisfactory way of calibration will be used.

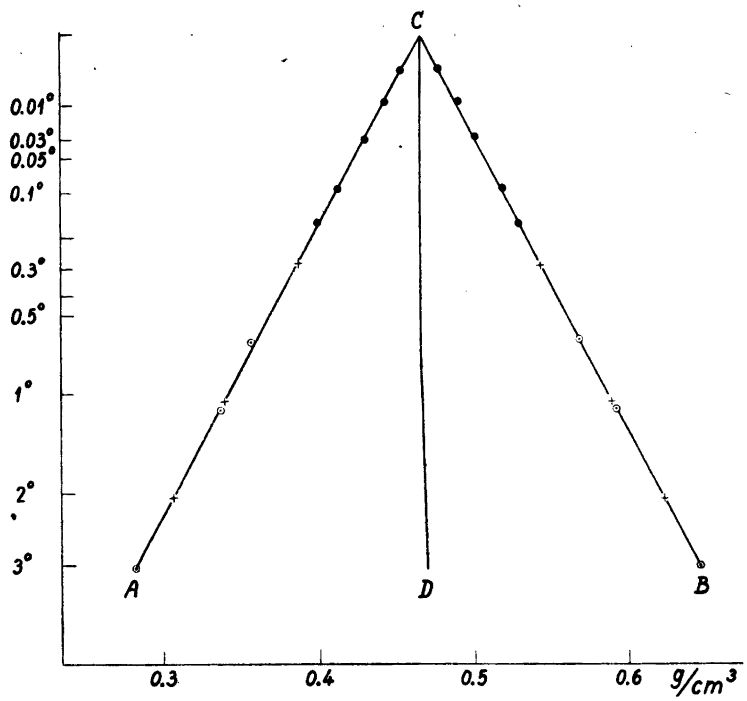

Fig. 2. Top of Coexistence Curve.

C is the critical point. The ordinate is in the scale of $\Delta T^{0.357}$.
AC and BC are the vapour and liquid parts of the coexistence curve, CD is the rectilinear diameter.
Down to $\Delta T = 1°$ *CD is a straight line. Below it has slightly positive curvature. The lines AC and BC should have corresponding curvature. For simplification, however, the curves are drawn as straight lines and the points below* $\Delta T = 1°$ *herefore slightly corrected. The 6* ⊙ *marked points correspond to the values of MBM, the 6* × *marked points to those used for calibration of the measurements and the* ● *marked points correspond to the determined densities of vapour and liquid at the surface at the temperatures 1.4, 9.6, 18, 90, 158 millidegrees below the critical.*

The Top of the Coexistence Curve

A. Michels, B. Blaisse, and C. Michels [5] (in the following abbreviated to MBM) have found that the coexistence curve for carbon dioxide at temperatures up to about 1° below the critical can be represented by the equation $d_l - d_v = C \cdot \Delta T^{0.357}$ where d_l and d_v are the densities of liquid and vapour and ΔT the distance from the critical temperature. Furthermore, the Matthias rule of rectilinear diameter was found to be correct up to the temperature mentioned. Above this temperature the coexistence curve was found to be less regular. If, however, the coexistence curve is supposed to be regular all the way up to the critical point, it will, when drawn with the temperature scale $\Delta T^{0.357}$ as ordinate, be represented by two symmetrical straight lines

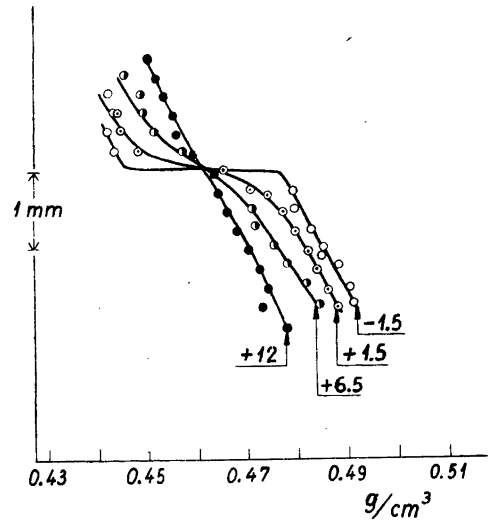

Fig. 3. Mass Distributions Close To the Critical Point.

The temperature of the thermostat was raised in steps to the temperatures of 1.5 millidegrees below and 1.5, 6.5 and 12 millidegrees above the critical. The curves show the mass distribution in the middle section of the tube. The line marked 1 mm corresponds to 1 mm height difference in the tube. The horizontal part of the curve −1.5 corresponds to the density difference at the surface.

meeting at the critical point. Down to 1° below the critical temperature the inclination of the rectilinear diameter is of no practical importance. In Fig. 2 the coexistence curve is drawn in the way just described. The 6 centered circles correspond to the values of MBM for the temperatures 2.99°, 1.11° and 0.63° below the critical with a minor correction for the lower two. The 6 points marked by crosses correspond to those used for the calibration as described above. The filled circles thus correspond to my measurements of the coexistence curve up to 1.4 millidegrees below the critical temperature.

Mass distribution close to the critical point

In the second series carried out in the autumn of 1952 the tube was heated in steps of 3 millidegrees per hour past the critical temperature. Some of the mass distribution curves obtained are shown in Fig. 3. In the third series the tube was cooled from a temperature some millidegrees above the critical, past the critical temperature in steps of about 1 millidegree per 20 minutes. In Fig. 4 the mass distribution curves for the temperatures + 1.2 and —1.4 millidegrees are shown. A mass distribution curve made at a temperature between these two is not shown on the figure, but runs, as is to be expected, in between the curves + 1.2 and — 1.4.

The agreement between the curves + 1.5 and — 1.5 in Fig. 3, and + 1.2 and — 1.4 in Fig. 4, is as good as can be expected. In the second series (Fig. 3)

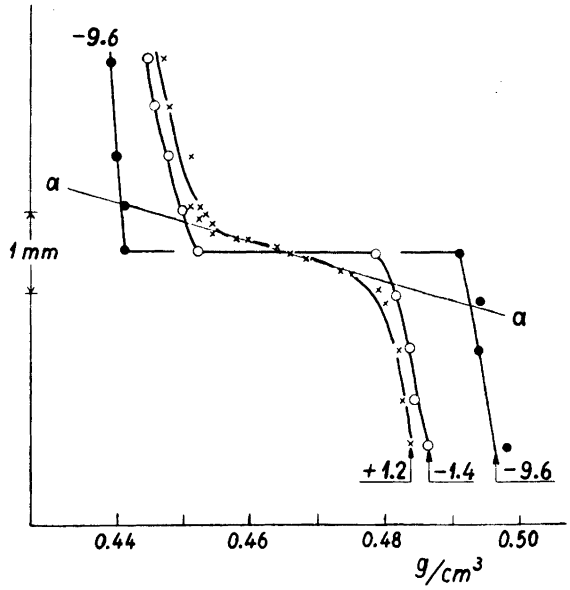

Fig. 4. Mass Distributions Close to the Critical Point.

The temperature of the thermostat was lowered in steps to 1.2 millidegrees above and 1.4 and 9.8 millidegrees below the critical. Ordinate and abscissa as in Fig. 3.

The straight line (a—a) represents a calculated tangent of the mass distribution curve +1.2 at the inflexion point.

the inner diameter of the tube was 6 mm. For optical reasons this diameter is too large for really good measurements of a very sharp density gradient. Moreover, as the calibration of the apparatus was not carefully done, the density scale of Fig. 3 is uncertain by about ± 10 %.

The line *a—a* in Fig. 4 is the calculated tangent of the isotherm + 1,2 millidegrees of carbon dioxide at the inflexion point. The calculation is based on the data given by MBM in their Fig. 9 and the average pressure increase downward in the tube of 0.000046 atm. per mm. In addition, the tangents for the isotherm + 7 millidegrees and + 90 millidegrees have been calculated and found to be in adequate agreement with the mass distribution curves + 7 and + 90 in Figs. 5 and 6.

Fig. 4 also shows the liquid and vapour curves at — 9.8. The inhomogeneity of the two phases can be seen.

The critical temperature

In the second series (Fig. 3) the critical temperature was determined as the temperature at which the surface was no longer seen as a horizontal, brilliantly reflecting disc. In the third series (Fig. 4) the density gradient was visible at the temperature + 1.2 millidegrees. Moreover, the photograph shows clearly

the smooth transition between the densities in the upper and lower part of the tube. In the next step it was not possible to determine visually whether a very large density gradient or a real surface existed. Nor did the photograph allow this determination. The temperature of this step was chosen as the critical in this series. At the temperature — 1.4 millidegrees, however, the brilliantly reflecting disc of the surface was clearly visible and, moreover, the photograph showed definitely the density step at the surface.

The critical temperature was found to be 31.04°, the same as found by MBM.

Mass distributions at equilibrium

Already in the first series the mass distributions were determined in the tube at a temperature (+ 25 millidegrees) which was reached by cooling as well as by heating. The mass distributions were found to be similar, but definitely different from the distributions which were found at higher and lower temperatures. Corresponding experiments which, however, showed poorer agreement between the final mass distributions at + 20, are reported in the next paragraph.

Identical mass distributions by heating and by cooling have never been obtained, most probably because insufficient time has been allowed for equilibration at the temperatures selected.

More precise experiments will be carried out when the accuracy of the system is improved. Any serious hysteresis effect, however, would have been detected in the great number of mass distribution measurements which have been carried out.

Mass transportation phenomena

When the mass distribution in the tube, at temperatures below the critical, is in equilibrium, the surface will appear at certain heights in the tube, dependent upon the temperature and the ratio between the interior volume of the tube and the mass of the enclosed substance.

On rapid heating, however, the surface will start an unexpected movement upwards in the tube. The reason for this may be that the thermal expansion of the liquid is faster than the mass transfer from liquid to vapour. In the case of heating rapidly past the critical temperature the surface will degenerate at a position too high in the tube.

Fig. 6 demonstrates that a similar phenomenon takes place when the tube is rapidly heated from + 7 up to + 20 millidegrees above the critical. Curve + 7 shows the mass distribution in a tube which had remained at this temperature over night. Curve *a* shows the mass distribution 10 minutes after the temperature was raised up to + 20. The density gradient has moved upwards in the tube. Curve + 20 shows the mass distribution in the tube after having been kept for 3 hours at this temperature.

On the other hand, by rapid cooling of the tube past the critical temperature, the reappearence of the surface will take place in a lower position in the tube than expected. By cooling the tube to a temperature above the critical

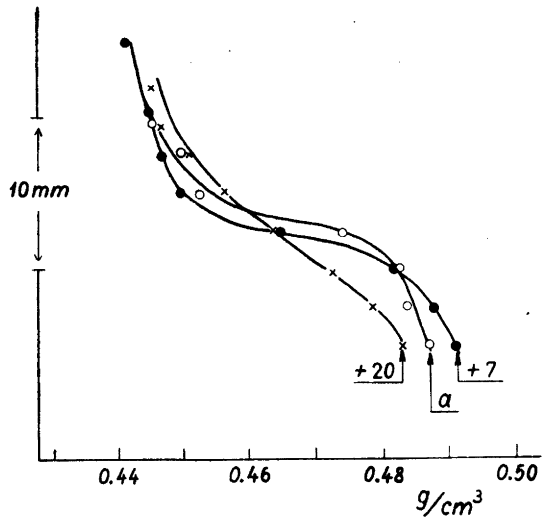

Fig. 5. Mass Transportation.

Ordinate and abscissa as in Fig. 3. The temperature of the thermostat was raised from 7 millidegrees above the critical (curve +7) to 20 millidegrees above the critical. Curve a shows the mass distribution when tube had remained 10 minutes at this temperature, curve +20 shows the mass distribution after 3 hours.

a similar phenomenon seems to take place. In Fig. 6, curve + 90 shows the mass distribution in the tube after the tube had stayed at + 90 millidegrees over night. By lowering the temperature the density of the lower part of the tube increases almost immediately and a density gradient is built up which moves very slowly upwards in the tube — *cf.* curves *a* and *b*. Curve + 20 shows the mass distribution after 48 hours at 20 millidegrees above the critical.

Fog

Fog formation, commonly found by cooling the tubes past the critical region, may be connected with the mass transportation phenomena. In the tubes of the two first series heavy fog was formed by cooling and gave the impression of being a subcooling phenomenon. Fog was generally not produced by heating. However, when the tube was slowly heated past the critical temperature (second series Fig. 3), a light fog was observed in the region of a sharp density gradient.

In the tubes prepared for the last series, and with the care described above, no fog formation at all was produced by either heating or slow cooling. On cooling rapidly past the critical temperature, however, a 5 mm high white belt introduced the reappearance of the surface.

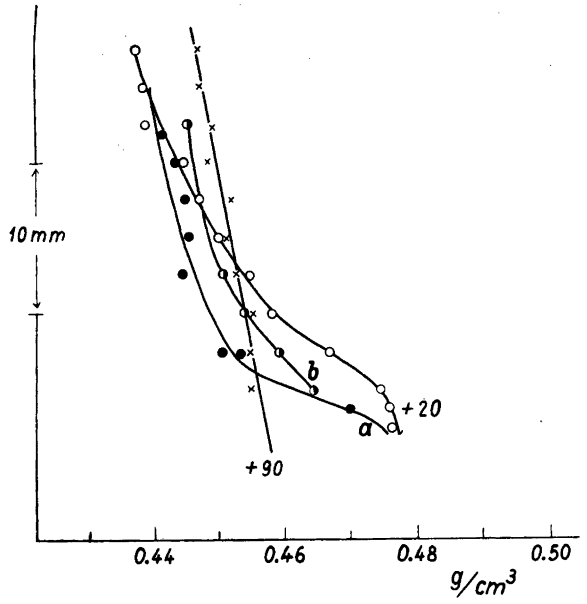

Fig. 6. Mass Transportation.

Ordinate and abscissa as in Fig. 3. The temperature of the thermostat was lowered from 90 millidegrees above the critical (curve +90) to 20 millidegrees above the critical. Curve a shows the mass distribution immediately after the thermometer had reached the new temperature, curve b the mass distribution 3 hours later. Curve +20 shows the mass distribution when the tube had remained for 48 hours at 20 millidegrees above the critical.

SUMMARY

The initial purpose of this work was to study the supposed deviation from classical behaviour of a substance (carbon dioxide) enclosed in a vertical tube of adequate filling and heated within the critical region. However, no anomalies could be observed. The mass distributions were as expected if determined by isotherms of the classical type. Furthermore, the coexistence curve has a rounded top. The measurements of mass distribution in vertical tubes can very likely provide a method for the determination of the isotherm in the very neighbourhood of the critical point.

Although the equipment has not yet reached the desired perfection for precise quantitative determinations, it appears most likely that the isotherms have been determined and further that the top of the coexistence curve conforms to the "cubic rule".

The carbon dioxide, which was prepared with the greatest caution, did not show the commonly observed fog-formation when heated or cooled in the critical region.

Measurements in the close neighbourhood of the critical point require a very good thermostat. A new thermostat with the temperature control based

on the thermal expansion of the thermostat water itself has been designed and built. For the measurements of the mass distributions in the vertical tubes the refractivity of the enclosed carbon dioxide could be measured at any desired level. For this purpose a cylindrical lens method was developed.

First of all I wish to thank Professor Dr. Odd Hassel for his interest in this work and for his permission to let me work in his laboratory. Thanks are also due to several members of the staff for valuable discussions, and to Dr. K. W. Hedberg, Pasadena, temporarily in Oslo.

I am indebted to my father for the economical support which allowed the accomplishment of this work. I also thank "*Almenvidenskapelige Forskningsråd*", which has paid the wages of an instrument maker put at my disposal. Mr. Amund Helgesen I thank for his loyal interest and a series of ideas in the design of the equipment.

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Optically Active α,α'-Diethylglutaric Acids and their Anhydrides

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The α,α'-diethylglutaric acid, $HOOC \cdot CH(C_2H_5) \cdot CH_2 \cdot CH(C_2H_5) \cdot COOH$, was first described by Dressel [1] who apparently had in hand a mixture only of the isomeric forms. Auwers [2] succeeded in isolating the high-melting acid in a pure state while the other was obtained as a low-melting mixture with the first one. Reformatsky [3] later prepared both acids in the pure state and found the melting points 119.5—120° and 93.5—94.5°, respectively. By treatment with acetyl chloride the high-melting acid was transformed into a liquid anhydride but no anhydride of the low-melting acid was described.

No report has been given of an optical resolution of any of the acids and it could not therefore be decided which was the meso- and which the racemic form. We have prepared both the inactive acids according to the method used by Auwers [2] and found the same melting points as given by Reformatsky. All attempts to resolve optically the high-melting acid by fractional crystallization of an alkaloid salt failed. On the other hand we were able by means of the brucine salt, and also the cinchonidine salt, to resolve the low-melting acid into active components having a specific rotation in ethanolic solution of about ± 30.3° and m.p. 75.5—76.5°. From the form of the melting point curve between the antipodes it can be concluded that the inactive acid is a true racemic form.

The active acids were easily converted into the corresponding active anhydrides without any racemization taking place. The sign of the rotation changed, however, when passing from an active acid into its anhydride and *vice versa*. The highest specific rotation found for an anhydride was —24.6°. The anhydrides were obtained as liquids which could be distilled in a vacuum without diminishing their optical rotation.

EXPERIMENTAL

Preparation of the inactive acids

The ethyl ester of propane-1,1,3,3-tetracarboxylic acid was prepared mainly according to Knoevenagel [4] by condensation of malonic ester and formaldehyde using diethylamine as a catalyst. The yield was about 50 %. The method used by Dressel [1] was also tried, *viz.* the reaction between sodium malonic ester and methylene iodide. The yield was about the same but as the first method was the easier to carry out it was preferred.

The ethylation of the propanetetracarboxylic ester was effected by treatment with sodium ethoxide and ethyl iodide as described by Auwers [2]. The crude reaction product was obtained in a yield of about 50 % as a colourless crystalline mass with m.p. 58—61° which was used directly for further reactions. If desirable it could be recrystallized from ether and was then obtained as clear crystals with m.p. 60—61°.

The hydrolysis of the ethyl-substituted tetracarboxylic ester and the subsequent decarboxylation to dicarboxylic acid were carried out in one operation, mostly in the following way. The ester (20 g) was dissolved in concentrated sulphuric acid (35 ml) and adding water (35 ml) most of the ester separated as a layer on top of the acid. The mixture was then refluxed until practically all ester had disappeared which took 25—30 hours. On cooling a mixture of the acids was obtained as a crystalline mass in a yield of 90—95 %.

The separation of the two inactive acids from the crystalline mass melting from 70° to 100° was a rather difficult and tedious process. Several methods were tried but the only useful one was repeated fractional crystallization alternately from water and benzene. As an example may serve the following. A solution of 10 g of the crystalline mass in 140 ml water was decolorized by boiling with charcoal. On cooling crystals rich in the high-melting form and having m.p. 113—117° soon separated and were filtered off the next day. The mother liquor left at room temperature for a couple of days gave a new fraction of crystals having the m.p. 85—90°. The mother liquor from these crystals was concentrated somewhat when more crystals rich in the high-melting acid were obtained. This process was continued until in all 4 g of an acid rich in the highmelting form and 4 g rich in the low-melting form had been obtained. The material from several experiments was collected and the further purification carried out by continued fractional crystallization either from water or from benzene. The purest high-melting acid had m.p. 119—120°.

0.2061 g required 21.92 ml 0.1 *N* NaOH, M 188.1; calc. M 188.2.

The isolation of the pure low-melting acid was successful only when the initial separation of the mixture of the acids resulted in a fraction sufficient rich in the low-melting acid. Then repeated crystallizations from water and benzene in the end led to an acid with m.p. 93.5—94.5°.

0.1740 g required 18.48 ml 0.1 *N* NaOH, M 188.2

Both acids were very soluble in ether, acetone, methanol, ethanol, chloroform and glacial acetic acid.

In the case of α,α'-dimethylglutaric acid Möller [5] found that the meso-form but not the racemic form gave an anhydride on treatment with acetyl chloride (quite unaccountably he at the same time found that both the active components of the racemic form were transformed into anhydrides under the same conditions). If the isomeric forms of the α,α'-diethylglutaric acid behaved in the same way it should be possible to separate the meso-form from the racemic form by means of acetyl chloride. It was, however, found that both the inactive isomerides of α,α'-diethylglutaric acid on heating with acetyl chloride easily gave the corresponding anhydrides. The anhydrides formed liquids which boiled at 154—155°, at 15 mm Hg. Starting with an acid-mixture having m.p. 88—91° the acid-mixture recovered from the distilled anhydrides had m.p. 90—91° which showed that no separation of the isomerides was effected in this way.

Resolution of the racemic acid

In a warm solution of 10 g α,α'-diethylglutaric acid with the m.p. 93.5—94.5° in 240 ml water 21 g of brucine (a little less than 1 mole alkaloid to 1 mole acid) were dissolved in portions. Placing the solution in a refrigerator and inoculating with crystals from a preliminary experiment 10.4 g brucine salt of the (+)-acid crystallized in the course of two days. After recrystallizing once from water the salt was dissolved in hydrochloric acid and the active acid extracted with ether.

$$[\alpha]_D^{20} = + 28.74° \quad (p = 4.02 \text{ in abs. ethanol})$$

On concentrating the mother liquor some more salt of the (+)-acid was obtained. From the rest of the solution a levorotatory acid (3 g) was obtained having a specific rotation of — 19.4°.

In another case, starting with 14 g of racemic acid, the least soluble brucine salt was recrystallized 6 times from water. The acid isolated from this salt had the specific rotation + 29.5°. A new treatment with brucine did not lead to an increase of the rotation.

In the mean time it had been found that the cinchonidine salt (1 mole alkaloid to 1 mole acid) crystallized well from acetone. Also in this case the salt of the (+)-acid crystallized first. In a series of experiments in which the cinchonidine salt had been recrystallized 1, 3 and 6 times from acetone the following specific rotations for the acid were found: + 29.03°, + 29.65° and + 30.75°. A final treatment of the acid + 30.75° with cinchonidine resulted in an acid with rotation +30.3°. This active acid on recrystallization from water was obtained in well-developed flat prisms with m.p. 75.5—76.5°.

$$[\alpha]_D^{20} = + 30.33° \ (p = 4.0 \text{ in abs. ethanol})$$

In order to determine the specific rotation at various concentrations it was necessary to measure the variation of the specific gravity with concentration. The results obtained, using the racemic acid, are given in Table 1 in which p denotes g acid in 100 g ethanolic solution.

Table 1. Specific gravity of solutions of racemic acid in ethanol.

p	d_4^{20}
0	0.791
3.94	0.801
10.21	0.816
13.52	0.824
21.17	0.843
39.15	0.889

According to these figures the relationship between specific gravity and percentage is practically linear.

The specific rotation of the (+)-acid at various concentrations will be found in Table 2.

Table 2. Specific rotation of (+)-α,α'-diethylglutaric acid in abs. ethanol.

p	d_4^{20}	l	α_D^{20}	$[\alpha]_D^{20}$
4.014	0.801	2	+ 1.95°	+ 30.33°
10.29	0.816	2	5.09	30.30
13.26	0.824	2	6.63	30.33
21.26	0.844	2	10.92	30.44
40.86	0.893	1	11.36	31.13
49.09	0.914	1	14.12	31.47
57.41	0.936	1	17.34	32.26

The isolation of pure levorotatory acid was successful only once. In that case 0.9 g of a levorotatory acid isolated from the light soluble cinchonidine salt was dissolved in 10 ml hot water and 1 ml 2 N hydrochloric acid added to the cooled solution. The next day a crystalline acid was filtered off consisting mostly of racemic acid (m.p. 89—91°). When the filtered solution had been left for another day some rather big rhombic crystals had separated (0.2 g). Recrystallized from water the m.p was 75.5—76.5°.

$$[\alpha]_D^{20} = - 30.34° \ (p = 3.54 \text{ in abs. ethanol})$$

By other experiments the (—)-acid isolated always had a lower rotation (—20 to —27°). As shown by the melting point curve in Fig. 1 the inactive acid is a true racemic form.

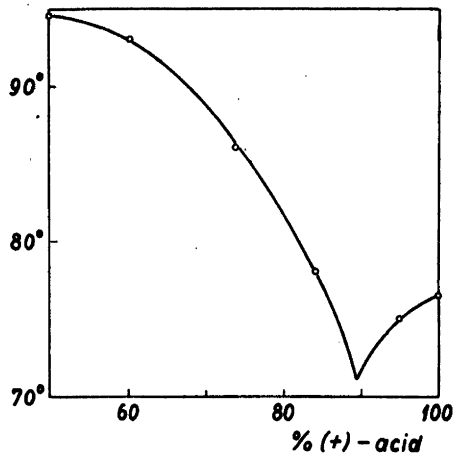

Fig. 1. Melting points of mixtures of (+)- and (−)-α,α'-diethylglutaric acids

The optically active anhydrides

The active acid (0.5 g) to which had been added acetyl chloride (3 g) was heated to 50—60° for 2 hours. After removing excess of acetyl chloride the anhydride was distilled in a vacuum. None of the anhydrides would crystallize. Starting with pure (+)-acid a levorotatory anhydride with the following rotation was obtained:

$$[\alpha]_D^{20} = -24.64° \ (p = 4.975 \text{ in dioxan})$$

The specific rotation of the acid recovered from this anhydride was + 30.56°, showing that no racemization had taken place during the preparation of the anhydride. From a (−)-acid with specific rotation − 20.24° an anhydride was prepared having the rotation + 13.75° (p = 4.36 in abs. ethanol). The acid recovered from this anhydride had the specific rotation − 19.91°.

SUMMARY

Of the two inactive α,α'-diethylglutaric acids with m.p. 119—120° and 93.5—94.5°, respectively, the low-melting was found to be the racemic form. It was resolved into optically active components having the specific rotations ± 30.3°. The two inactive as well as the active acids were converted into the corresponding anhydrides. On preparing the anhydride of an active acid the sign of the rotation changed but no racemization took place. The highest specific rotation observed for an anhydride was —24.6°.

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The Reaction between Acetylacetone and *p*-Benzoquinone

III. ω, ω, ω', ω'-Tetraacetyl-*p*-xyloquinone

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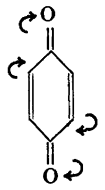
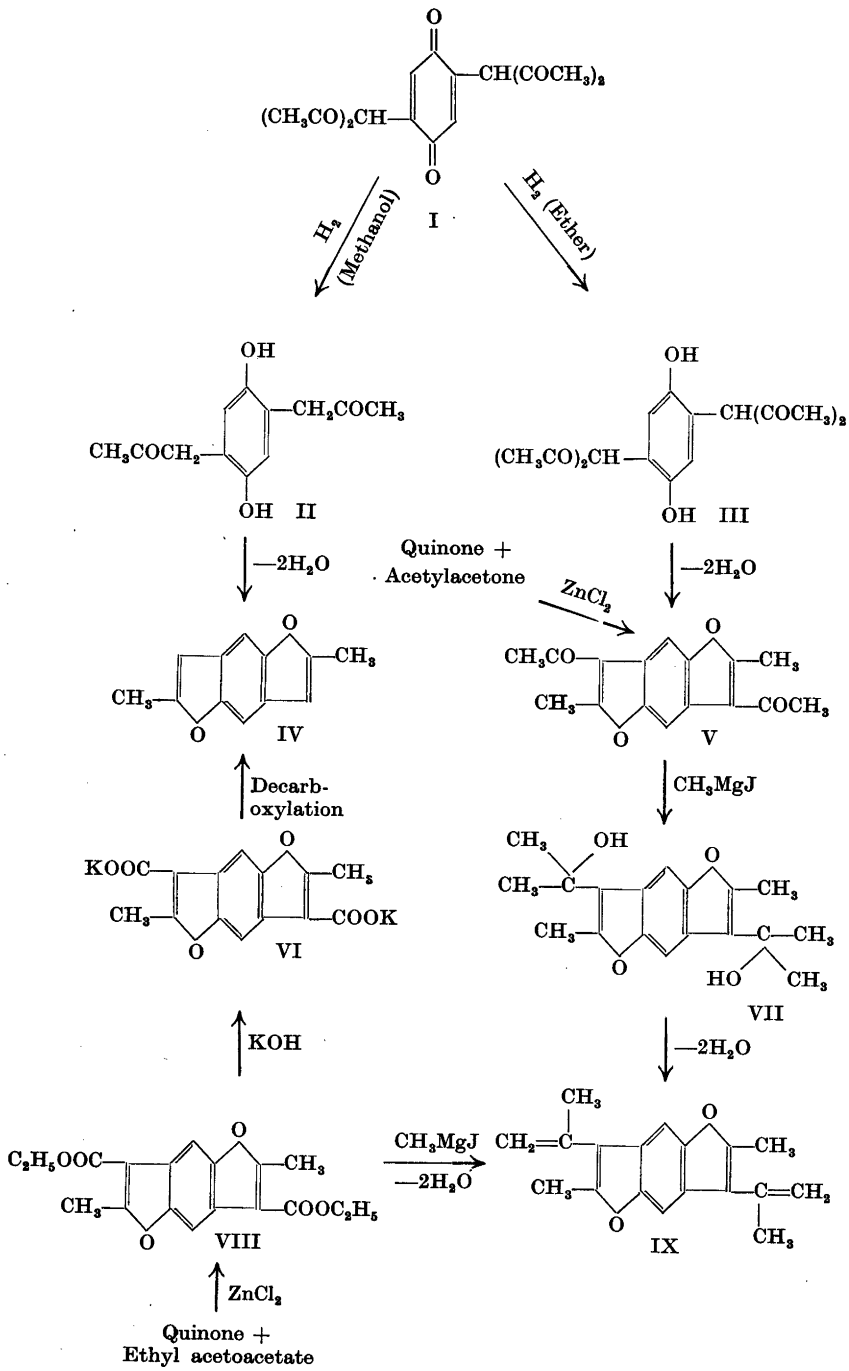
As previously communicated [1] a quinone $C_{16}H_{16}O_6$ was formed in a base-catalyzed reaction between acetylacetone and *p*-benzoquinone where apparently one mole of the quinone and two moles of the diketone participated.

In *p*-benzoquinone the mesomeric (and in reaction electromeric) shifts indicated on the figure are supposed to take place leaving small negative charges on the oxygen atoms and corresponding positive charges on the carbon atoms in the 2- and 5-positions. In *p*-benzoquinone are thus 2,5-substitutions with nucleophilic reagents very common. We were therefore led to the assumption that the acetylacetone anion could have reacted with the quinone in this way which would result in a product $C_{16}H_{16}O_6$ with the structure I. As is always the case in nucleophilic substitutions an oxidizing agent must be present, in our case *p*-benzoquinone itself. In the present paper evidence shall be furnished for the correctness of this assumption.



Being a quinone $C_{16}H_{16}O_6$ was easily reduced by the action of catalytically activated hydrogen. Though always consuming about one mole of hydrogen the reaction took different courses in different solvents. In absolute ethyl ether a product $C_{16}H_{18}O_6$ (III) could be obtained, obviously the hydroquinone corresponding to I. III could easily be oxidized back to I if treated with *p*-benzoquinone in ethanol. When hydrogenated in absolute methanol, however, $C_{16}H_{16}O_6$ yielded a substance $C_{12}H_{14}O_4$ (II). II and III gave a positive iodoform reaction, could be acetylated to give the diacetates and reacted with 2,4-dinitrophenylhydrazine but yielded no defined hydrazones. The substances gave strong colour when dissolved in dilute alkali, II gave a deep red and III a violet colour both of which became brown when the solutions were kept for a while. On distilling the methanol from the hydrogenation mixture a small quantity of acetaldehyde could be found in the distillate, probably arising from the acetyl groups split off during the hydrogenation. On the other hand II when boiled with 2 *N* hydrochloric acid yielded acetone, other fragments of the molecule could not be isolated. Boiled with undiluted acetyl chloride II


O
CH(COCH3)2
(CH3CO)2CH
O
I
H2 (Methanol)
H2 (Ether)
OH
CH2COCH3
CH3COCH2
OH II
OH
CH(COCH3)2
(CH3CO)2CH
OH III
—2H2O
Quinone +
Acetylacetone
ZnCl2
—2H2O
O
CH3
CH3
O IV
O
CH3CO
CH3
CH3
COCH3
O V
Decarb-
oxylation
CH3MgJ
KOOC
CH3
CH3
COOK
O VI
CH3
OH
CH3—C
CH3
O
CH3
C—CH3
HO
CH3
VII
KOH
—2H2O
C2H5OOC
CH3
CH3
COOC2H5
O VIII
CH3MgJ
—2H2O
CH3
CH2=C
CH3
O
CH3
C=CH2
CH3
O IX
ZnCl2
Quinone +
Ethyl acetoacetate

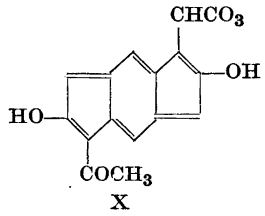
split off two molecules of water giving a compound $C_{12}H_{10}O_2$ from which no simple functional derivatives could be prepared. The substance was very stable, *e.g.* distilling unchanged at ordinary pressure. It is assumed to result from a ring closure with elimination of water from the tautomeric form of II, hence the formula IV is ascribed to it. A similar dehydration and ring closure could be effected with III by the action of cold concentrated sulphuric acid. The resulting product had the composition $C_{16}H_{14}O_4$ (V) and gave a *bis*-2,4-dinitrophenylhydrazone. The iodoform reaction, however, was doubtful and attempts to oxidize $C_{16}H_{14}O_4$ to its corresponding dibasic acid were not succesful.

We have previously reported [2] that acetylacetone and *p*-benzoquinone react in presence of anhydrous zinc chloride to give a mixture of two substances. One of these substances was soluble in alkali and identified as 2-methyl-3-acetyl-5-hydroxybenzofuran. The other substance has now been found to be identical with $C_{16}H_{14}O_4$ (V).

In order to connect IV and V with a known substance and with each other we repeated Pechmann's [3] and Ikuta's [4] synthesis consisting in the condensation of ethyl acetoacetate with quinone in presence of anhydrous zinc chloride. One of the products of this condensation is the benzodifuran derivative VIII which has the same ring skeleton as our compounds IV and V. The corresponding potassium salt VI was prepared by treating VIII with alcoholic potassium hydroxide. This salt was easily decarboxylated to give a compound $C_{12}H_{10}O_2$ identical with IV.

Further the diketone $C_{16}H_{14}O_4$ (V) reacted easily with methyl magnesium iodide to give the corresponding tertiary alcohol $C_{18}H_{22}O_4$ (VII). By heating to 100° *in vacuo* this alcohol as expected lost two moles of water, the product having the composition $C_{18}H_{18}O_2$ and to which we ascribed the structure IX. The same compound could be prepared by reacting Pechmanns ester VIII with an excess of methyl magnesium iodide in xylene. We were not able to isolate the intermediate tertiary alcohol in this case.

Ionescu [5] who also studied the reaction between acetylacetone and *p*-benzoquinone isolated a substance $C_{16}H_{12}O_4$ to which he assigned the structure X. This substance should according to Ionescu result from a ring closure of I thus breaking the C = O double bond of the quinone. He did not, however, isolate I but assumed it to be an intermediate in the reaction. We have not so far been able to effect a ring closure with elimination of water from I leading to X.



X

EXPERIMENTAL

(Melting points not corrected)

$\omega,\omega,\omega',\omega'$-Tetraacetyl-*p*-xyloquinone (I)

To a solution of *p*-benzoquinone (2 g) in very pure acetylacetone (20 ml) was added dry pyridine (about 20 drops). The reaction mixture was left for 4 hours, the separated red product was filtered off and washed with cold ethanol. Yield *ca.* 0.25 g. If the size of the

batches was increased the yield invariably was relatively lower. Recrystallized from ethanol the substance had m.p. 212–213° dec.

$C_{16}H_{16}O_6$	Calc.	C	63.16	H	5.29
	Found	»	63.15	»	5.30

Hydrogenation of I in ethyl ether

I (1.0 g) was suspended in dry ethyl ether (100 ml) to which platinum oxide (0.1 g) was added. Due to the variations in vapour pressure of the ether with the differences in temperature the amount of hydrogen used was difficult to determine but is estimated to 300 ml (1 200 mm Hg) in 8 hours; calc. for one mole of hydrogen 263 ml. The hydrogenation vessel was filled with nitrogen and the platinum oxide filtered off under flushing with nitrogen. The ether was removed in a vacuum desiccator with concentrated sulphuric acid. The solid white residue (0.88 g) was recrystallized twice from acetone-water and had m.p. 199°. It consisted of fine, colourless needles and gave a strong violet colour with aqueous ferric chloride.

$C_{16}H_{18}O_6$	Calc.	C	62.73	H	5.93
	Found	»	62.70	»	6.10

Reoxidation to I: $C_{16}H_{18}O_6$ (0.2 g) was dissolved in ethanol and boiled for a few minutes with an excess of *p*-benzoquinone. The reaction mixture was evaporated to dryness. The red residue was washed with ethanol and recrystallized from the same solvent, m.p. 210°, mixed m.p. with I showed no depression.

$C_{16}H_{16}O_6$	Calc.	C	63.16	H	5.29
	Found	»	62.87	»	5.51

Acetylation: $C_{16}H_{18}O_6$ (0.1 g) was dissolved in acetyl chloride (10 ml) and refluxed for 45 minutes. The reaction mixture was poured into cold water the separated solid substance being filtered off and recrystallized twice from methanol. Small plates of m.p. 220–221°.

$C_{20}H_{22}O_8$	Calc.	C	61.53	H	5.69
	Found	»	61.50	»	5.92

Hydrogenation of I in methanol

A suspension of I (5.0 g) in dry methanol (100 ml) to which platinum oxide (0.5 g) had been added took up 337 ml hydrogen (1 200 mm Hg) in 35 minutes; calc. for one mole of hydrogen 263 ml. After filtering the methanol was evaporated *in vacuo* above calcium chloride. The residue which consisted of a yellow oil and a white crystalline substance weighed 4.35 g. The oil was removed by washing with ethyl acetate and the solid substance (2.2 g) was recrystallized from dilute ethanol and sublimated *in vacuo*. M.p. 193–195°.

$C_{12}H_{14}O_4$	Calc.	C	64.80	H	6.35
	Found	»	64.49	»	6.48

Methoxyl content nil.

In an experiment similar to that above the methanol was distilled off through a Vigreux column. The residue consisted chiefly of $C_{12}H_{14}O_4$. To a part of the distillate was added a solution of 2,4-dinitrophenylhydrazine in dilute hydrochloric acid. Immediately a hydrazone separated which after recrystallization from glacial acetic acid had m.p. 165°.

$C_8H_8N_4O_4$	Calc.	C	42.86	H	3.58	N	25.02
	Found	»	43.22	»	3.69	»	25.80

Mixed melting point with the 2,4-dinitrophenylhydrazone of acetaldehyde showed no depression.

Acetylation: To a solution of acetyl chloride in toluene (10 ml *ca.* 1.5 molar) was added dry pyridine (2 ml) and $C_{12}H_{14}O_4$ (0.18 g). The mixture was heated on a water bath for 20 minutes and the excess of acetyl chloride was removed by shaking with water. The toluene layer was dried with calcium chloride and evaporated to dryness. The residue (0.20 g) was recrystallized from dilute alcohol and had m.p. 126°.

$C_{16}H_{18}O_6$	Calc.	C	62.73	H	5.93
	Found	»	62.65	»	5.92

Treatment with HCl: $C_{12}H_{14}O_4$ (0.1 g) was heated with a solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid on a water bath for 30 minutes. A light yellow substance had separated which after recrystallization from alcohol had m.p. 126°.

$C_8H_{10}N_4O_4$	Calc.	N	23.53
	Found	»	23.59

Mixed melting point with 2,4-dinitrophenylhydrazone of acetone showed no depression.

Ring closure of the hydrogenated product II

II (1.2 g) was dissolved in acetyl chloride (28 ml) and refluxed for one hour. After cooling to room temperature the reaction mixture was poured on ice and the separated product recrystallized twice from dilute methanol. M.p. 112—113°.

$C_{12}H_{10}O_2$	Calc.	C	77.38	H	5.43	M	186.2
	Found	»	77.50	»	5.33	»	182

Ring closure of the hydrogenated product III

III (0.24 g) was dissolved in cold concentrated sulphuric acid (30 ml) and the solution after a few minutes poured into cold water. The white amorphous precipitate (0.18 g) was recrystallized twice from alcohol. M.p. 247—248°.

$C_{16}H_{14}O_4$	Calc.	C	71.08	H	5.23
	Found	»	70.82	»	5.23

Dinitrophenylhydrazone: The derivative was prepared in the usual way by heating the carbonyl compound with 2,4-dinitrophenylhydrazine dissolved in 2 *N* hydrochloric acid. Recrystallized from nitrobenzene it had m.p. 250° (dec.)

$C_{28}H_{22}N_8O_{10}$	Calc.	C	53.45	H	3.49	N	17.78
	Found	»	54.09	»	3.61	»	17.24

Acetylacetone and *p*-benzoquinone in presence of anhydrous zinc chloride

The reaction was carried out as described by Bernatek [2]. The alkali-insoluble part of the reaction mixture was recrystallized twice from glacial acetic acid. M.p. 248°. Mixed melting point with $C_{16}H_{14}O_4$ from the above experiment showed no depression.

Ethyl acetoacetate and *p*-benzoquinone in presence of anhydrous zinc chloride

The reaction was carried out as described by v. Pechmann [3] and Ikuta [4]. The slightly soluble benzodifuran derivative (VIII) was isolated from the reaction mixture and treated with dilute aqueous sodium hydroxide in order to remove any hydroxybenzofuran. The reaction product which was a diester was hydrolysed by refluxing for two hours with alcoholic potassium hydroxide (10 %), the separated potassium salt being collected and dried.

Decarboxylation: The dry potassium salt was heated in a wide test tube over a luminous flame. The decomposition took readily place and a substance distilled off, condensed and crystallized in the upper part of the test tube. Recrystallized from dilute methanol and treated with charcoal this substance had m.p. 113°.

$C_{12}H_{10}O_2$	Calc.	C	77.38	H	5.43
	Found	»	77.29	»	5.43

Mixed melting point with $C_{12}H_{10}O_2$ (obtained from the ring closure of II) showed no depression.

The methyl ketone V and methyl magnesium iodide

To a solution of methyl magnesium iodide (from 0.2 g of magnesium) in dry ether was added the methyl ketone (1.1 g). After the vigorous reaction had subsided the reaction mixture was refluxed for 15 minutes and decomposed by ice and dilute hydrochloric acid. The ethereal layer was dried and evaporated the residue weighing 1.0 g. Recrystallized several times from dilute methanol, treated with charcoal and finally recrystallized twice from carbon tetrachloride it had m.p. 138—139° (dec.)

$C_{18}H_{22}O_4$	Calc.	C	71.48	H	7.28
	Found	»	71.17	»	7.27

The tertiary alcohol VII thus formed was sublimated *in vacuo* at 100° (bath temperature). It evidently split off water and the sublimated product had m.p. 110—111°. Mixed melting point with the reaction product from the next experiment showed no depression.

v. Pechmanns ester and methyl magnesium iodide

To a solution of methyl magnesium iodide (from 0.2 g of magnesium) in dry ether (10 ml) was added a suspension of the ester (0.6 g) in dry toluene (10 ml). The mixture was refluxed for 30 minutes and decomposed and worked up as usual. The reaction product was sublimated *in vacuo* and recrystallized several times from acetone-water. M.p. 112°.

$C_{18}H_{18}O_2$	Calc.	C	81.20	H	6.76
	Found	»	81.02	»	6.89

SUMMARY

$\omega,\omega,\omega',\omega'$-Tetraacetyl-*p*-xyloquinone has been found to result from a base-catalyzed reaction between acetylacetone and *p*-benzoquinone. Dependent upon the working conditions the quinone could be hydrogenated with or without loss of acetyl groups. The corresponding hydroquinones easily lost two molecules of water obviously undergoing a ring closure thus being transformed into benzodifuran derivatives. These benzodifuranes have been connected with substances of known structure.

The authors' thanks are due to Professor Dr. Endre Berner for helpful advice and for his interest in the work. One of us (E.B.) also wants to thank *Grosserer Alf Bjerckes legat* for a grant.

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The Preparation of S-Alkylthiuronium Picrates and a New Method for the Estimation of *tertiary* Alcohols

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In a recent paper one of us (L.S. [1]) published an investigation of S-alkylthiuronium picrates and styphnates and found in accordance with Wild [2] that tertiary alkyl halides behave anomalously when refluxed with thiourea in ethanolic or methanolic solution, S-ethyl or -methyl thiuronium picrate being formed instead of S-*tertiary* alkylthiuronium picrate when the reaction mixture is treated with picric acid. This is probably due to a rapid reaction between the tertiary alkyl halide and the solvent by a S_1-mechanism, proceeding more rapidly than the reaction between the tertiary alkyl halide and thiourea and yielding a tertiary alcohol and the alkyl halide corresponding to the solvent alcohol, which then reacts with thiourea giving the primary alkyl derivative. Using the corresponding tertiary alcohols as solvents, however, the preparation of S-*tertiary* alkylthiuronium picrates was successful [1].

At the same time the other of us (S.V.) investigated the possibility of characterising tertiary alcohols as S-alkylthiuronium picrates, converting the tertiary alcohols into halides by shaking 1 vol. of the alcohol with 5 vol. of concentrated hydrochloric acid or constant boiling hydrobromic acid for some minutes, separating the non-aqueous layer and using it as alkyl halide. When preliminary experiments proved successful we agreed upon a common investigation of the reaction, the main difference between the techniques used by us being that one of us was using absolute ethanol or methanol, the other 35—80 % ethanol as a solvent, 35 % ethanol being used for the lower tertiary alkyl halides, 65 % or 80 % ethanol for the higher members of this series.

The general procedure used now has been: 1.52 g (0.02 mole) of thiourea are dissolved in 4 ml of water. 3 ml of ethanol and 0.02 mole of alkyl halide are added and the mixture is refluxed on the steam bath until the alkyl halide-layer has disappeared and 15 min. further (usually 2—3 hours); (*cf.* the preparation of S-benzylthiuronium chloride, Veibel and Lillelund [3].

In some instances it was not possible to reach the point where the non-aqueous layer disappeared completely, possibly because the alcohol had not been completely converted into alkyl halide. In such cases the refluxing was continued for 5 hours and then interrupted.

The refluxed solution is poured into 200—300 ml of a 1 % aqueous solution of picric acid. A precipitate of the S-alkylthiuronium picrate was formed instantaneously. After ½ hour the precipitate was isolated by suction and recrystallised from ethanol or dilute ethanol. Usually one recrystallisation suffices for getting analytically pure picrates.

Table 1 gives a summary of the alcohols examined. In the table is indicated when standard procedure has been followed and when more ethanol than the 3 ml prescribed for the standard procedure has been used. Besides, the table indicates the time of refluxing and the yield obtained.

In other experiments the possibility of substituting 4 ml of concentrated hydrochloric acid for the 4 ml of water prescribed for the standard procedure and 0.02 mole of the carbinol for 0.02 mole of the alkyl halide was examined.

Table 1. Preparation of S-alkylthiuronium picrates from tertiary alcohols.

Alcohol	Procedure	Time of reflux min.	Yield %	Radical name
Trimethylmethanol 2-Methylpropanol-2	Standard	45	48	*Tert.* butyl
Dimethyl-ethyl-methanol 2-Methylbutanol-2	Standard	90	43	1,1-Dimethylpropyl
Methyl-diethyl-methanol 3-Methylpentanol-3	Standard	160	30	1-Methyl-1-ethyl-propyl
Triethylmethanol 3-Ethylpentanol-3	Standard	120	22	1,1-Diethylpropyl
Methyl-ethyl-propyl-methanol	20 ml ethanol	150	11	1-Methyl-1-ethyl-butyl
3-Methylhexanol-3	20 ml ethanol	240	16	
Dimethyl-butyl-methanol 2-Methylhexanol-2	20 ml ethanol	180	19[1]	1,1-Dimethylpentyl
Dimethyl-*tert.*butyl-methanol 2,3,3-Trimethylbutanol-2	10 ml ethanol	300	20[2]	1,1,2,2-tetramethyl-propyl
Methyl-ethyl-*iso*propyl-methanol	20 ml ethanol	300	15[3]	1-Methyl-1-*iso*propyl-propyl
2,3-Dimethylpentanol-3	standard	240	5[4]	
Methyl-ethyl-phenyl-methanol 2-Phenylbutanol-2	No reaction neither as chloride nor as bromide			1-Methyl-1-phenyl-propyl
Methyl-ethyl-benzyl-methanol 1-Phenyl-2-methyl-butanol-2	No reaction			1-Methyl-1-benzyl-propyl
Ethyl-diphenyl-methanol 1,1-Diphenylpropanol-1	No reaction			1,1-Diphenylpropyl
Triphenylmethanol	No reaction			Triphenylmethyl

[1] Contaminated with traces of S-ethylthiuronium picrate

[2] The alcohol shaken with constant boiling hydrobromic acid instead of concentrated hydrochloric acid

[3] Contaminated with considerable amounts of S-ethylthiuronium picrate

[4] Not contaminated with S-ethylthiuronium picrate

This procedure had to be abandoned, as the S-alkylthiuronium picrates isolated were strongly contaminated with picric acid.

As alkyl iodides react more rapidly than alkyl chlorides it was tried to add potassium iodide to the reaction mixture. This procedure, too, was abandoned as the precipitate obtained consisted mainly of potassium picrate. Sodium iodide is presumably preferable to potassium iodide, sodium picrate being much more soluble in ethanol and water than potassium picrate.

In Table 2 melting points of and nitrogen-determination in picrates not previously described are reported. All melting points are determined by plunging capillary tubes into a bath previously heated to some degrees under the m.p. All m.p.'s are corrected.

The above mentioned assumption about the reaction between a tertiary alkyl halide and the solvent, ethanol, seems to be corroborated by the appearance of S-ethylthiuronium picrate as a contamination of the S-*tertiary* alkylthiuronium picrate, when 80 % ethanol was used as a solvent instead of 35 % ethanol. The formation of the ethyl derivative is of course most probable in the more concentrated ethanol solution, but it is also noteworthy that a decrease in dielectric constant will considerably retard the rate of the reaction between the tertiary alkyl ion and thiourea (S_1-reaction) but somewhat accelerate the S_2-reaction between ethyl chloride, rapidly formed from the halide and the solvent, and thiourea. S-Ethylthiuronium picrate is thus obtained as a by-product in the case with lower dielectric constant, *i. e.* with 80 % ethanol as a solvent (Table 1).

Table 2. Melting points of and nitrogen determination in some S-tertiary alkylthiuronium picrates.

Radical name	M.p.	% N	
		found	calculated
1-Methyl-1-ethyl-propyl	127°	18.17	17.98
1,1-Diethyl-propyl	113°	17.50	17.37
1-Methyl-1-ethyl-butyl	124°	17.38	17.37
1,1-Dimethyl-pentyl	108°	17.18	17.37
1,1,2,2-Tetramethyl-propyl	125°	17.39	17.37
1-Methyl-1-*iso*propyl-propyl	122°	17.18	17.37

For the formation of S-*primary* alkylthiuronium picrates a S_2-reaction, *i. e.* a second order substitution reaction, was assumed responsible. Its velocity is no doubt dependent on the proportion of thiourea present in the *iso*-form and this, on the other hand, is dependent on the solvent used, solvents with a high dielectric constant favouring the *iso*-form. A corroboration of this assumption was obtained by comparing the yield obtained when *n*-butyl chloride was refluxed with thiourea, one time using the standard procedure and the other time using 80 % ethanol as a solvent (Table 3).

Table 3. Comparison of the yield of the reaction between n-butyl chloride and thiourea in 35 % ethanol and in 80 % ethanol.

Time of refluxing in minutes		Yield in %	
35 % ethanol	80 % ethanol	35 % ethanol	80 % ethanol
	30		3
	60		7
	120		12
150		15	
	180		18
210		30	
	360		29 [1]

[1] Contaminated with S-ethylthiuronium picrate.

It is seen that the velocity is somewhat greater in 35 % ethanol than in 80 % ethanol and besides, that here, too, the conversion of the alkyl chloride used into ethyl chloride may be observed in 80 % ethanol when the mixture is refluxed for a very long time.

The fact that the 4 last mentioned tertiary alcohols in Table 1 do not react with thiourea may be due to the failure of converting the alcohols into halides by shaking them with concentrated hydrobromic or hydrochloric acid. The reason may also be that the tertiary halides in question do not react with thiourea. For triphenylmethanol we tried to use preformed pure triphenylmethyl chloride instead of with hydrochloric acid treated alcohol, but no S-alkylthiuronium picrate was obtained, even so.

It is, however, well known that triphenylmethyl chloride will react easily with alcohols, forming triphenylmethyl ethers. This can possibly explain the failing reaction with thiourea. But the problem can also be discussed from another point of view and an explanation of the result given by comparison with the behaviour of triphenylmethane thiol. This thiol, which contains a C—S-linkage of the same kind as the one in the thiourea derivative wanted, splits easily away the mercapto group, forming triphenylcarbenium ions. In water solution thus triphenylmethanol and hydrogen sulphide are formed. The conclusion is of course that aryl groups strongly weaken the C—S-linkage so that, applied to the reaction between triphenylmethyl chloride and thiourea, even if a formation of the arylsubstituted alkylthiuronium halide takes place by a S_1-mechanism, such a substance may by prolonged heating be converted into the corresponding alcohol or its ethyl ether. Thus the thiourea derivative cannot be isolated.

In the above mentioned paper [1] one of us drew attention to the variation of the melting points of the S-alkylthiuronium picrates and styphnates with the number of carbon atoms. We have now supplemented this material with the picrates described in this paper, and from Table 4 can be seen that also the newly prepared picrates fit well into the regularity found previously but, besides, that the melting points are dependent on the symmetry of the carbon chain.

Table 4. Melting points of S-alkylthiuronium picrates.

Radical	M.p.	Radical	M.p.
CH_3-	224°	4 C-group	
2 C-group		$CH_3-CH_2-CH_2-CH_2-$	177°
CH_3-CH_2-	188°	$CH_3-CH_2-CH_2-CH(CH_3)-$	155°
$CH_3-CH(CH_3)-$	196°	$CH_3-CH(CH_3)-CH_2-CH_2-$	173°
$CH_3-C(CH_3)_2-$	151°	$CH_3-CH_2-CH_2-C(CH_3)_2-$	124°
3 C-group		5 C-group	
$CH_3-CH_2-CH_2-$	177°	$CH_3-CH_2-CH_2-CH_2-CH_2-$	155°
$CH_3-CH_2-CH(CH_3)-$	166°	$CH_3-CH(CH_3)-CH_2-CH_2-CH_2-$	147°
$CH_3-CH_2-CH(CH_2-CH_3)-$	159°	$CH_3-CH_2-CH_2-CH_2-C(CH_3)_2-$	103°
$CH_3-CH(CH_3)-CH_2-$	167°	6 C	
$CH_3-CH_2-C(CH_3)_2-$	131°	$CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-$	157°
$CH_3-CH_2-C(CH_3)(CH_2-CH_3)-$	127°	7 C	
$CH_3-CH_2-C(CH_2-CH_3)_2-$	113°	$CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$	143°
$CH_3-CH(CH_3)-C(CH_3)(CH_2-CH_3)-$	122°	8 C	
$CH_3-C(CH_3)_2-C(CH_3)_2-$	125°	$CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$	134°

The S-alkylthiuronium picrates are not only well fitted as derivatives for characterisation of tertiary alcohols, they are, too, useful for the estimation of these alcohols, which till now have been difficult to estimate, due to the difficulty with which they are acetylated or converted into derivatives containing easily estimatable groups.

It is well known that salts of not too strong acids behave as bases when they are dissolved in glacial acetic acid and titrated with a standard solution of perchloric acid in glacial acetic acid (see *e. g.* Markunas and Riddick [4]). Berger [5] has recently shown that S-benzylthiuronium salts of carboxylic acids may be estimated in this way. The S-alkylthiuronium picrates, too, can be titrated with perchloric acid. The titration can be carried out electrometrically or with crystal violet as an indicator. The picrate ion may also be used as an indicator, the alkylthiuronium picrates dissolving in glacial acetic

Table 5. Equivalent weights of some S-alkylthiuronium picrates.

Alcohol	E_{salt}		$E_{alcohol}$	
	found	calc.	found	calc.
n-Butanol	359.4	361.3	72.2	74.1
Trimethylmethanol	359.9	361.3	72.7	74.1
Dimethyl-ethyl-methanol	374.6	375.4	87.4	88.2
Methyl-diethyl-methanol	387.9	389.4	100.7	102.2
Triethylmethanol	402.4	403.4	115.2	116.2
Methyl-ethyl-propyl-methanol	401.4	403.4	114.2	116.2
Dimethyl-butyl-methanol	402.7	403.4	115.5	116.2
Dimethyl-*tert.*butyl-methanol	401.7	403.4	114.5	116.2
Methyl-ethyl-*iso*propyl-methanol	405.2	403.4	118.0	116.2

acid with an intense yellow colour which during the titration fades, and when the equivalent amount of perchloric acid has been added a colourless solution results; picric acid dissolving in glacial acetic acid without colour. Table 5 shows some results found for the equivalent weight of some S-alkylthiuronium picrates. From these values the equivalent weight of the alcohols may be found by subtracting 287.2.

It is seen that the number of carbon atoms in the alcohol is determined with sufficient exactitude.

For preparative purposes, too, the S-*tertiary* alkylthiuronium derivatives are useful. It is well known that a convenient method for preparing thiols consists in preparing S-alkylthiuronium salts and treating these compounds with sodium hydroxide. Through the S-*tertiary* alkylthiuronium salts, now investigated, tertiary alkane thiols thus become readily available.

SUMMARY

S-*tert.* Alkylthiuronium picrates have been investigated and a method for their preparation is indicated.

The mechanism of the reaction is discussed in order to find an explanation of the fact that the preparation of S-*tert.* alkylthiuronium salts is possible when aqueous ethanol is used as a solvent, but not when the solvent is absolute ethanol.

A method for the estimation of tertiary alcohols by use of S-*tert.* alkylthiuronium picrates is described.

Attention is drawn to the possibility of preparing tertiary alkane thiols from the S-*tert.* alkylthiuronium salts.

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Über eine Beziehung zwischen der Tollens'schen und der Formaldehyd-Olefin-Reaktion. Die Umsetzung von Formaldehyd mit Propionaldehyd und Cyclohexanon *

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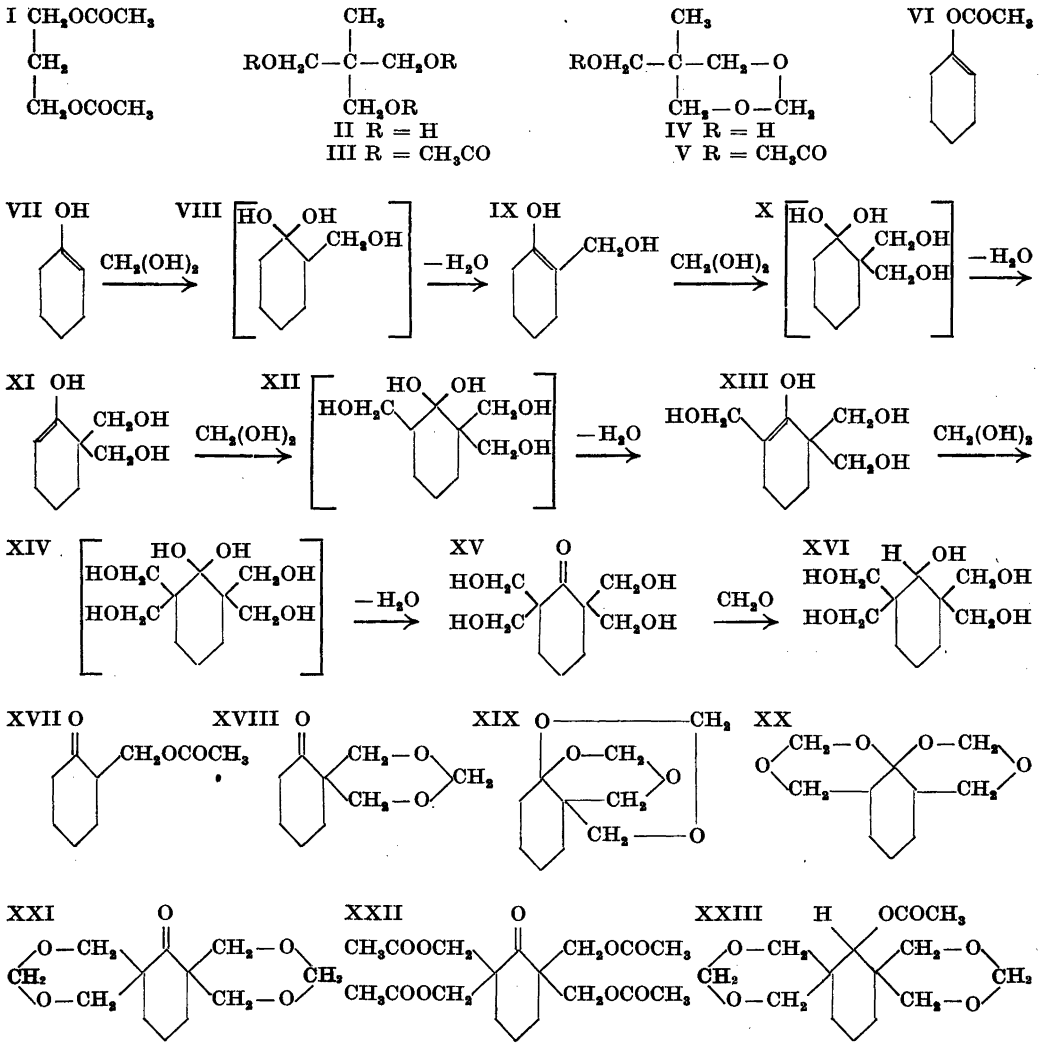
Bei der Umsetzung von Formaldehyd mit Äthylen in Eisessig-Schwefelsäure entstand, wie wir früher zeigten, neben dem normalen Reaktionsprodukt *Trimethylenglykol-diacetat* (I) als anomales Reaktionsprodukt das *Pentaglycerin-triacetat* (III). Dessen Bildung wurde von uns damals durch die Hypothese gedeutet, dass sich aus dem normalen Primärprodukt zunächst Propionaldehyd bildet, der dann im Sinne einer Tollens-Reaktion mit Formaldehyd weiterreagiert [1]. Während jedoch der Übergang des Trimethylenglykols in Propionaldehyd experimentell belegt ist [2], war eine Tollens-Reaktion bis zu diesem Zeitpunkt in saurem Medium nicht beobachtet worden. Baker, dem unsere Arbeit aus dem Jahre 1946 nicht bekannt war, vermochte zunächst die Natur des anomalen Reaktionsproduktes nicht zu deuten [3]; er schloss sich aber später unserer Auffassung über dessen Konstitution an [4].

In Verbindung mit der vorliegenden Reaktionsanomalie haben wir schon in den Jahren 1944/45 die Frage behandelt, ob enolisierbare Carbonylverbindungen — dank ihrer Enoldoppelbindung — mit Formaldehyd in saurem Medium den Olefinen bzw. deren Derivaten wesensgleich reagieren, d.h. einem »Glykolaufbau» durch Formaldehyd zugänglich sind. Als Untersuchungsobjekt schien uns das Cyclohexanon bzw. dessen leicht zugängliches *Enolacetat* (VI) besonders geeignet. Wir dachten dabei — ähnlich wie für die Formaldehyd-Olefin-Reaktion gezeigt [5] — an einen stufenweisen Aufbau in dem Sinne, dass sich an die *Enolform des Cyclohexanons* (VII) ** Formaldehydhydrat unter Bildung des unbeständigen *2-Methylol-cyclohexanon-hydrates* (VIII) anlagert, das unter Wasserabspaltung sofort in die *Enolform* (IX) übergeht, die ihrer-

* Vorgetragen auf der 8. Nordischen Chemikertagung in Oslo am 15.6.1953. XV. Mitt. über Formaldehyd-Olefin-Reaktionen.

** In den Formeln VII bis XIV sind alle Carbonylformeln in der Enolform und die labilen Hydrate in eckigen Klammern geschrieben. Der Formaldehyd wurde der Übersichtlichkeit halber als Methylenglykol formuliert.

seits wieder einem erneuten Angriff durch Formaldehydhydrat ausgesetzt ist unter Bildung des *2,2-Dimethylol-cyclohexanonhydrates* (X). Dieses Wechselspiel zwischen Wasserabspaltung und Formaldehyd-Aufbau könnte sich im Sinne der Formeln XI bis XV wiederholen, bis beim *2,2,6,6-Tetramethylol-cyclohexanon* (XV) die Reaktion dadurch zum Stillstand kommt, dass in dieser Verbindung keine Möglichkeit zur Enolisierung mehr vorhanden und ein weiterer »Glykolaufbau» unmöglich geworden ist. Vom 2,2,6,6-Tetramethylolcyclohexanon aus wäre lediglich noch der durch Tollens bekannt gewordene und durch Formaldehyd bewirkte Reduktionshub zum *2,2,6,6-Tetramethylol-cyclohexanol* (XVI) denkbar. —



Man erkennt ohne weiteres, dass sich die Interpretation als ein Glykolaufbau an der Enoldoppelbindung in jeder Stufe mit den bekannten Effekten der Tollens'schen Reaktion deckt und für den speziellen Fall des Cyclohexanons zu den gleichen Verbindungen führt, wie sie von Mannich und Brose [6] sowie von Gault und Steckl [7] zum grössten Teil experimentell dargestellt worden sind.

Für einen Reaktionsverlauf in dem hier angedeuteten Sinne scheint die Tatsache zu sprechen, dass sowohl bei der Umsetzung des freien Cyclohexanons als auch des Cyclohexenylacetates [8] mit Formaldehyd in Eisessig-Schwefelsäure ein bisher unbekannter Bismethylenäther der Summenformel $C_{10}H_{16}O_4$ vom Schmp. 87—89° entsteht, der sich zweifellos von der *Hydratform des 2,2-Dimethylol- oder des 2,6-Dimethylol-cyclohexanons* ableitet. Er reagiert nicht mit Carbonylreagenzien und hat wahrscheinlich die Formel XIX oder XX. Ausserdem isolierten wir einen anderen bisher ebenfalls unbekannten *Monomethylenäther des Dimethylol-cyclohexanons* von der Summenformel $C_9H_{14}O_3$ und dem Schmp. 39—40°. Diese Verbindung gibt ein 2,4-Dinitrophenylhydrazon und ein Semicarbazon und hat daher wahrscheinlich die Formel XVIII. Beide Methylenäther entstanden auch bei Versuchen, in denen Cyclohexanon und Formaldehyd im Molverhältnis ca. 1 : 1 umgesetzt wurden. Dieses berechtigt zu dem Schluss, dass Methylolderivate des Cyclohexanons mit Formaldehyd rascher reagieren als das Cyclohexanon selbst, was auch mit der Tatsache übereinstimmt, dass in diesen Fällen reichliche Mengen Cyclohexanon zurückgewonnen wurden. — Da uns bei dieser Untersuchung die Darstellung und Isolierung weiterer Reaktionsprodukte unwesentlich erschien und es uns nur darauf ankam, die Reaktionsfähigkeit von Carbonylverbindungen gegenüber Formaldehyd in saurem Medium bündig darzutun, haben wir in einem weiteren Versuch das Cyclohexanon unter sonst gleichen Bedingungen mit einem grossen Überschuss an Formaldehyd (Molverhältnis 1 : 10) umgesetzt, um dadurch gleich zu dem von Mannich und Brose beschriebenen *Bismethylenäther-acetat* (XXIII) vorzustossen. Wie sich zeigte, machte die Reaktion jedoch nach Entstehung des von den gleichen Autoren beschriebenen *Bismethylenäthers des 2,2,6,6-Tetramethylol-cyclohexanons* (XXI) halt. Eine Reduktion der Carbonylgruppe erfolgte also unter diesen Bedingungen nicht. Zwecks Identifizierung wurde der Bismethylenäther in das bekannte *Tetraacetat* (XXII) übergeführt.

Bei Versuchen, die genannten Methylenäther des Di- und Tetramethylolcyclohexanons durch Erwärmen mit Mineralsäuren in die zugrunde liegenden Alkohole zu verwandeln, fiel uns auf, dass hierbei mehr Formaldehyd abgespalten wurde, als dem als Methylenäther gebundenen Formaldehyd entspricht. Beim Destillieren des Bismethylenäthers des 2,2,6,6-Tetramethylolcyclohexanons mit 20-prozentiger Salzsäure unter Zusatz von etwas Aluminiumchlorid wurden im Destillat 4,45 Mol Formaldehyd per Mol Bismethylenäther mit Dimedon nachgewiesen. Wir haben aus diesem Grunde eine kleine Menge des 2,2,6,6-Tetramethylol-cyclohexanons nach Mannich und Brose dargestellt und mit Salzsäure destilliert. Auch in diesem Falle konnte im Destillat eine reichliche Menge Formaldehyd als 2,4-Dinitro-phenylhydrazon gefällt werden, ohne dass es bei der geringen Substanzmenge möglich war, andere Spaltstücke (Cyclohexanon oder dessen einfachere Methylolderivate) zu fas-

sen. — Freudenberg hat im Zusammenhange mit Ligninstudien neben zahlreichen anderen Modellsubstanzen auch aromatisch substituierte β-Ketonalkohole bzw. deren Äther oder Ester der Destillation mit starker Schwefelsäure unterworfen und dabei z.T. erhebliche Mengen Formaldehyd erhalten [9]. Die vorliegenden Versuche zeigen, dass die Eigenschaft, unter den erwähnten Bedingungen Formaldehyd abzuspalten, nicht auf aromatische Systeme beschränkt ist, sondern auch anderen mehrwertigen β-Keton-alkoholen gemeinsam zu sein scheint.

Die Richtigkeit der eingangs erwähnten Annahme über die anomale Bildung des Pentaglycerin-triacetates, die neuerdings auch von Prins [10] am Acetaldehyd geprüft und wahrscheinlich gemacht worden ist, fand ihre endgültige Bestätigung durch das nun vorliegende Untersuchungsergebnis über die Umsetzung des Propionaldehyds mit Formaldehyd in Eisessig-Schwefelsäure: Wie gefunden wurde, entsteht dabei tatsächlich das *Pentaglycerin-triacetat* bzw. dessen *Methylenäther-derivat* (V). Sieht man davon ab, dass sich unter den vorliegenden Bedingungen Acetate bilden, lässt sich die Reaktion — wie beim Cyclohexanon ausgeführt — auch hier zwanglos als ein »Glykolaufbau» an der Enoldoppelbindung formulieren. Auffallend ist, dass bei Aldehyden im Anschluss an den vollständigen Methylolaufbau die Carbonylgruppe auch in saurem Medium zur Alkoholgruppe reduziert wird, während dieses nach unseren bisherigen Erfahrungen bei Ketonen nicht der Fall ist.

EXPERIMENTELLER TEIL

Unter Mitarbeit von Maria-Magdalena Schatz [8] und Gustav Havre

Umsetzung des Cyclohexanons mit Formaldehyd

I. Molverhältnis ~ 1 : 4.

In Anlehnung an den früher ausgeführten Versuch (Molverhältnis ca. 1 : 1) [8] wurde eine Mischung aus 121 g Paraformaldehyd, 100 g Eisessig und 5 ml konz. Schwefelsäure bei einer Anfangstemperatur von 37° unter mechanischem Rühren portionsweise mit einer Lösung von 100 g Cyclohexanon in 100 ml Eisessig versetzt. Nachdem die Temperatur spontan auf 72° gestiegen war, wurde von aussen gekühlt. Das braune Reaktionsgemisch wurde in Äther aufgenommen und die Ätherlösung mit gesättigter Sodalösung in üblicher Weise neutral gewaschen und über Natriumsulfat getrocknet. Nach Entfernen des Äthers erhielt man bei der Destillation als Hauptfraktion ein farbloses Öl vom Sdp. $_{12}$ 132—134°, das bald teilweise krystallisierte. Die Krystalle wurden durch Absaugen vom Öl *A* befreit und mehrmals aus Alkohol umkrystallisiert. Ausbeute 16 g. Schmp. 87—89°.

Bismethylenäther des Dimethylol-cyclohexanonhydrates (XIX bzw. XX)

$C_{10}H_{16}O_4$ (200,2)	Ber.	C 59,98	H 8,05	
	Gef.	» 59,99	» 8,10	

Aus dem Öl *A* schieden sich beim Stehen in der Kälte grosse farblose Krystalle aus. Diese wurden mehrmals aus Alkohol umkrystallisiert: Schmp. 39—40°. *Methylenäther des 2,2-Dimethylol-cyclohexanons* (XVIII)

$C_9H_{14}O_3$ (170,2)	Ber.	C 63,53	H 8,23	
	Gef.	» 63,53	» 8,26	

2,4-Dinitro-phenylhydrazon aus Methanol Schmp. 198—200°

$C_{15}H_{18}O_6N_4$ (336,3)	Ber.	C 51,45	H 5,18	N 16,00
	Gef.	» 51,48	» 5,25	» 16,37

Semicarbazon aus Methanol Schmp. 235—237,5°

$C_{10}H_{17}O_3N_3$ (217,3)	Ber.	C 52,84	H 7,54	N 18,49
	Gef.	» 52,99	» 7,57	» 19,08

II. Molverhältnis ~ 1 : 10.

Eine Mischung aus 30 g Paraformaldehyd, 40 ml Eisessig, 1 ml konz. Schwefelsäure und 10 ml Cyclohexanon wurde unter Rückflusskühlung und gelegentlichem Umschwenken des Kolbens allmählich zum Sieden erhitzt und einige Minuten gekocht. Nach dem Erkalten schieden sich Krystalle aus, die abgesaugt und aus Alkohol umkrystallisiert wurden. Ausbeute 8 g. Schmp. 151° (Mannich u. Brose geben den Schmp. 150° (korr.) an).

Bismethylenäther des 2,2,6,6-Tetramethylol-cyclohexanons (XXI)

$C_{12}H_{18}O_5$ (242,3) Ber. C 59,49 H 7,49
Gef. » 59,55 » 7,48

Überführung in das Tetra-acetat (XXII). Eine Probe des Bismethylenäthers wurde mit überschüssigem Essigsäureanhydrid und einigen Tropfen konz. Schwefelsäure mehrmals abgedampft. Der Rückstand wurde in Äther aufgenommen und die Ätherlösung neutral gewaschen. Die beim Verdampfen des Äthers hinterbleibende krystalline Substanz wurde mehrmals aus Aceton umkrystallisiert. Farblose Krystalle vom Schmp. 140° (in Übereinstimmung mit den Angaben von Mannich und Brose)

$C_{18}H_{26}O_9$ (386,4) Ber. C 55,95 H 6,78
Gef. » 55,84 » 6,73

Abspaltung von Formaldehyd aus 2,2,6,6-Tetramethylol-cyclohexanon. 1,1 g 2,2,6,6-Tetramethylol-cyclohexanon (dargestellt nach Mannich und Brose) wurde mit 250 ml 20 %-iger Salzsäure destilliert. Es wurden 200 ml Destillat aufgefangen. Das trübe Destillat wurde ausgeäthert. Wässrige Flüssigkeit *A*. Nach Verdampfen des Äthers hinterblieb eine geringe Menge Öl von cyclohexanonähnlichem Geruch, das mit salzsaurem 2,4-Dinitrophenylhydrazin einen Niederschlag gab. Dessen Menge reichte nicht zur weiteren Untersuchung. Die wässrige Flüssigkeit *A* gab nach Zusatz von salzsaurem 2,4-Dinitro-phenylhydrazin einen kräftigen Niederschlag des Formaldehyd-2,4-dinitrophenylhydrazons, das durch Schmelz- und Mischschmelzpunkt identifiziert wurde.

In einem anderen Versuch wurde der *Bismethylenäther des 2,2,6,6-Tetramethylol-cyclohexanons* in analoger Weise mit Salzsäure unter Zusatz von etwas Aluminiumchlorid destilliert und der abgespaltene Formaldehyd im Destillat mit Dimedon gefällt. Der Niederschlag wurde abgesaugt und nach dem Trocknen gewogen. Wie die Rechnung ergab, wurden bei dem Versuch 4,45 Mol Formaldehyd per Mol Bismethylenäther abgespalten.

Umsetzung des Cyclohexenyl-acetates mit Formaldehyd. Zu einer Lösung von 40.8 g Paraformaldehyd und 0,5 ml konz. Schwefelsäure in 270 ml Eisessig liess man bei einer Anfangstemperatur von 38° allmählich 136 g Cyclohexenylacetat unter mechanischem Rühren hinzutropfen. Im Verlaufe von 1 Stunde stieg die Temperatur auf 65°, und die Flüssigkeit färbte sich dunkel. Das Reaktionsgemisch wurde in Äther aufgenommen und die Ätherlösung in üblicher Weise gewaschen und getrocknet. Bei der Destillation erhielt man neben unverändertem Cyclohexenyl-acetat und einigen geringfügigen Zwischenfraktionen (2-Acetoxymethyl-cyclohexanon (XVII) ?) ca. 9 g eines farblosen Öles, das im wesentlichen beim Sdp.$_{12}$ 132° überging und nach dem Erkalten zum Teil krystallisierte. Die krystalline Substanz erwies sich als identisch mit dem aus Cyclohexanon erhaltenen *Bismethylenäther des Dimethylol-cyclohexanonhydrates* (XIX bzw. XX).

Umsetzung des Propionaldehyds mit Formaldehyd. Eine Mischung von 104 g Paraformaldehyd, 350 g Eisessig und 10 ml konz. Schwefelsäure wurde bis zur völligen Lösung des Paraformaldehyds erhitzt und bei 80° mit 40 g Propionaldehyd versetzt. Es trat sofort Reaktion unter Dunkelfärbung und Temperaturanstieg auf 102° ein. Im Anschluss wurde eine Zeitlang unter Rückflusskühlung gekocht, bis die Temperatur bei 111° konstant blieb. Das Reaktionsgemisch wurde in Äther aufgenommen und die Ätherlösung neutral gewaschen. Nach dem Entfernen des Äthers erhielt man 97,3 g Öl. Dieses gab bei der Destillation zwei Fraktionen (unbedeutende Zwischenläufe nicht mitgerechnet): 1) Sdp.$_9$ 93°, 57,8 g, farbloses Öl, V.Z. 261, $n_D^{22} = 1{,}4439$, $D_4^{22} = 1{,}1002$; 2) Sdp.$_9$ 148–159° 4 g, farbl. Öl.

Die Fraktion 1) gibt beim Erwärmen mit einer Lösung von 2,4-Dinitro-phenylhydrazin in verd. Salzsäure einen gelben Niederschlag, der sich durch Schmelz- und Mischschmelzpunkt als das 2,4-Dinitrophenylhydrazon des Formaldehyds erwies. — Bei der Umesterung mit methylalkoholischer Salzsäure erhielt man *Pentaglycerin* (II) vom Schmp. 200–201,5°

$C_5H_{12}O_3$ (120,1) Ber. C 49,98 H 10,00
Gef. » 49,94 » 10,12

und wahrscheinlich den unreinen *Methylenäther-alkohol* (IV) als Öl. Bei der Fraktion 1) handelt es sich daher um das

Methylenäther-acetat des Pentaglycerins (5-Methyl-5-acetoxymethyl-1,3-dioxan) (V) MR (ber.) 41,88, (gef.) 42,05. V.Z. (ber.) 322.

Die Fraktion 2) besteht aus unreinem *Pentaglycerin-triacetat* (III). Sie wurde durch Umesterung in das freie *Pentaglycerin* übergeführt.

ZUSAMMENFASSUNG

An den Beispielen Propionaldehyd und Cyclohexanon bzw. Cyclohexenylacetat wird gezeigt, dass Carbonylverbindungen auf Grund ihrer Enoldoppelbindung — wie die Olefine — einem »Glykolaufbau» durch Formaldehyd in saurem Medium zugänglich sind. In Eisessig-Schwefelsäure entstand aus Propionaldehyd das Pentaglycerin-triacetat und dessen Methylenäther-derivat, aus Cyclohexanon bildeten sich zwei unbekannte und ein bekannter Methylenäther des Di- bzw. Tetramethylol-cyclohexanons. — Die Bildung des Pentaglycerin-triacetates bei dem früheren Äthylenversuch erklärt sich nun als logische Konsequenz des der Formaldehyd-Olefin-Reaktion zugrunde liegenden Aufbauprinzips. — Andererseits erscheint nach diesen Befunden die sog. Tollens-Reaktion nicht mehr als selbständiger Reaktionstypus, sondern als ein spezieller Zweig der Formaldehyd-Olefin-Reaktion.

Dem *Fridtjof Nansen-Fond* dankt man für finanzielle Unterstützung.

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*iso*Thiocyanates VI. A Synthesis of Cheiroline (γ-Methylsulphonylpropyl *iso*Thiocyanate)

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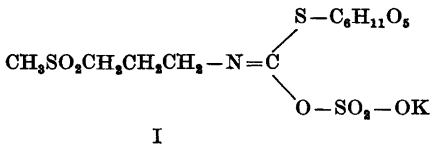
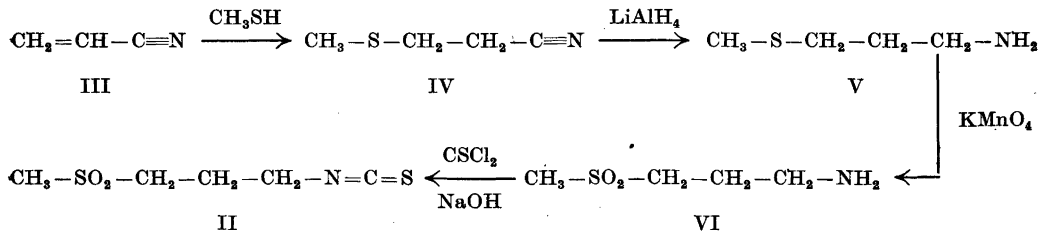
From seeds of the wall-flower (*Cheiranthus cheiri* L.) Schneider and Schütz [1] isolated a crystalline *iso*thiocyanate glucoside (I), named glucocheiroline. The aglucone, cheiroline, had been shown in an earlier communication by Schneider [2] to possess the remarkable structure (II) which was proved by degradation and confirmed by synthesis. The latter proceeded through several steps, many of which gave rather poor yields and involved a great number of troublesome and time-consuming operations.



I

II

In the course of our studies of naturally occurring *iso*thiocyanates a need arose for a supply of cheiroline (II). Here we wish to report our preparative route to cheiroline which we believe represents an improvement over Schneider's synthesis. Contrary to the latter the present synthesis proceeds from readily accessible starting materials in few steps, all of which give satisfactory yields. The reactions involved are outlined in the following scheme.



Following the directions by Hurd and Gershbein [3], methanethiol was added to acrylonitrile (III) to give β-methylthiopropionitrile (IV) in 90 % yield. The latter was smoothly reduced with lithium aluminium hydride in ethereal solution to γ-methylthiopropylamine (V) in *ca.* 70 % yield. To our knowledge, this reaction represents the first example of reduction with lithium aluminium hydride of a nitrile containing a sulphide-grouping. It is of interest to note that no concomitant fission of the carbon-sulphur linkage was observed, indicating the inertness of the reagent towards the sulphide-grouping, which is known to be easily hydrogenolysed by other reducing reagents, such as Raney nickel. Furthermore, it appeared that satisfactory yields were obtained only when at least one molecular proportion of the hydride was applied. Although this requirement is not easily reconcilable with the stoichiometric equation, the present finding is in accord with the results of Amundsen and Nelson [4]. They showed that only half the hydrogen of lithium aluminium hydride is available for the reduction of nitriles to primary amines. The oxidation of (V) to the sulphoneamine (VI) was performed in 76 % yield, essentially as described by Schneider [2]. For the conversion of the amine (VI) into cheiroline (II) the traditional methods of *iso*thiocyanate syntheses were at disposal. By far the best and most convenient approach, however, proved to be a modification of the method of Dyson *et al.*[5,6]. Whereas their standard procedure involved the simple reaction between thiocarbonyl chloride and the amine, we found it advantageous to add slightly less than two equivalents of sodium hydroxide to the mixture, resulting in a fast reaction as inferred from the disappearance of the red colour of the thiophosgene. Cheiroline (II) was obtained in 73 % yield as a crystalline product, melting only about one degree below a specimen for analysis, prepared by a further recrystallisation from ethyl ether. The pure cheiroline appeared as colourless crystals, m.p. 46—47°, with physical and chemical properties in accord with the statements in Schneider's paper [2].

Cheircline was transformed into its thiourea (VII), m.p. 116—117°, upon reaction with ethanolic ammonia. Again, the derivative possessed properties agreeing well with those reported by Schneider for his N-γ-methylsulphonylpropylthiourea. For further characterisation, cheiroline was brought into reaction with aniline, *p*-toluidine and α-naphthylamine, respectively, to give the corresponding substituted thioureas, (VIII)—(X), (*cf.* Table 1).

Table 1.

Amine	Formula	Composition	M.p., °C	Analyses			
				Nitrogen		Sulphur	
				Calcd.	Found	Calcd.	Found
Ammonia	VII	$C_5H_{12}O_2N_2S_2$	116—117[a]	14.28	14.31	32.66	32.44
Aniline	VIII	$C_{11}H_{16}O_2N_2S_2$	138 [b]	10.29	10.37	23.54	23.38
p-Toluidine	IX	$C_{12}H_{18}O_2N_2S_2$	150	9.78	9.81	22.39	22.20
1-Naphthylamine	X	$C_{15}H_{18}O_2N_2S_2$	182—183	8.69	8.69	19.89	19.85

[a] Ref.[2] reports 116°. [b] Ref.[2] reports 136°.

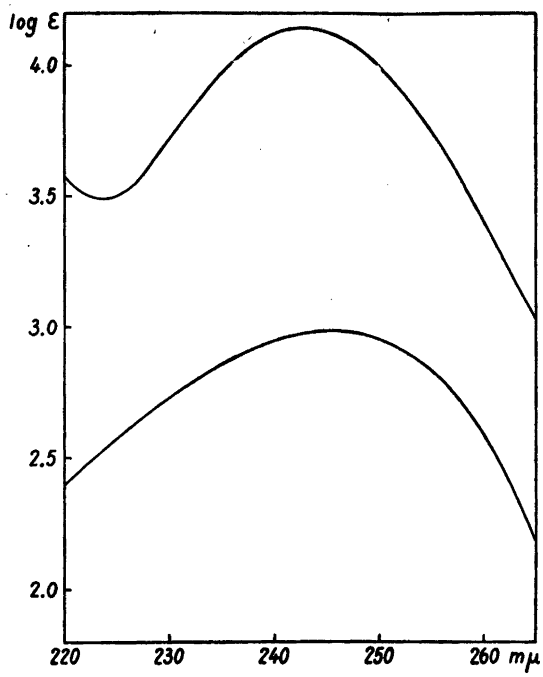


Fig. 1. Ultra-violet absorption spectra, determined in ethanol solutions. Lower curve: cheiroline (II). Upper curve: N-(γ-methylsulphonylpropyl)-thiourea (VII).

$CH_3—SO_2—CH_2—CH_2—CH_2—NH—CS—NH_2$ VII

$CH_3—SO_2—CH_2—CH_2—C\equiv N$ XI

$CH_3—SO_2—CH_2—CH_2—CH_2—NH—CS—NH—R$

VIII, R = C_6H_5
IX, R = p-C_6H_4-CH_3
X, R = α-$C_{10}H_7$

In the preliminary phases of the present work β-methylthiopropionitrile (IV) was oxidised in glacial acetic acid with hydrogen peroxide to the heretofore unknown β-methylsulphonylpropionitrile (XI). Attempts to reduce the nitrile-grouping of (XI) with lithium aluminium hydride failed, presumably because of its slight solubility in ether and tetrahydrofuran. In experiments, conducted in a Soxhlet-apparatus over an extended period of time, the formation of methanethiol was definitely established, indicating the occurrence of hydrogenolysis of the carbon-sulphur linkage during the long exposure to the reagent.

The ultra-violet absorption spectra in ethanol of cheiroline and its thiourea-derivative (VII) are presented in Fig. 1. The broad maximum of rather low extinction in the spectrum of cheiroline is characteristic of the N=C=S-grouping. The thiourea absorption data are equally typical when compared with the curves for analogous mono-substituted derivatives (*cf.* Ref. 7). Both spectra clearly indicate the sulphonyl-grouping as a spectroscopically non-interfering part of the molecules.

EXPERIMENTAL*

β-Methylthiopropionitrile (IV). This substance was prepared from methanethiol and acrylonitrile in benzene solution, essentially as previously described [3]. It proved advantageous to increase the amount of catalytically acting sodium methoxide to 1 g per 27.5 g of methanethiol. A 90 % yield of the colourless nitrile was obtained, b.p. 96° at 14 mm.

γ-Methylthiopropylamine (V). In a three-necked flask, equipped with a mercury-sealed stirrer, separatory funnel, reflux condenser and an inlet for dry nitrogen, was placed a solution of 16.0 g (0.42 mole) of lithium aluminium hydride in 350 ml of dry ether. The flask was swept with nitrogen and a solution of 30.3 g (0.30 mole) of β-methylthiopropionitrile in 60 ml of anhydrous ether added from the separatory funnel under vigorous stirring in the course of half an hour. The reaction mixture was then refluxed for one hour in order to complete the reaction and thereafter cooled in an ice-bath. From the funnel were carefully added 20 ml of water, 20 ml of 15 % sodium hydroxide and 70 ml of water, successively. The precipitate was removed by filtration with gentle suction and thoroughly washed with several portions of ether. The ethereal phase was dried over potassium hydroxide, the ether removed over a small column and the residue distilled *in vacuo*. 21.5 g (68 %) of γ-methylthiopropylamine distilled as a colourless liquid of unpleasant smell, b.p. 66—67° at 14 mm.

Upon treatment of the amine with ethereal hydrogen chloride, the crystalline hydrochloride was obtained, m.p. 132°. It served to establish the identity of the amine (Schneider [3] reports the m.p. 136° for the amine hydrochloride).

γ-Methylsulphonylpropylamine (VI). A solution of 15.7 g of γ-methylthiopropylamine in 36 ml of 4.13 *N* hydrochloric acid and 500 ml of water was oxidised with a solution of 31.6 g of potassium permanganate in 1 000 ml of water containing 48.5 ml of 4.13 *N* HCl, as described by Schneider [3]. 19.7 g (76 %) of the sulphoneamine hydrochloride were obtained, m.p. 147.5—148.5° (Schneider [3] reports the m.p. 146°).

An additional amount of material (2.1 g) could be gained from the mother liquor.

Cheiroline (II). The following procedure was found to give the most satisfactory results. To an ice-cooled solution of 4.54 g of thiocarbonyl chloride in chloroform (50 ml) was added slowly and under constant shaking a solution of 6.86 g of γ-methylsulphonylpropylamine hydrochloride in 40 ml of 1 *N* sodium hydroxide. Then an additional amount of 68 ml of 1 *N* NaOH was added in small portions to the two-layer mixture and the latter vigorously shaken for about one hour. The red colour of the thiocarbonyl chloride rapidly faded to a yellow one. The chloroform layer was removed and the aqueous phase extracted with a small portion of fresh chloroform. The solvent was removed in a stream of air whereupon the cheiroline spontaneously crystallised. The crystals were dissolved in chloroform (17 ml), treated with Darco-60 and the suspension filtered through a thin layer of Celite. To the filtrate were added 50 ml of petroleum ether (b.p. 60—100°), whereupon an oil separated which immediately crystallised on seeding to give 5.15 g (73 %) of cheiroline, m.p. 44—46°. An additional recrystallization from ethyl ether gave analytically pure cheiroline as beautiful, colourless crystals. M.p. 46—47° (Schneider [3] reports the m.p. 46—48°).

$C_5H_9O_2NS_2$ (179.3)	Calc.	N 7.81	S 35.76
	Found	» 7.84	» 35.68

Reactions between cheiroline and amines (VII)—(X). As a standard method for the preparation of cheiroline-thioureas the following procedure was adopted. Cheiroline and a slight excess of the appropriate amine were dissolved in ethanol. The mixtures were gently heated on the water-bath, cooled and the products collected by filtration. The derivatives were recrystallized from ethanol before analysis. The results are summarized in Table 1.

β-Methylsulphonylpropionitrile (XI). To a cooled solution of 17.0 g of β-methylthiopropionitrile in 200 ml of glacial acetic acid were slowly added 75 ml of 30 % hydrogen peroxide. After standing at 0° for about two hours the reaction mixture was heated on the steam-bath for another two hours. Next day, the solution was concentrated to a small

* All melting points are uncorrected and determined in capillary tubes in an electrically heated block.

volume *in vacuo* and placed in the ice-box. On prolonged keeping here, the solution deposited a crop of large, colourless prisms (8.0 g). Further concentration of the mother liquor and chilling gave an additional crop of crystals (9.0 g) of slightly lower purity. The sulphonylnitrile was soluble in water, insoluble in ether and could be recrystallised from ethanol. An analytical specimen melted at 71—72°.

$C_6H_7O_2NS$ (133.2)	Calc.	C 36.07	H 5.30	S 24.07
	Found	» 36.20	» 5.36	» 24.12

Due to its slight solubility in ether a reduction of the nitrile with lithium aluminium hydride in a Soxhlet-apparatus was attempted. Only after refluxing for more than 200 hours the substance had been completely removed from the thimble. Upon working up the mixture in the usual way no well-defined products could be obtained. A considerable formation of methanethiol was easily recognisable, indicating the occurrence of secondary reactions.

Absorption spectra. The ultra-violet absorption spectra reproduced in Fig. 1 were determined on a Beckman model DU quartz spectrophotometer in 1 cm cells.

SUMMARY

An improved synthesis of cheiroline, the aglucone of the *iso*thiocyanate glucoside of wall-flower seeds, has been developed. It implies fewer steps than the original synthesis and proceeds from readily accessible starting materials in a good over-all yield.

Two new reaction products of cheiroline and aromatic amines are described.

Microanalyses were performed in this laboratory by Mr. W. Egger. The authors are indebted to Dr. O. Rosenlund for a generous gift of thiocarbonyl chloride.

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Studies Related to Naturally Occurring Acetylene Compounds. XI. Further Investigations on the Composition of Essential Oils from the Genus *Erigeron*

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Some years ago Sörensen and Stavholt [1] described in this journal investigations of 5 essential oils of fleabanes *(Erigeron) viz. E. acris* L., *E. borealis* Simm., *E. canadensis* L., *E. politus* Fr. and *E. uniflorus* L. The main constituents of these oils were matricaria ester (I) and lachnophyllum ester (II).

$$CH_3-CH=CH-C\equiv C-C\equiv C-CH=CH-COOCH_3 \quad (I)$$

$$CH_3-CH_2-CH_2-C\equiv C-C\equiv C-CH=CH-COOCH_3 \quad (II)$$

With the object of establishing whether these acetylenic compounds were characteristic of the genus *Erigeron*, we have in the past years tried to investigate as many fleabane oils as possible. As only the oil of *E. canadensis* L. is commercially available all had to be prepared from cultivations of foreign *Erigeron*-species in Tröndelagen and we are greatly indepted to Mr. Jens Roll-Hansen, curator of Statens Forsöksgaard Kvithamar and to his skilled staff for the great work laid down by them in the cultivation of most of our material. In the season 1951 and 52 some additional *Erigerons* were cultivated at *Norges Tekniske Högskole*, Trondheim.

All of the hitherto investigated 32 fleabanes turned out to contain acetylenic compounds. All but one did contain either matricaria (I) or lachnophyllum (II) ester or, more commonly, mixtures of these two compounds. The relative proportions were estimated spectroscopically. The results are summarised in Table 1.

Since earlier investigations had shown [2] that the oils from the roots of some plants belonging to the *Compositae* differed remarkably from the oils from the rest of the plant, the plants have mostly been divided into three parts: flowers, stem + leaves and root. This division is of course not a sharp one, the phyllaries for example may in some species be rather herbaceous, further the

Table 1. Acetylenic Compounds in the Genus Erigeron.

Section and Species:	Relative Concentrations Matricaria Ester: Lachnophyllum E		
	Flowers	Leaves	Roots
Section *Euerigeron* A.Gr.			
1. *E. aurantiacus* Rgl.	100 : 0	100 : 0	48 : 52
2. *E. candidus* Widder	100 : 0	100 : 0	42 : 58
3. *E. Coulterii*, Porter et Coult.	100 : 0	27 : 73	100 : 0
4. *E. compositus* Pursh, var. *glabratus* Macoun	43 : 57	42 : 58	39 : 61
5. *E. compositus* Pursh, var. *typicus* Payson f. *discoideus*	35 : 65	34 : 66	44 : 56
6. *E. compositus* Pursh. var. *discoideus* A. Gray	56 : 44	54 : 46	44 : 56
7. *E. elatior* Greene	100 : 0	100 : 0	56 : 44
8. *E. eriocephalus* J. Vahl	whole plant 18 : 82		
9. *E. glabellus* Nutt.	100 : 0	100 : 0	100 : 0
10. *E. glaucus* Ker.	100 : 0	53 : 47	17 : 83
11. *E. nematophyllus* Rydb.	70 : 30	65 : 35	28 : 72
12. *E. polymorphus* Scop.	61 : 39	50 : 50	100 : 0
13. *E. speciosus* D.C.	100 : 0	100 : 0	100 : 0
14. *E. superbus* Greene ex Rydb.	80 : 20	69 : 31	62 : 38
15. *E. subtrinervis* L.	100 : 0	100 : 0	100 : 0
16. *E. uniflorus* L.s.s.	2 : 98	2 : 98	45 : 55
Section *Olygotrichium* Cronq.			
17. *E. flagellaris* A.Gr.	whole plant 10 : 90		
18. *E. philadelphicus* L.	100 : 0	100 : 0	100 : 0
Section *Phalacroloma* Cronq.			
19. *E. annus* Pers.	8 : 92	31 : 69	48 : 52
20. *E. strigosus* var. *Beyrichii* (Fische & Mey) A.Gr.	55 : 45	50 : 50	53 : 47
21. *E. strigosus* var. *typicus* Cronq.	11 : 89	17 : 83	56 : 54
Section *Trimorphaea* Cass.			
22. *E. acris* L. var. *brachycephalum* H. Lindb.	20 : 80	16 : 84	16 : 84
23. *E. atticus* Vill.	100 : 0	100 : 0	59 : 41
24. *E. borealis* Vierh. et Simm.	100 : 0	—	—
25. *E. droebachiensis* O. F. Müll.	8 : 92	11 : 89	11 : 89
26. *E. eriocephalus* Regel et Smalh.	4 : 96	7 : 93	24 : 76
27. *E. Mairei* Braun-Blanquet	60 : 40	60 : 40	47 : 53
28. *E. politus* Fr.	56 : 44	70 : 30	59 : 41
Section *Caenotus* Nutt.			
29. *E. canadensis* L.s.s.	48.6 : 51.4	56 : 44	100 : 0
» subsp?	100 : 0	100 : 0	—
30. *E. montevidensis* Baker	5 : 95	33 : 67	100 : 0
31. *E. linifolius* L.	62 : 38	56 : 44	42 : 58
Section *Conyzastrum* Boiss.			
32. *E. khorassanicus* Boiss	—	—	Matricarianol!

division between stem and root had to be done rather arbitrarily. Sometimes the proportions between the two esters are rather remarkably constant through the whole plant (see Nos. 4—6 *E. compositus* and No. 13 and No. 15 *E. speciosus* and *E. subtrinervis*). In other cases, as will be seen from Table 1, the propor-

tions may vary considerably and in both directions (see for example No. 10 *E. glaucus* and No. 30 *E. montevidensis*).

No regularity has been found between these variations and the botanical subdivision of the genus *Erigeron*. O. Hoffmann [3] in 1897 divided the genus *Erigeron* into 5 sections *viz.*:

Sections of Hoffmann	Further sections of Cronquist
I *Oritrophium*	
II *Leptostelma*	
III *Euerigeron*	*Olygotrichium*, *Phalacroloma*, *Wyomingia*, *Pycnophyllum*
IV *Trimorphaea*	
V *Caenotus*	

Cronquist [4], who recently revised the North-American species of *Erigeron*, transferred section V *Caenotus* to the genus *Conyza* and further introduced 4 new sections into the section *Euerigeron* of Hoffmann.

Of the altogether 32 *Erigerons* investigated on the presence of acetylenic compounds none belongs to the sections I and II of Hoffmann, 7 belongs to section *Trimorphaea*, 3 to section *Caenotus* and 21 to the large section *Euerigeron*. Of these 21 some 18 belongs to the section *Euerigeron* in the narrower sense given to it by Cronquist, 2 belongs to his section *Olygotrichium* and 3 to his section *Phalacroloma*, whereas no representative has been available in his sections *Wyomingia* and *Pycnophyllum*.

There has been obtained one example of the section *Conyzastrum* of E. Boissier [5] *viz. E. khorassanicus* Boiss. This section of Boissier is not treated either by Hoffmann or by Cronquist. We understand that some modern botanists refer this section to the genus *Conyza*, whereas others are of the opinion that this section botanically belongs to *Erigeron*. The essential oil of *E. khorassanicus* differs fundamentally from that of the 31 other fleabanes investigated by us. The essential oil from the flowers and the green parts of the plant was spectroscopically devoid of acetylenic compounds. In fact the U.V.-spectra had only a "stepout" at short wave-lengths and no selective absorption. The essential oil from the root showed a characteristic selective absorption in U.V., but none of the observed maxima did agree with either matricaria or lachnophyllum ester. The maxima observed coincide with those of matricarianol (III) = deca-diene-2 : 8-diyne-4 : 6-ol-1. This alcohol was synthesized in 1951

$$CH_3-CH=CH-C\equiv C-C\equiv C-CH=CH-CH_2OH \qquad \text{(III)}$$

by Bruun *et al.*[6] in both the 2-*trans*-8-*trans* (m.p. 104°) and the 2-*trans*-8-*cis* (liq.) modifications. In 1951 Dagny Holme [7] isolated for the first time a liquid compound with the U.V.-spectrum of matricarianol from the root of *Aster tripoleum* L. Recently Holme *et al.*[8] firstly isolated the acetate of 2-*trans*-8-*trans* matricarianol from the leaves and roots of *Grindelia arenicola*, and later the same alcohol from *G. stricta*.

2-*trans*-8-*trans* Matricarianol synt.[6]	λ_{max},	3 122	2 930	2 765	2 615	2 300	ÅU
2-*trans*-8-*cis*- » »	»	3 132	2 938	2 755	2 612	2 320	»
Oil from root of *Erigeron khorassanicus*	»	3 130	2 940	2 785	2 620	2 290	»

So far only 7 species of *Aster* have been investigated in this laboratory, and of these only *A. tripoleum* has given pure *trans-trans*-matricarianol after saponification; the acid moiety of the ester is still unknown. All the 7 species of *Aster* so far investigated are lacking acetylenic compounds in the flowers and herbaceous parts; if present, the acetylenic compounds do occur in the root exactly as in *Erigeron khorassanicus*. It is hoped that it will be possible later on to obtain some further representatives of the section *Conyzastrum* as well as some real *Conyza* species, which might establish the chemical boundaries between the botanically somewhat overlapping genera *Aster-Erigeron-Conyza*.

It is remarkable that the 3 representatives of the section *Caenotus* investigated by us all behave chemically as real *Erigerons*. This section *Caenotus*, the members of which are found in North- and South-America, no doubt morphologically approaches *Conyza*, and for that reason was transferred to this genus by Cronquist. This view is opposed by their chemistry which indicates that they are real *Erigerons*.

It is hoped that a further investigation of some of the most intermediate representatives may introduce distinct chemical boundaries and so give some hints to the genesis of these genera. The indications furnished by the results of the present investigation are obviously not in accordance with the view of most botanists [4,9] *viz.* that *Conyza* is derived from *Erigeron*, whereas *Erigeron* and *Aster* are parallel groups.

Artificial mixtures of equal parts of matricaria and lachnophyllum esters are liquid at ordinary temperature and difficult to separate. If any component is dominating M : L $< 30 : 70$ or M : L $> 70 : 30$ the main component can easily be isolated in pure condition by crystallisations from ethanol-water or petroleum ether. If the liquid 2-*trans*-8-*cis*-matricaria ester is present, as in some *Amellus* oils [10], the isolation of the pure components is very troublesome. With the exception of *E. compositus* we have encountered no difficulties in the isolation of the crystalline esters and so we have no indications for the presence of 2-*trans*-8-*cis* matricaria ester in fleabane oils. As will be seen from the experimental part some of the commonly cultivated *Erigerons* (as *E. speciosus* and *E. strigosus*) are excellent sources of matricaria and lachnophyllum esters.

EXPERIMENTAL

Section *Euerigeron* A. Gr.

1. *Erigeron aurantiacus* Rgl. Seed samples: *Hortus Universitatis Lousonnensis, Botanischer Garten München-Nymphenburg, Botaniska Trädgården*, Lund, Sweden. Cultivated Kvithamar 1950/51.

	kg	Essential oil ‰	ME / LE
Flowers	1.0	2.6	100 : 0
Leaves	6.7	0.5	100 : 0
Roots	0.9	3.6	48 : 52

The essential oils from the flowers and from the leaves solidified spontaneously, m.p. of the crude matricaria ester 33°.

2. *Erigeron candidus* Widder. Seed sample: *Hortus Universitatis Lousonnensis*. Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	ME / LE
Flowers	34	2.5	100 : 0
Leaves	166	0.9	100 : 0
Roots	30	7.4	42 : 58

The oils from the flowers and from the leaves solidified in the cold, m.p. of the crude matricaria ester 34° and 32° C.

3. *Erigeron Coulterii*, Porter et Coult. Seed sample: *Hortus Botanicus Hauniensis*. Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	% ME	% LE
Flowers, air-dried	305	1.8	30	—
Leaves, » »	1180	0.76	7	19.3
Roots, » »	220	6.7	81	—

4. *Erigeron compositus* var. *glabratus* Macoun. Four different cultivations have been separately investigated. Seed sample: a) *Botanischer Garten München-Nymphenburg*, b) *Hortus botanicus Hauniensis*, c) *Botaniska Trädgården*, Lund, Sweden, d) *Botanischer Garten der Universität Graz*. All cultivations at Kvithamar 1950/51.

	g	Essential Oil ‰	ME / LE
a) Flowers	33	2.3	45 : 55
Leaves	234	7.5	44 : 56
Roots	23	4.5	46 : 54
b) Flowers	36	2.4	44 : 56
Leaves	360	8.3	44 : 56
Roots	33	1.7	33 : 67
c) Flowers	43	2.0	42 : 58
Leaves	390	4.4	43 : 57
Roots	63	1.4	36 : 64
d) Flowers	8	1.9	39 : 61
Leaves	200	3.9	37 : 63
Roots	15	1.2	41 : 59

The variations in the composition of the oils from different cultivations and from different parts of the plant are rather small. All these oils remain liquid at −15° and the isolation of crystalline matricaria ester succeeded only after several recrystallisations at −30°.

5. *Erigeron compositus* var. *typicus* Payson f. *discoideus*. Seed sample: *Hortus Universitatis Lousonnensis*. Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	ME / LE
Flowers	29	1.9	35 : 65
Leaves	200	2.3	34 : 66
Roots	25	1.4	44 : 56

These oils remained liquid at −15°, compare 4.

6. *Erigeron compositus* var. *discoideus* A. Gray. Seed sample: a) *Hortus botanicus Hauniensis*. Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	ME / LE
Flowers	33	2.7	50 : 50
Leaves	535	6.2	52 : 48
Roots	84	1.5	43 . 57

The essential oils remained liquid at −15°.

b) *Royal Botanic Gardens, Edinburgh,* cultivated Trondheim 1951.

	g	Essential oil ‰	% ME	% LE
Flowers	132	0.9	46	27
Leaves	380	1.7	40	30
Roots	30	2.0	35	44

7. *Erigeron elatior* Greene. Seed sample: *Hortus Universitatis Lousonnensis,* Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	ME / LE
Flowers	37	0.5	100 : 0
Leaves	135	0.75	100 : 0
Roots	200	1.8	56 : 44

8. *Erigeron eriocephalus* J. Vahl see *E. uniflorus* L. No. 16.

9. *Erigeron glabellus* Nutt. Cultivations were carried out at Kvithamar on 4 different seed samples: a) *Botanischer Garten München-Nymphenburg,* b) *Hortus Botanicus Bergianus,* Stockholm, c) 75 = ? and d) *Hortus Universitatis Lousonnensis.* a) b) and c) gave essential oils which in all parts of the plant contained only matricaria ester as chromophoric substance. All oils solidified and afforded pure matricaria ester after one crystallisation.

	kg	Essential oil ‰	% ME	% LE
d) Flowers	1.51	0.40	13	26
Leaves	4.65	0.09	14	9
Roots	0.98	0.92	77	—

The essential oil from the root of d) gave a high yield of crystalline matricaria ester, m.p. crude 33° undepressed by authentic matricaria ester. Whether sample d) is somewhat mixed up botanically through hybridisation or there really are variations in the composition of *E. glabellus* oils has not been investigated further.

10. *Erigeron glaucus* Ker. Seed sample: *Hortus Botanicus Hauniensis.* Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	ME / LE
Flowers	720	2	100 : 0
Leaves	4000	1.4	53 : 47
Roots	260	1.9	17 : 83

The oil from the flowers solidified at room temperature and a single crystallisation from petroleum ether furnished pure matricaria ester. The oil from the leaves deposited from petroleum ether solution crystalls which after one recrystallisation also was pure matricaria ester. The essential oil from the roots was diluted with a little 96 % alcohol and the crystalls which separated at 0° recrystallised from alcohol. M.p. and mixed m.p. with lachnophyllum ester 31°.

11. *Erigeron nematophyllus* Rydb. Seed sample: *Hortus Universitatis Lousonnensis,* cultivated Kvithamar 1950/52.

	g	Essential oil ‰	% ME	% LE
Flowers	15	2.5	70	30
Leaves	160	5.7	41	22
Roots	8	5.4	22	65

12. *Erigeron polymorphus* Scop. Seed sample: a) *Royal Botanic Gardens, Kew.* Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	% ME	% LE
Flowers air-dried	350	1.1	11	7
Leaves » »	1400	0.5	4	4
Roots » »	225	3.2	66	—

b) From a seed sample marked with the name of *E. uniflorus (Bot. Garten München-Nymphenburg)* developed genuine *E. polymorphus* (Kvithamar 1950/51). The whole plant, 740 g, was distilled without separation, essential oil 1.4 ‰, the U.V. curve of the oil was indistinguishable from the prototype of matricaria ester. The oil solidified at room temperature and afforded matricaria ester m.p. 33° after one single crystallisation from petroleum ether.

13. *Erigeron speciosus* D.C. Seed samples: a) *Royal Botanic Gardens, Kew*, b) *Botanischer Garten der Universität Graz*, c) *Botaniska Trädgården*, Lund, Sweden, d) *Botanischer Garten München-Nymphenburg*, all four cultivated at Kvithamar 1950/51. As with most of the *E. glabellus* samples all parts of the four cultivations of *E. speciosus* gave oils which contained only the matricaria ester and in high percentage. One single crystallisation afforded pure matricaria ester.

14. *Erigeron superbus* Greene ex Rydb. The seed sample originated from the *Royal Botanic Gardens, Kew*, and was named *E. macranthus* without author designation. The plant which developed was the most splendid fleabane in our cultivations and as far as can be decided from the scanty comparison material available the plant must be *E. superbus* Greene ex Rydb.≡ *E. macranthus* subsp. *mirus* A. Nels. Cultivated Trondheim 1951/52.

	g	Essential oil ‰	% ME	% LE
Flowers	178	0.69	80	20
Leaves	700	0.20	55	25
Roots	210	2.5	58	35.5

15. *Erigeron subtrinervis* L. Seed samples: a) and b) *Botanischer Garten München-Nymphenburg* (under two other, obviously false, designations) c) *Botanicus Lovaniensis* d) *Hortus Universitatis Lousonnensis*, all cultivated at Kvithamar 1950/51. With a) b) and c) the concentrations were not estimated, all U.V.-spectrograms of the crude oils from all parts of the plant were identical with that of matricaria ester; with d) the content of matricaria ester was estimated.

	kg	Essential oil ‰	% ME
Flowers	1.1	0.18	19
Leaves	3.5	0.11	5
Roots	0.93	1.5	83

All the essential oils from the *E. subtrinervis* samples solidified at zero degree and gave pure matricaria ester after one single crystallisation.

16. *E. uniflorus* L.

In our first paper on the composition of the essential oils of *Erigeron* species [1] the results were given of an investigation of the species *E. uniflorus* L. collected in the Dovre region of Southern Norway. Mr. J. Lid, curator of the Botanical Museum at the University of Oslo had the kindness to direct our attention to the fact that two main types of *E. uniflorus* occurs in the Norwegian mountains, the one agreeing with *E. uniflorus* L.s.s., the other identical with the plant described by J. Vahl in Flora Danica [11] as *E. eriocephalus*. A study of the two descriptions revealed that the material reported on in our first paper was in all probability a mixture of *E. uniflorus* s.s. and *E. eriocephalus* J. Vahl. Some botanical excursions confirmed the view of Mr. Lid. When *E. boreale* Vierh. & Simm. is absent, there is mostly no difficulty in keeping the two types apart. *E. eriocephalus*, which reaches the highest altitudes available in southern Norwegian mountains, retain the curved spreading phyllaries with woolly-villous hairs when cultivated at sea level in Trondheim.

E. uniflorus s. s. with adpressed phyllaries are in the mountains mostly somewhat hairy on the lower parts of the phyllaries, practically glabrous at lower altitudes. From a seed sample named *E. Schleicheri* (without author designation) a fleabane developed in our garden which obviously had nothing to do with either *E. Schleicheri* Moritz ≡ *E. alpinus* L. var. *intermedius* (Schleicher) Gremli or with *E. Schleicheri* Gremli ≡ *E. glandulosus* Hegetschev, but turned out to be a nearly glabrous form of *E. uniflorus*. With this type and another sample from seeds from Hortus Universitatis Lousonnensis and a char-

acteristic collection of *E. eriocephalus* J. Vahl from Vinstradalen, Opdal, Sör-Tröndelag, a reinvestigation of the essential oils was carried out.

8. *E. eriocephalus* J. Vahl.

The air-dried material weighed 4 g which furnished 14 mg essential oil, that is 3.5 ‰. ME = 11.7 %, LE = 52.7 %.

16. *E. uniflorus* L.s.s. a) Seed sample: *Botanicka Zahrada University Karlovy*, Praha, cultivated Trondheim 1951.

	g	Essential oil ‰	% ME	% LE
Flowers	1.7	3.5	1.2	57.5
Leaves	91	2.1	2.0	98
Roots	23	4.4	44	55

Only the U.V.-spectrum of the oil from the root extends into visible light and so the origin of "Compositcumulene I" is the root of *E. uniflorus* L. Recalculated on the whole plant the relation is: LE : ME = 5.1 whereas *E. eriocephalus* J. Vahl gives 4.5. The two types thus cannot be separated chemically.

b) Seed sample: *Hortus Universitatis Lousonnensis*, cultivated Kvithamar 1950/51. Whole plant 105 g, essential oil 4.0 ‰, ME : LE = 11 : 89.

The oil solidified in the cold; from an alcoholic solution pure lachnophyllum ester crystallised immediately, m.p. 31.2° undepressed by authentic lachophyllum ester.

Section *Olygotrichium* Cronq.

17. *Erigeron flagellaris* A. Gr. Seed sample: *Botanischer Garten München-Nymphenburg*. Cultivated Kvithamar 1950. The whole plant distilled with steam. U.V.-spectrum of the essential oil gave ME : LE = 10 : 90; pure lachnophyllum ester was obtained by repeated crystallisations from 96 % alcohol.

18. *Erigeron philadelphicus* L. Seed sample: *Botanischer Garten München-Nymphenburg*, cultivated Kvithamar 1950/51.

	kg	Essential oil ‰	% ME
Flowers	0.98	0.25	37
Leaves	3.8	0.08	11.5
Roots	0.69	1.4	93

All three U.V.-curves were within the limits of the experimental errors identical with that of matricaria ester. The oil from the root solidified and gave pure matricaria ester after one crystallisation from petroleum ether.

Section *Phalacromola* Cronq.

19. *Erigeron annus* Pers. Seed sample: *Hortus Botanicus Bergianus*. Cultivated Kvithamar 1950/51.

	kg	Essential oil ‰	% ME	% LE
Flowers	1.5	3.5	6	66
Leaves	3.5	0.20	1.7	3.8
Roots	0.78	0.33	32	35

The oil from the flowers deposited white crystals from alcohol, m.p. 31°, mixed m.p. with lachnophyllum ester 31.5°, test with a 1 : 1 mixture with matricaria ester: liquid at 20°.

20. *Erigeron strigosus* var. *Beyrichii* (Fisch & Mey) A. Gr. Seed sample: *Jardin Botanique Rouan*. Cultivated Kvithamar 1950/51.

	g	Essential oil ‰	% ME	% LE
Flowers, air-dried	97	1.4	3.6	3
Leaves » »	700	0.5	4.0	4
Roots » »	8	0.05	20	18

21. *Erigeron strigosus* var. *typicus* Cronquist. Seed samples: a) *Hortus Botanicus Bergianus*, Stockholm. b) *Royal Botanic Gardens*, Kew; both cultivated Kvithamar 1950/51.

	kg	Essential oil ‰	% ME	% LE
a) Flowers	3.25	1.5	7.5	75
Leaves	3.5	0.31	4.2	23
Roots	0.92	0.88	18.5	22
b) Flowers	2.3	1.9	5	34
Leaves	3.6	0.38	8	34.5
Roots	1.0	0.76	56	29

The oils from the flowers solidified in the cold and gave pure lachnophyllum ester after some crystallisations from alcohol.

Section *Trimorphaea* Cass.

22. *Erigeron acris* L. var. *brachycephalus* H. Lindb. *Erigeron acris* L.s.s. was reported on in our first paper on fleabane oils and turned out to contain large amounts of oil, rich in lachnophyllum ester. Mr. Knut Stokke, Drammen, had the kindness to send us an air-dried sample of a fleabane found by him in the vicinity of Kragerö, Southern Norway, and supposed by the discoverer to be *E. politus* Fr. Since three different strains of *E. politus* Fr. had given very consistent results (cf. Ref. No. 28) — dominance of matricaria ester in all parts of the plant — whereas the *Erigeron* of Mr. Stokke chemically comes very close to the investigated strains of *E. acris* L. a redetermination was demanded, the more so since *E. politus* Fr. has a rather sharp southern limit in the mountains of Torpa, some 200 km north of Kragerö. The only alternative seems to be the subspecies *brachycephalus*, described by the Finnish botanist Harald Lindberg. This subspecies has been ignored in Norwegian flora and no distinguishing marks between *E. politus* Fr. and *E. acris* var. *brachycephalus* H. Lindberg have been worked out.

	g	Essential oil ‰	% ME	% LE
Flowers, air-dried	110	0.7	2	7.8
Leaves, stems and roots	260	0.16	10	53

Both oils solidified in the cold and gave pure lachnophyllum ester from ethanol solution.

23. *Erigeron atticus* Vill. Seed samples: a) *Botanischer Garten München-Nymphenburg*, b) *Hortus Universitatis Lousonnensis*. Cultivated Kvithamar 1950/51.

	kg	Essential oil ‰	% ME	% LE
Flowers	3.07	0.57	26	—
Leaves	4.2	0.25	20	—
Roots	1.22	0.35	56	39

The oils from flowers and leaves solidified at room temperature and furnished pure matricaria ester from petroleum ether solution.

24. *Erigeron borealis* Vierh. et Simm. data taken from [1], only the flowers were collected.

25. *Erigeron droebachiensis* O. F. Müll. In 1782 Flora Danica described in Fasciculus XV, Tab. DCCCLXXIV a fleabane discovered by O. F. Müller in the vicinity of the village of Dröbak as in many respects so different from *E. acris* L.s.s. that it ought to be given the rank of a species. Norwegian floras have, however, either included *E. droebachiensis* into *E. acris* or they have only given it the rank of a subspecies under *E. acris*.

In contrast to Norwegian botanists their American colleagues have upheld the distinction between these two species, which of course is confirmed by the fact that *E. acris* L.s.s. does not occur in U.S.A., whereas *E. droebachiensis* O. F. Müller is regarded as

synonymous to *E. acris* var. *asteroides* (Andrz. ex Bess) DC, which is widely spread in Northern U.S.A.[4].

Through the most kind assistance of Professor Dr. T. Braarud, Oslo, and Mr. Knut Stokke, we had the opportunity to inspect this species in the original locality and to obtain good samples for chemical investigations. In our opinion the morphological differences between *E. droebachiensis* and *E. acris* s.s. are considerable, and there did not seem to exist intermediate types. *E. acris* s.s. occurs in the immediate surroundings in its typical form.

	g	Essential oil ‰	% ME	% LE
Flowers	110	0.7	4.0	49
Leaves & Roots	260	1.6	9	75

Pure lachnophyllum ester was obtained from both oils by crystallisation from alcohol.

26. *Erigeron eriocephalus* Regel et Smalh. In 1877 the cited authors described a fleabane from Turkestan and gave it the designation "*eriocephalus*", obviously overlooking the older work of J. Vahl, which already had occupied this name. The plant of Regel and Schmalhans belongs to the *E. acris* group. Seed sample *Hortus Botanicus Hauniensis.* cultivated Kvithamar 1950/51.

	g	Essential oil ‰	% ME	% LE
Flowers air-dried	200	1.9	2	42.5
Leaves » »	700	0.7	2.5	32
Roots » »	32	1.7	17	54.5

The oils from flowers and leaves solidified in the cold and afforded pure lachnophyllum ester after crystallisations from ethanol.

27. *Erigeron Mairei* Braun-Blanquet. Seed sample: *Botanicka Zahrada.* Cultivated at N.T.H. 1951/52.

	g	Essential oil ‰	% ME	% LE
Flowers	126	1.5	44	30
Leaves	380	1.1	60	40
Roots	120	2.7	47	53

28. *Erigeron politus* Fr. was mentioned in our first paper. The material originated from Vinstradalen, Dovre and was separated into flowers and non-floral parts. The essential oil from both gave U.V. curves nearly identical with that of matricaria ester. Two cultivations have been carried out at Kvithamar 1950/51. Seed samples: a) *Hortus Botanicus Bergianus*, Stockholm, b) *Hortus Universitatis Lousonnensis.*

	g	Essential oil ‰	ME / LE
a) Flowers	190	2.5	50 : 50
Leaves	820	1.3	70 : 30
Roots	154	6.3	60 : 40
b) Flowers	750	1.8	62 : 38
Leaves	820	1.3	71 : 29
Roots	154	6.3	58 : 42

All essential oils solidified in the cold and gave pure matricaria ester from petroleum ether solution.

Section *Caenotus* Nutt.

29. *Erigeron canadensis* L. This widespread weed was first investigated through a sample of the commercially available essential oil which, besides large amounts of terpene hydrocarbons, contained small amounts of matricaria ester (0.2 %). Owing to the danger of contamination with oils from other plants in the distilled material we further tried to

cultivate *E. canadensis* from a seed sample from Texas. This plant did not flower in Trondheim; the stem and leaves gave an oil devoid of selective absorption. From the oil of the root was isolated a dehydromatricaria ester m.p. 112.5°, also found in the oil from the root of *Artemisia vulgaris* [2]. The constitution of this dehydromatricaria ester might be that of the 2-*cis* isomer of

$$H_3C-C\equiv C-C\equiv C-C\equiv C-CH=CH-COOCH_3$$

These deviating results gave rise to an investigation of other samples of this plant. Cultivations at Kvithamar 1950 from two different seed samples were unsuccessfull as the plant would not flower and all died during the winter 1950/51 in accordance with the general habit of *E. canadensis* to be annual.

Further cultivations at Trondheim were also unsuccessfull until it was discovered that *E. canadensis* is a short day plant, readily developing flowers in Trondheim, when kept in darkness for 10 hours a day. Further one quite different strain occurring wild near Oslo has been investigated.

a) *E. canadensis* s.s. Seed sample: *Hortus Botanicus Hauniensis.* Cultivated Trondheim 1952 with a 14 hour night.

	g	Essential oil ‰	% ME	% LE
Flowers	65.5	1.7	48.6	51.4
Leaves	611	1.0	45	35
Roots	180	1.1	90	—

b) *E. canadensis* subsp.? On "Kadettangen" in Sandvika near Oslo there has for many years existed a colony of a small species of *Erigeron*, by Norwegian botanists determined to be *E. canadensis* L. The tiny plant, which to an amateur botanist looks very different from most drawings of *E. canadensis* and all the specimens seen in culture or in exsiccata, was collected August 1951 and air-dried. On our refusal to accept this species as *E. canadensis* the plant has been carefully inspected by Dr. Carl Blom, Gothenburg and Johs. Lid, Oslo, who both assure us that the plant is really *E. canadensis* L. We are indebted to both of them for the solicitude with which this question has been settled.

	g	Essential oil ‰	% ME	% LE
Flowers air-dried	14	3.1	64	—
Leaves » »	30	0.84	65	—

Both oils solidified in the cold and furnished pure matricaria ester from petroleum ether.

The roots, which only weighed 9 g, were extracted with acetone to look for the presence of the somewhat thermolabile dehydro ester m.p. 112.5°. The acetone extract was evaporated at room temperature in a vacuum (185 mg) and showed only a very weak U.V.-absorption. Distillation at 0.001 mm afforded 2.2 mg distillate with the U.V.-curve of pure matricaria ester, content 12 %.

The two investigated strains of *E. canadensis* do not agree in the composition of the essential oils, and since most of the investigated different strains of other fleabanes have agreed remarkably well, we do still suppose that a study of the microspecies of *E. canadensis* would be desireable.

What is much more important is that both of the two strains investigated this time have a composition of the essential oil in accordance with the general picture arrived at for real fleabane oils, whereas the non-flowering Texas-plant investigated 1950 has so far found no chemical relatives. The leaves of that plant reminded very much of those of *E. canadensis;* the stem was more slender and somewhat woody and in one respect it really showed a deviation not met with any other of our cultivations of *E. canadensis;* the Texas plant developed subterraneous runners. We are trying to obtain new seed samples of this plant, because the occurence of dehydromatricaria ester m.p. 112.5° in the roots makes a safe botanical determination desireable.

30. *Erigeron montevidensis* Baker. Seed sample: *Hortus Botanicus Hauniensis.* Cultivated Trondheim 1951 in hothouse.

	g	Essential oil ‰	% ME	% LE
Flowers	9.5	6.8	2	35
Leaves	1 630	2.4	13	27
Roots	300	3.5	78	—

The oil from the flowers furnished pure lachnophyllum ester, the oil from the roots pure matricaria ester.

31. *Erigeron linifolius* L. Seed sample: *Hortus Botanicus Hauniensis.* Cultivated Trondheim 1952 in hothouse.

	g	Essential oil ‰	% ME	% LE
Flowers	153	1.6	57	34.5
Leaves	3 770	0.4	45.5	35
Roots	870	0.2	42	58

Section *Conyzastrum* Boiss.

32. *Erigeron khorassanicus* Boiss. Seed sample: *Hortus Botanicus Hauniensis.* Cultivated in hothouse, Trondheim 1951/52.

	g	Essential oil ‰	% ME	% LE
Flowers	28.5	0.8	—	—
Leaves	190	0.2	—	—
Roots	105	0.5	—	—

All maxima of the oil from the roots were in agreement with those of matricarianol (III). The concentration calculated as free alcohol from the 4 most long-waved maxima was 43, 41, 40, 40, mean 41 %. The amount of essential oil was too small to allow an isolation of the chromophore.

SUMMARY

The essential oil of some further 27 species of the genus *Erigeron* have been investigated as to their content of acetylenic compounds. With the exception of one species *(E. khorassanicus)* belonging to the section *Conyzastrum* of Boissier — which seems to contain matricarianol — all the other fleabane oils contained the matricaria ester (I) or the lachnophyllum ester (II), both mostly in varying mixtures. Since the only exception belongs to a section, which by the botanists has been placed now in *Conyza*, now in *Erigeron*, it seems likely to conclude that all true *Erigerons* shall have the acetylenic esters I or II in all parts of the plant. It is remarkable that the investigated members of Section *Caenotus* Nutt. chemically behave as true *Erigerons*. Modern botanists have been inclined to transfer the whole section *Caenotus* to *Conyza*.

We are greatly indebted to Professor Dr. K. Fægri, the Botanical Department, University of Bergen and the Botanical Garden of the University of Copenhagen, who most kindly have furnished the seed samples. Some university garden samples gave rise to numerous cultivations of plants with quite false names. We are utmostly grateful to Mr. Olav Gjærevoll, curator of the botanical Museum in Trondheim for the valuable assistance he has rendered with the redeterminations of the plants. Grants from *Norges Almenvitenskapelige Forskningsråd*, which have made it possible to carry through these investigations, are gratefully acknowledged.

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Studies Related to Naturally Occuring Acetylene Compounds. XII. The Synthesis of Methyl *n*-Deca-2 : 4-diynoate

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In the tenth communication [1] of this series some di- and tetrahydro deiivatives of the matricaria ester (I) were synthesised with the object of obtaining comparison material for the identification and isolation of such compounds from essential oils. This work was so far immediately successful as the *cis*-isomer of the α,β-dihydromatricariaester (II) turned out to be rather widely distributed — mostly as a minor constituent — in oils of the *Compositae* [2].

$$CH_3-CH=CH-C\equiv C-C\equiv C-CH=CH-COOCH_3 \quad (I)$$

$$CH_3-CH=CH-C\equiv C-C\equiv C-CH_2-CH_2-COOCH_3 \quad (II)$$

$$CH_3-CH_2-CH_2-CH_2-CH_2-C\equiv C-C\equiv C-COOCH_3 \quad (III)$$

As mentioned in the tenth communication we had also tried to synthesise the ester (III) = methyl *n*-deca-2 : 4-diyn-oate. The Glaser coupling from hept-1-yne and propiolic acid methyl ester, however, failed completely, because propiolic acid methyl ester was removed very rapidly by self-coupling.

The reason for our particular interest in (III) was the presence of an unknown compound in the oils from roots of some *Helipterum* species. The crude oils show sharp peaks of low intensity in positions close to but significantly different from the maxima of (II), *viz.*

λ_{max}, II	2 810	2 650	2 514	2 381	2 275	ÅU
» Crude *Helipterum* oils	2 862	2 650	—	—	—	»

Since a carbomethoxy-group has turned out to be about equivalent to one double bond [3], the possibility existed that the chromophore present in (III) was responsible for the maxima in the *Helipterum* oils. The synthesis of (III) was finally accomplished by introduction of the carboxyl group in amyldiacetylene by a Grignard reaction. Amyldiacetylene was synthesised analogously to the procedures given by Armitage *et al.*[4] for the synthesis of monoalkylacetylenes, *cf.* Experimental part.

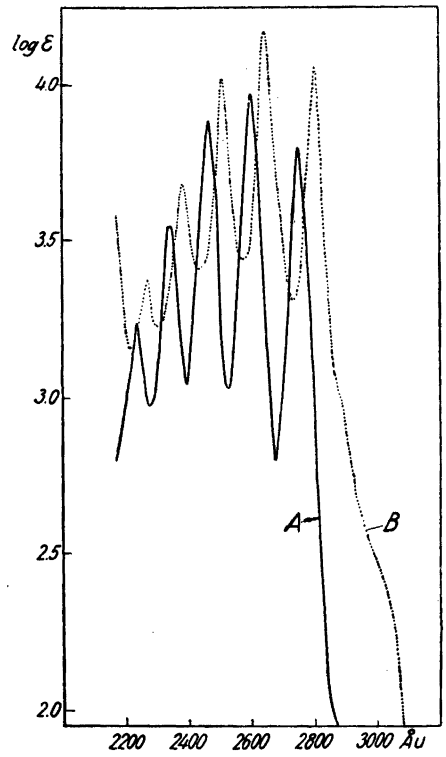

Fig. 1. Ultraviolet absorption in hexane of A: methyl n-deca-2 : 4-diyn-1-oate, B: methyl n-deca-8-ene-4 : 6-diyn-1-oate (α,β-dihydro matricaria ester).

Methyl *n*-deca-2 : 4-diyn-oate was a colourless liquid with freezing point below zero. The free acid and the methyl ester (III) showed a beautiful fine-structure spectrum in U.V., *cf.* Fig. 1, curve A. The maxima of III, however, are displaced some 50 ÅU towards *shorter* wave-lengths relative to II, whilst the unknown chromophore from *Helipterum* was displaced about 50 ÅU towards *longer* wave-lengths. The carbomethoxy group thus seems to be a little less active than one double bond. With the broad and undistinct spectra such as those given by the shorter polyenes — *cf.* the ethylenic analogue of III-sorbic acid — with only one broad maximum at 2 610 ÅU — such small differences cannot easily be verified. With the remarkable fine-structure of acetylenic compounds this difference is easily established and may be useful by the identification of unknown chromophores. So far the maxima shown by (III) have not been observed in any of the essential oils studied in this laboratory.

It is hoped to obtain enough *Helipterum* oils in the season 1953 to get insight into the structure of the substance with the unknown chromophore.

For comparison with very long-waved chromophores found in some *Coreopsis*-oils amyldiacetylene was coupled to diamyl-tetraacetylenes according to [5]. This tetra-acetylene is rather stable; the spectral properties was found in excellent agreement with the data given for dialkyltetra-acetylenes by Armitage *et al* [5].

EXPERIMENTAL

1. *Amyl-diacetylene.* In 1 l. liq. ammonia sodamide was prepared in the usual way [6] from 35 g of sodium metal and 0.4 g ferric nitrate. To the solution was added slowly 62 g of freshly distilled 1 : 4-dichloro-2-butyne during 1.5 hours, then followed by 75 g of *n*-amylbromide, also freshly distilled, during 1 hour. After 3.5 hours 200 ml ether was added and the ammonia slowly evaporated. To the semi-solid residue some ammonium-chloride was added and then 500 ml ether. The ether solution was dried, evaporated carefully and the residue distilled at 3 mm. Amyldiacetylene was a colourless liquid which soon discoloured in air. B.p. 46–48° at 3 mm, $n_D^{19} = 1.4808$, yield 19 g (31 %).

2. *Methyl* n-*deca-2 : 4-diynoate.* From 16 g of ethylbromide the Grignard compound was prepared in the usual way. The solution was cooled to −25° and freshly distilled amyl-diacetylene added drop by drop during 40 min. with vigorous stirring. The reaction mixture was left until it had reached room temperature and was then refluxed for 2 hours. The solution of amyl-diacetylene magnesium bromide was then cooled to −50° and a stream of dry carbon dioxide passed through for 3 hours. A thick dark liquid separated and the temperature rose to −30°. A few lumps of solid carbon dioxide were added and the reaction mixture left for 10 hours. After addition of a solution of 50 g sodiumbisulphate in 100 g of water and cooled to −15° the mixture was extracted with ether. The ether solution was evaporated finally at the oil pump at 25°. The residue solidified and the acid parts were taken up in soda solution and the acids set free with cold sulphuric acid and extracted with petroleum ether. Some volatile acids were evaporated at 20°/0.001 mm and the residue crystallised from petroleum ether. The deca-2 : 4-diyn-1-carboxylate crystallised in colourless needles which very rapidly turned yellow even at 0° C, and because of this marked unstability the acid was immediately converted to the methyl ester. 1 g of the acid in 50 ml of methanol and 1.5 ml of conc. sulphuric acid was left for 5 days in the cold and the ester worked up in the usual way. Yield 0.65 g.

Methyl *n*-deca-2 : 4-diynoate distilled as a colourless liquid, b.p. 40°/0.001 mm.

$C_{11}H_{14}O_2$ (178.1)	Calc.	C 74.1	H 7.92
	Found	» 73.8, 73.8	» 7.89, 8.14

U.V.-spectrum (Fig. 1, curve A):

in hexane	ε_{max}	6 240	9 340	7 770	3 550	1 740
	λ_{max}	2 755	2 602	2 463	2 342	2 234
	$\nu_{max} \times 10^{12}$	1 088.9	1 153.0	1 218.0	1 281.0	1 342.9
	$\Delta\nu_{max} \times 10^{12}$	64.1	65.0	63.0	61.9	
in alcohol	ε_{max}	5 630	7 080	6 320	3 240	1 700
	λ_{max}	2 772	2 610	2 474	2 360	2 258
The free acid in alcohol:						
	λ_{max}	2 750	2 600	2 465	2 345	—

3. *Octadeca-6 : 8 : 10 : 12-tetrayne.* From 4 g of ethyl bromide the Grignard compound was prepared in the usual way. To this solution amyldiacetylene (4.5 g) was added slowly at −25°, the reaction mixture was left until it had attained room temperature and then refluxed for 2 hours. To the cooled solution 8.4 g of anhydrous cupric bromide was added in small portions; finally the solution was refluxed for half an hour. Decomposed with ice and the hydrocarbon extracted with ether and distilled at 80°/0.0001 mm. Bright yellow oil, which crystallised at about −30°.

Spectrum in hexane:

ε_{max}	369	572	1 140	2 170	1 870	396 000	300 000
λ_{max}	3 557	3 330	(3 075)	2 885	2 718	2 386	2 272
$\nu_{max} \times 10^{12}$	843.4	900.9	975.6	1 039.9	1 103.8	1 257.3	1 320.4
$\nu\Delta_{max} \times 10^{12}$	57.5	74.7	64.3	63.9		63.1	

Given by Armitage *et al.* for dialkyl-tetra-acetylenes [5]:

λ_{max}	3 550	3 295	3 070	2 875	2 380	2 270	2 170

SUMMARY

Methyl *n*-deca-2 : 4-diyn-oate (III) has been synthesised by carboxylation of amyldiacetylene. The U.V.-spectrum of this ester demonstrates that the carbomethoxy group is a little less active than one double bond when conjugated with acetylenic bonds.

Grants from *Norges Almenvitenskapelige Forskningsråd*, which made it possible to carry out these investigations, are gratefully acknowledged.

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Quantitative Determination of Acetaldehyde in the Presence of Crotonaldehyde

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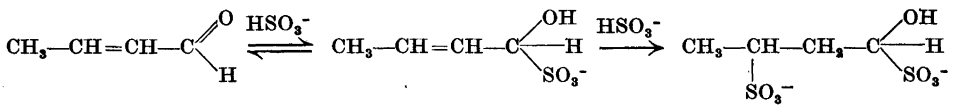
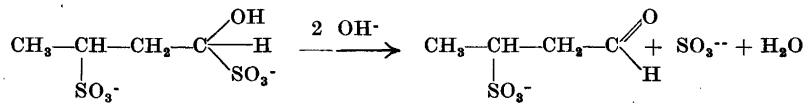
Numerous reagents, such as ammonia, hydrocyanic acid, and sodium bisulphite, which ordinarily do not react with the ethylenic linkage are known to add to the double bond in the α,β-unsaturated aldehydes and ketones [1]. The reaction of crotonaldehyde with bisulphite can be written as follows:



I II

The formation of the "normal" aldehyde bisulphite compound (I) takes place rather quickly. The addition of bisulphite to the ethylenic linkage proceeds comparatively slowly. When the "disulphonic" acid (II) is treated with weak alkali, the 1-sulphonic acid group is removed, whereas the 3-sulphonic acid group in the resulting sulphonic acid (III), remains unaffected:



II III

The bisulphite-addition compound of acetaldehyde is also decomposed in alkaline medium.

The method described in this paper is based on the facts given above. A mixture containing acetaldehyde and crotonaldehyde is treated with sodium bisulphite to form the bisulphite-addition compounds of these aldehydes. Subsequently, the mixture is made alkaline and the liberated acetaldehyde distilled off.

PROCEDURE

Ten to a hundred milliliters of an aqueous solution containing crotonaldehyde (<0.5 g) and acetaldehyde is transferred to a 200 ml Erlenmeyer flask and 25 ml approximately 10 % sodium bisulphite is added. The flask is tightly stoppered and allowed to stand overnight at 70—80° C. After cooling, 30 ml 1 *M* sodium bicarbonate is added and the liberated acetaldehyde distilled off to yield about 25 ml distillate (total distillation time approximately 15 min.). The end of the condenser tube dips below the surface of 10 ml 2 % sodium bisulphite contained in a volumetric flask which is cooled with ice-water. The amount of bisulphite in the collecting flask given above is sufficient provided that not more than 50 mg acetaldehyde is present. Otherwise the amount of bisulphite has to be increased. After standing for about ½ hour at room temperature acetaldehyde is determined according to the iodometric bisulphite method [2] or the colorimetric method with *p*-hydroxydiphenyl [3]. The bisulphite method is suitable for samples containing more than 10 mg of acetaldehyde. The colorimetric method is used at concentrations ranging from 0.0002 to 0.002 mg acetaldehyde per ml.

EXPERIMENTS AND RESULTS

In order to investigate whether crotonaldehyde reacts completely with sodium bisulphite and whether the sulphonic acid (III) remains unattacked during the distillation, experiments were performed with crotonaldehyde without the addition of acetaldehyde.

500 mg (7.13 mmoles) of crotonaldehyde (Eastman), redistilled *in vacuo* (b.p. 20°/30 mm), was treated according to the procedure described above. The iodometric determination indicated that "the bound bisulphite" in the distillate did not exceed 0.005 mmole. Fuchsine sulphurous acid gave no colour with a distillate to which no bisulphite had been added. On the other hand, if crotonaldehyde, redistilled at atm. pressure (b.p. 102.7°) was used, "the bound bisulphite" reached about 0.1 mmole and a bisulphite-free distillate gave a faint colour reaction with fuchsine sulphurous acid. The ultraviolet absorption spectrum of the bisulphite-free distillate, however, differed distinctly from that of crotonaldehyde. These observations indicate that distillation of commercial crotonaldehyde at atmospheric pressure may yield products containing carbonyl compounds as impurities.

Table 1.

Time of reaction (hours)	Added			Found	
	Sodium bisulphite 10 per cent (ml)	Crotonaldehyde (mg)	Acetaldehyde (mg)	Acetaldehyde (mg)	Relative error per cent
24	10	100	83.00	83.00 *	± 0.0
24	10	200	83.00	84.04 *	+ 1.3
24	10	250	83.00	81.84 *	− 1.4
24	10	250	41.50	42.24 *	+ 1.8
24	10	400	83.00	83.60 *	+ 0.7
17	25	50	10.00	9.75 *	− 2.5
17	25	500	10.00	10.38 *	+ 3.8
21	25	100	10.00	9.95 **	− 0.5
21	25	250	10.00	10.32 **	+ 3.2
7	25	500	10.00	9.95 **	− 0.5
21	25	250	1.000	1.001 **	+ 0.1

* According to the iodometric bisulphite method [2].
** According to the colorimetric method with *p*-hydroxydiphenyl [3].

In a further series of experiments, mixtures of crotonaldehyde (redistilled *in vacuo*) and acetaldehyde were treated according to the procedure described above and the acetaldehyde estimated in the distillates. As can be seen in Table 1 satisfactory values were obtained even in the presence of large amounts of crotonaldehyde.

SUMMARY

Acetaldehyde can be determined quantitatively in mixtures of acetaldehyde and crotonaldehyde by heating the mixture with bisulphite solution and subsequently distilling off the acetaldehyde in the presence of sodium bicarbonate.

The author wishes to thank Prof. O. Samuelson for his great interest in this work and for valuable discussions. The investigation has been supported by a grant from the *Swedish Institute (Svenska Institutet för Kulturellt Utbyte med Utlandet)*.

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Systematic Micro Detection of Arsenic, Bromine, Carbon, Chlorine, Fluorine, Iodine, Nitrogen, Phosphorus, and Sulfur in Organic Compounds, by a Modified Lassaigne's Method

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The elements which it is most frequently necessary to detect in organic compounds are: carbon, hydrogen, oxygen, nitrogen, sulfur, halogen, phosphorus, silicon, and arsenic, and less commonly, *e. g.* mercury, boron and antimony. There is so far no analytical scheme covering all these elements and usually only two to four of the elements are identificated after each combustion.

The large number of ways in which *nitrogen* may be bound in organic compounds makes a specific detection of this element difficult. Thus the choice of method for nitrogen combustion is the chief consideration in systematic analysis and dictates whether or not other elements may be detected after a single combustion. If nitrogen compounds are heated with *calcium oxide*, ammonia is liberated. Emich [1] used a mixture of calcium oxide and zinc, which is advantageous, among other things, for the simultaneous detection of sulfide and halogen. Bennett, Gould, Swift and Niemann [2] have developed Emich's test to a rapid systematic micro method, covering the elements: nitrogen, chlorine, bromine, iodine, sulfur, arsenic, and phosphorus. The method has been further investigated by Widmark [3], who has shown that disturbing cyanide also can be produced at the combustion.

When heated with carbon and *alkali metals* or magnesium, nitrogen compounds form cyanide, which can readily and specifically be detected as Prussian blue. Lassaigne [4] ignited the sample with sodium in an open vessel. Sodium has sometimes been replaced by potassium, which has a lower melting point and is more reactive. Castellana [5] used a mixture of magnesium and potash, with which the reaction is intensified if sugar [6] is also added. The risk of a reaction between magnesium and nitrogen from the air cannot, however, be neglected [7].

In the method proposed here *1—5 mg* of the test substances are combusted with *sodium* at 500—590° in *sealed ampoules*. The small amount of sample introduced makes the combustion in sealed tubes possible with only little danger of explosion. Indeed, the ampoule very seldom breaks even when explosives are analysed. Moreover, as the combustion should be carried out in an iron furnace, an explosion causes no harm.

In the systematic analysis which follows the combustion, water is added to the content of the ampoule, and the elements or groups of elements are *removed* from the solution as gases or precipitates. Hence, the detection of one element does not lower the sensitivity of the analysis of an element following in the scheme. Only for the detection of fluorine must the solution be divided.

Each element can be detected in the presence of all the others with the exception of phosphorus, which still cannot be detected in the presence of arsenic.

The analytical method developed is so simple that the analyses could be performed in an ordinarily equipped school laboratory. The main part of the specific equipment and the reagents are purposely made so durable and stable that they could be obtained *ready-made* commercially or be prepared in the laboratory and afterwards preserved for future analytical work.

PRINCIPLES OF THE ANALYTICAL SCHEME

Upon combustion the different elements of the test substance react with sodium. Arsenic forms arsenide, phosphorus phosphide, sulfur sulfide, and halogen halide. Nitrogen is converted to cyanide, the carbon source being either the organic compound itself or the xylene vapor which the ampoule contains. When the ampoule is opened and water added AsH_3 and PH_3 escape. The gases pass $AgNO_3$-impregnated silica gel giving different colours. The carbon forms a black deposit. A part of the alkaline solution is tested for fluorine and the remainder acidified with sulfuric acid, and boiled to free it from H_2S and HCN, but the halides are retained. The gases are taken up on alkaline ferrous sulfate paper, and the halogens are precipitated from the solution as silver salts and separated.

Not included in this systematic scheme are the important elements hydrogen, oxygen, and silicon and the less common elements such as antimony, boron, and mercury. The possibility of extending the method is being studied.

REAGENTS AND EQUIPMENT

Combustion ampoules. Seal off a piece of Pyrex tubing (outer diameter 6.5—7 mm, inner diameter 2—2.5 mm) of 16 cm length to form two equal round-bottomed tubes. Cut sodium wire (1.2 mm diameter; anal. pure) in 1 cm pieces under xylene. Rapidly wipe one piece free of liquid and transfer it to the bottom of the tube, warm for ½ hour at 95° in an oven and immediately heat in a flame about 1 cm from the sodium (without melting the metal), draw out, and, after a few seconds, melt off so that a 3—4 cm long peak is obtained (Fig. 1 a). A stock of such ampoules may conveniently be prepared. Reserve ampoules with thick peaks for solids.

Hydrogen cyanide reaction paper. Press a small round filter paper (*e. g.* Whatman No. 54) between a hole in a polystyrene plate and a bored stopper (Fig. 2 a). Place 1—2

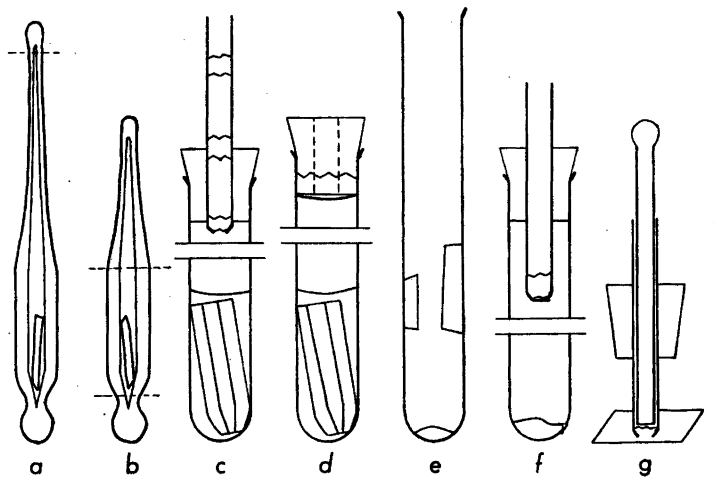

Fig. 1. a) Ampoule with sodium. b) Charged ampoule. c) Alkaline solution, with $AgNO_3$-$PbAc_2$-gel tube. d) Acid solution, with alkaline $FeSO_4$-paper. e) Silver halide precipitate, with fluorescein and starch papers. f) Fluorine test with moist glass wool. g) Fluorine test upon Zr-alizarinpaper.

drops of $FeSO_4$ solution (1 part saturated solution + 1 part water) on the paper and dry at max. 70°. Add 1 drop of 3 *M* NaOH and dry in the same way. Carefully loosen the cork with adhering paper from the plate. An active paper should have an even light brown colour and can be kept for a few weeks in a closed vessel.

Zirconium-alizarin paper. Place a filter paper in a solution of 0.2 g $ZrOCl_2 \cdot 8H_2O$ in 100 ml 4 *M* HCl for a few minutes, dry and dip twice into a solution of 0.01 g alizarin in 100 ml ethanol, and again dry. Cut the evenly red-violet part of the paper into pieces of ½ × 1 cm, and extract with small portions of ether until the ether solution becomes only weakly yellow in colour (about 5 times). The papers may be kept for a long time.

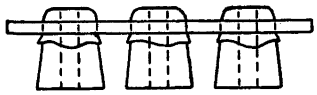

Fig. 2. a) Polystyrene plate (60 × 25 × 2 mm) with holes (diameter 8.5 mm) for bored stoppers with alkaline ferrous sulfate papers.

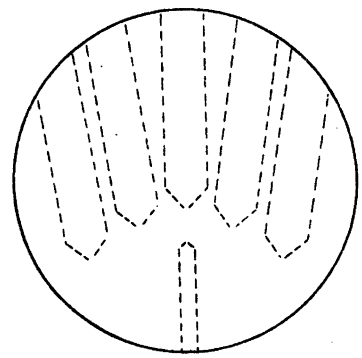

Fig. 2. b) Iron furnace (diameter 125 mm, height 50 mm) covered with 2 mm asbestos, 5 holes (diameter 15 mm, depth 55 mm) for asbestos tubes, one hole (diameter 7 mm, depth 50 mm) for a termo-couple.

Table 1. Equipment box for students' laboratories.

Bored stoppers	Sodium ampoules	Fluorescein papers	Zirconium alizarin papers	Round papers
Rubber plugs	Asbestos tubes Polystyrene plate Stainless wire Glass rod for fluorine test	Starch papers	Gel tubes	Fluorine tubes
Emich tubes File			Phosphate papers	Div.

Fluorescein paper. Impregnate filter paper with a saturated solution of fluorescein in 50 % ethanol. The papers may be kept for a long time.

Starch paper. Impregnate filter papers with 1 % starch solution. The dried papers may be kept for several months.

Gel tube. Constrict one end of a 4 cm long glass tubing with an inner diameter of about 3 mm. Place a small amount of fine glass wool at the constriction and add silica gel impregnated with lead acetate to a height of 1 cm and a similar layer of silver nitrate impregnated gel, the layers being separated by glass wool. (Fig. 1 c.) The tubes may be kept for more than a year when stored in a closed dark vessel.

Lead acetate gel. Stir silica gel (1 g, Davison, 0.1 – 0.4 mm), which has been oxidized in nitric acid, thoroughly washed and dried, moistened with a solution of 0.1 g $PbAc_2 \cdot 3H_2O$ in 1 ml of water, and dry.

Silver nitrate gel. Impregnate the gel (1 g) purified as above with 0.25 g silver nitrate in 1 ml of water. Store the dried gel in a dark closed glass vessel.

Asbestos tube. Cover a test tube (75 × 10 mm) with two layers of aluminium foil and bake on a 2 mm wet layer of asbestos and arm with 0.5 mm copper wire. Remove the glass tube and the aluminium foil after drying.

SUGGESTED ANALYTICAL METHODS

Addition of test substance. Shake the sodium to the bottom of the ampoule and cut off the top of the peak (Fig. 1 a).

Liquids and solutions. Warm the ampoule for a few seconds in a small flame. Attach a capillary containing the sample (*ca.* 5 μl) to the top of the ampoule. Cool so that the test liquid is drawn into the ampoule.

Solids. Press the top of the ampoule into the pulverized sample, and shake the column of substance (approximately 3 mm high) formed in the top down to the sodium. (Sometimes it is necessary to use a thin wire of stainless steel.) Three to four such fillings give sufficient substance.

Sealing the ampoule. Warm the tip about 1 ½ cm from the top, first very carefully in a small flame, so that any substance adhering to the glass melts and evaporates. The heating is then increased, causing the glass to melt. If a black line is formed, the ampoule will be properly sealed if the tip of the seal is drawn out again.

Glasses should be used during sealing and in handling the ampoule until it is again opened.

Combustion. Place the ampoule, sodium end downwards, in an asbestos test tube (70 × 12 mm) (see below), and heat for 15 minutes in a furnace (Fig. 2b) at 500—590°. The furnace should be heated with a Meker burner. Temperatures above 600° may cause explosion of the ampoule.

Arsenic and phosphorus

Remove the ampoule from the asbestos tube and cut off both ends as shown in Fig. 1 b. (Use a pair of tongs.) Smell the opened tube; *arsine* has an odor of garlic, *phosphine* of "carbide". Stir gently within the ampoule with a *dry* capillary which may then be used for taking out a fluorine test sample. Place the ampoule (sodium end downwards) in a test tube (75 × 10 mm) containing about ½ ml of water. Close the test tube immediately with a rubber stopper containing a gel tube (Fig. 1 c), the constricted end of which is turned downwards so that the escaping gas first passes the lead acetate layer. Tap the tube gently till all sodium has reacted with the water. Warm gently *.

Arsine gives, within two minutes, a black zone with a yellow top in the silver nitrate gel **. The yellow colour appears first. *Phosphine* gives a brown-black zone and the brown colour appears more clearly upon storing the gel tube. — The intensity of the zone colouration with phosphine is about one-tenth of that with arsine.

(Fluorine), sulfur and nitrogen

Remove the gel tube and boil the solution for 3 minutes and stir with the capillary previously used. Cool and transfer three drops of the solution to another test tube for the detection of *fluorine* (see below). Touch the emptied capillary against a drop of weak sodium nitroprusside solution; the trace of test solution remaining is sufficient to give violet colour if *sulfide* is present.

Add five drops of sulfuric acid (1 : 3) to the main solution and rapidly close the test tube with a hydrogen cyanide reaction paper (Fig. 1 d). Heat the tube and observe the reaction paper just before the liquid starts boiling. Hydrogen sulfide gives a black colour. After boiling for five minutes transfer the paper to a 20 ml test tube, add dilute sulfuric acid and warm. Iron hydroxide and sulfide are dissolved and *cyanide* is transformed to Prussian blue. In the presence of a large quantity of sulfur, the cyanide reaction may appear as a blue ring.

Carbon, halogen, and chlorine

Remove the ampoule, wash with a few drops of water and centrifuge the solution. Elementary *carbon*, liberated during the combustion, is deposited ***. Transfer the clear solution to a new test tube and add one or two drops of

* If the solution is boiled the whole gel column will become grey in colour.

** To check the arsine reactions, transfer the black zone (minimum 3 mm high) to a test tube, add 5 drops of conc. HCl and close the tube with a sodium sulfide paper. (Compare HCN reaction paper.) Boil for a short time. Yellow arsenic sulfide is formed on the paper.

*** To check if the deposit is carbon, 4 drops of concentrated HNO_3 are added to the black residue.

0.5 *M* silver nitrate solution. Centrifuge down the *halide* precipitate and decant the solution. Draw up the residual liquid with a capillary. Stir the precipitate for 2 minutes with 4 drops of 2 *M* NH_3. Most of the AgCl dissolves. Centrifuge and transfer all of the solution, with a capillary, to a new test tube. Add 2 drops of 4 *M* nitric acid, whereupon *silver chloride* precipitates.

Bromine and iodine

Dry thoroughly the remainder of the NH_3-treated precipitate in the test tube. It is essential that the walls also are completely dry. Mix the precipitate with a very little pulverized PbO_2 and moisten the mixture with about 20 μl sulfuric acid (1 : 3) from a capillary.

Fasten a fluorescein paper and a starch paper about 1 ½ cm from the precipitate in such a way that they are pressed out from opposite walls (Fig. 1 e). Careful heating liberates *bromine* to give the fluorescein paper an even red colour. The precipitate should still be black. Wait for 1 min. then increase the heating. The precipitate turns grey-white, and *iodine* is liberated, colouring the starch paper blue. Sometimes iodine forms a few very small red spots upon the fluorescein paper.

Fluorine

Heat the three drops of the alkaline solution to complete dryness. The walls of the tube must also be dry. Add 5 drops of concentrated sulfuric acid taking care that no acid contacts the upper parts of the tube. Close the tube with a rubber stopper containing a glass tube (empty gel tube). Place a small amount of moist fine glass wool in the constriction (Fig. 1 f). The reaction may fail to occur if there is too much water. Heat the tube for a few minutes at about 140°. Remove the stopper and press the water in the glass wool into a zirconium-alizarin paper by means of a small glass rod. (Fig. 1 g) *Fluorine* compounds destroy the red zirconium-alizarin lacquer and give a yellow spot.

DISCUSSION OF RESULTS

Detection of arsenic (sensitivity 1—5 μg As). When 5 μl of lewisite is heated with sodium for 15 min. at 100° in an ampoule, water added to the opened ampoule and the mixture heated for 3 min. to 80°, so much arsine is formed that a 1 mm high black shining zone with a yellow top is formed in the silver nitrate gel. If instead the lewisite and sodium are heated to 250° the whole gel column becomes black. A similar result is obtained with samples combusted for 15 min. at 400°, 450°, 500°, 550°, and 590° or for 3 min. at 550°. Combusted for 1 min. at 550° half the column blackens. In an analysis of 5 μl xylene solution containing 5 μg As as lewisite, a 1 mm yellow frontier with a scarcely visible black bottom is formed, and may be enlarged by a cautious suction from a pump; the same reaction is then obtained for 1 μg As as previously for 5 μg. Within 1 hour the main part of the black colour generally changes into lemon yellow. — If too much As_2O_3 (more than 10 mg) is com-

busted, the amount of sodium does not suffice to form Na_3As; the reaction in the gel tube will be very weak, and a black precipitate of As will form in the test tube after the addition of water.

Some unsaturated halogen compounds *e.g.* allyl bromide and hexachlorobenzene give sometimes a weakly grey-black zone (maximum 2 mm high) in the silver nitrate column. These reactions are devoid of the characteristic yellow or brown colours and can thus easily be distinguished from true arsine and phosphine reactions.

Detection of phosphorus (sensitivity 50 μg P). Tricresylphosphate (5 μl) has been combusted for 15 min. at 250°, 350°, 400°, 450°, 500°, 550°, and 590°. A temperature of 400° gives the maximum reaction in the $AgNO_3$ gel with a 4 mm black zone having a small yellow top. (The same reaction as for arsenic but less sensitive.) A temperature of 450° gives a black zone with a yellow brown top and 500—590° gives a dark brown zone about 2 mm high with a thin black bottom. The possibility of distinguishing between the arsine and phosphine reactions has governed the recommendation of the lower temperature limit (500°) for combustion. Otherwise satisfactory decomposition starts even at about 350°. The higher limit, 590°, is governed by the strength of the glass.

Tricresylphosphate (0.5 μl) heated for 5 min. at 550° gives a very weak brown zone, which is converted by suction to a 1 mm high black zone with a yellow top. Thus, the use of suction makes it impossible here to distinguish between arsenic and phosphorus. — PCl_3 (5 μl) heated for 15 min. at 550° blacken the whole gel column, but after an hour or two some brown particles appear, which can be recognized by careful examination.

The violent reaction between the water and the sodium remaining in the ampoule after the combustion seems to increase the sensitivity of the phosphine reaction in the gel. The shell surrounding the sodium should therefore be broken with a capillary.

The odour of phosphine, but not arsine, is distinctly noticeable when the ampoule is broken, with amounts under 50 μg. There is therefore a possibility of detecting smaller amounts of phosphorus than 50 μg.

Detection of sulfur (sensitivity 5—10 μg S). Organic sulfur compounds are so completely decomposed by potassium that Zimmermann[8] has developed a quantitative method for the determination of sulfur on this basis. Sodium is more convenient and has proved quite satisfactory for qualitative analyses. The low sulphate content in the glass does not interfere in the analyses.

Carbon disulfide (5 μl) combusted for 15 min. at 100° gives a clear sulfide reaction and temperatures above 250° do not seem to increase the reaction. The sulfide reaction appears for 2 μg S (as Na_2S solution) as a weak grey colouring of the dry, brown iron paper; 5—10 μg S give a clear black colour.

The small amount of alkaline solution which is left in the emptied capillary used for the fluorine test, is sufficient to give a positive sulfide reaction with nitroprusside solution, when the test tube originally contained more than 40 μg S.

Detection of nitrogen (sensitivity 10 μg N). As pointed out before the detection of nitrogen is the chief consideration in systematic qualitative analysis. Objections to Lassaigne's method are: a) low boiling substances evaporate before reaction with the sodium, b) diazo-, azo- and polynitro compounds

evolve nitrogen, c) some heterocyclic compounds do not react, d) halogen is difficult to detect in the presence of nitrogen.

Using the present method a cyanide reaction is obtained in all the three cases a)—c) mentioned. The azo and diazo compounds give a faint colour which is, however, quite visible. The heterocyclic compounds investigated give as strong a colour as other nitrogen compounds.

The carbon required for the formation of cyanide is provided by the decomposed organic compounds. The small amount of xylene which the ampoules contain, is, however, also sufficient to serve as a source of carbon. For example, combustion of $NaNO_2$, $NaNO_3$ and $Pb(NO_3)_2$ gives a spot of Prussian blue equal to that from about 10 μg N as NaCN.

Nitrobenzene reacts exothermically with sodium, forming cyanide even on heating to only 100°. A weak cyanide reaction is obtained when 5 μl quinoline are heated for 15 min. at 250°, but at 450° the colour is as strong as the corresponding one for nitrobenzene.

If the aqueous solution containing 2 mg CN from the combustion is boiled for 5 min. with 5 drops of sulfuric acid (1 : 3) all hydrocyanic acid but very little or no hydrogen halide escapes. Thus with this concentration of sulfuric acid the difficulty in detection of halogen (d) above is removed.

Sodium cyanide solution containing 0.3 μg N gives, on boiling with sulfuric acid, very faint blue spots on the paper. With 10 μg the whole area is coloured blue and with large amounts the paper becomes blue-black. The colour reaction from 0.5 ml solution containing 10 μg CN is not decreased by 5 mg NaF, NaCl, NaBr, NaI, $NaNO_3$ or NaH_2PO_2. The presence of sulfide decreases the cyanide colour and often causes it to take the shape of a blue ring. With 1 mg sulfide and 10 μg cyanide one test out of three failed. With higher cyanide content a colour was always obtained.

Detection of carbon (sensitivity *ca.* 200 μg C). During the combustion carbon is liberated from carbon compounds. The small amount of xylene which the ampoule contains is not enough to give a visible precipitate of carbon.

Compounds (5 μl test) containing much carbon give a black precipitate covering the whole bottom of the test tube. Some nitrogen compounds, *e. g.* tetrazine carboxylic acid and chloropicrin give visible precipitates after centrifuging. NaCN and NaCNS do not give precipitates of carbon.

Salts of some metals, *e. g.* SbOCl, Bi_2O_3, $SnCl_4$ and $PbSO_4$ and excessive amounts of As_2O_3 give similar black precipitates. Mercury compounds give a little shining ball of mercury. Phosphorus compounds give sometimes, at a combustion temperature over 450°, a brown deposit. However, all these precipitates are soluble in conc. nitric acid.

The following compounds with low carbon content gave black precipitates not soluble in conc. nitric acid: carbon tetrachloride, chloroform, chloropicrin, dichlorodifluoromethane, imidazole, nitromethane, sodium acid carbonate, sodium carbonate, sodium oxalate, tetrazine dicarboxylic acid, trifluorochloromethane, trifluorotrichloroethane.

The detection of carbon is beeing further investigated.

Detection of halogen (sensitivity 40 μg). Most halogen compounds investigated react exothermically with sodium at 100°. Increased temperature or time of combustion does not improve the reaction. It is very important that all the hydrocyanic acid is removed from the acidified solution, as otherwise silver cyanide will be precipitated upon the addition of silver nitrate.

Amounts of chlorine below 40 μg cannot be detected distinctly without aerating the solution, as an opalescence may be caused by the cyanide.

It is only possible to achieve the separation of bromine and iodine when the mixture is very slowly heated. The reactions may fail to appear if sulfuric acid or too much water contaminates the paper.

Detection of fluorine (sensitivity 30 μg). The fluorine reaction (Bennett [2]) has been carried out with 3 drops of the aqueous alkaline solution *without* distillation, but the reaction often gives incorrect results for compounds containing sulfur-nitrogen, and phosphorus-chlorine.

Satisfactory results are obtained only with a dried sample and by warming with concentrated sulfuric acid, the gases (HF and SiF_4) being taken up in moistened glass wool.

Zirconium-alizarin paper which has been made by impregnating first in zirconium solution and then in alizarin solution gives a stronger colour than that which has been impregnated in a mixed solution.

The red zirconium-alizarin lacquer is not bleached by the salts: NaCl, NaI, Na_2CO_3, Na_2SO_3, Na_2S, NaCl, and $Na_2C_2O_4$. Positive results are obtained with mixtures of 10 μg fluorine ion and 5 mg of the salts. The reaction is only disturbed by bromide ion, which in large amounts (over 500 μg) gives the same reaction as fluoride ion. In analyses of PBr_3, and $CHBr_3$ brown fumes of bromine are envolved which sometimes give a weak fluorine test. Sulfuric acid also gives the paper a yellow colour, but in the recommended procedure there is no risk of splattering.

Experiences from students' laboratories. The errors which appear in student analysis are usually concentrated in the first tests. A laboratory introduction confined to 1) boiling off HCN from 0.5 ml solution containing about 2 mg NaCN, 2) separation of Cl, Br and I in the same sample, 3) evaporation of sodium fluoride solution and reactions upon zirconiumalizarin paper, is advantageous in preventing students' errors.

SUBSTANCES TESTED

The substances investigated are tabulated for each element with subheadings for the different groups. Within the groups the substances are arranged according to increasing substitution. For the remainder, alphabetical order is used.

Arsenic compounds
Arsanilic acid
Arsenoacetic acid
Arsphenamine
Diphenyl chloroarsine
Diphenyl cyanarsine
Ethyl dichloroarsine
p-Hydroxyphenylarsonic acid
"Lewisite"
"Marpharside"
Neoarsphenamide
3-Nitro-4-hydroxyphenylarsonic acid
Phenylarsonic acid
Sodium arsonoacetate

Phosphorus compounds
Codeine phosphate
Diethoxychloro phosphate
Dimethylaminodichloro phosphate
Dimethylaminodiethoxy phosphate
Ethoxydichloro phosphate
Phenylphosphorus oxychloride
Phosphorus oxychloride
Phosphorus tribromide
Phosphorus trichloride
Sodium glucose phosphate
Sodium glycero phosphate
Sodium phenyl phosphate
Spermidin phosphate
Tricresyl phosphate
Triphenyl phosphate

DFP
Mintakol
Tabun
TEPP
Sarin

Sulfur compounds
Bis-(*a*-chloroethyl) sulfide
Carbon disulfide
Diphenyl sulfide
Diphenyl thiourea
2-Thiobarbituric acid
Thioglycolic acid
2-Thiosalicylic acid
Thiourea
Trithiane

Dimethyl sulfate
Diethyl sulfate
Potassium ethyl sulfate
Methanesulfonic acid
Trional

Benzenesulfinic acid
Benzenesulfonic acid
p-Toluenesulfonic acid
Benzenesulfonyl chloride
p-Toluenesulfonyl chloride
3,4-Dichlorobenzenesulfonyl chloride

Methyl menthylxanthate
Saccharin
Sulfapyridine
Sulfathiazole
Thionyl chloride

Thiophene
2-Methylthiophene
2,5-Dimethylthiophene

Amines
Methylamine, 6 % in benzene
Methylamine, 30 % in water
n-Propylamine
iso-Butylamine
iso-Amylamine
Diethylamine
1,3-Diamino-2-propanol
Tri-*n*-butylamine
Tri-*n*-amylamine
Triethanolamine
Tetramethylammonium iodide

Cyclohexylamine

Aniline
o-, *m*-, *p*-Chloroaniline
o-, *m*-, *p*-Bromoaniline
Mesidine
a-Naphthylamine
vic-Xylidine

Diphenylamine
n-Dimethylaniline
Triphenylamine

2-Aminopyrimidine
2-Amino-3-methylpyrimidine
2-Benzylamino-pyridine
Bis-(2-aminothiazole)

Amides
Acetamide
n-Iodo-acetamide
n-Dimethyl-formamide
Barbituric acid

Acetanilide
n-Diethylbenzamide
ω-Chloro-2,6-dimethylacetanilide

N-Acetyl-*p*-phenetidine

Nitro compounds
Chloropicrin
Ethyl nitrate
Nitromethane
Tetranitromethane
Nitroglycerin, 10 % in ethanol

Nitrobenzene
Nitroanthrone
3-Nitrophtalic acid
3-Nitroquinoline
2,4-Dinitrochlorobenzene
3,5-Dinitrobenzoic acid
2,4,6-Trinitrobenzoic acid
1,3,5-Trinitrobenzene
2,4,6-Trinitrotoluene
2,4,6-Trinitro-*m*-xylene
Picric acid

Ethyl nitrite
iso-Amyl nitrite

Nitroso compounds and oximes
Nitrosomethyl urea
Diphenylnitrosamine
sym-Nitroso-*m*-xylene
1-Nitroso-2-naphthol
2-Nitroso-1-naphthol

Dimethylglyoxime
Cyclohexanone oxime
Benzoin oxime
Camphor oxime
a-Pinene oxime
Δ^3-Carene oxime

Cyano compounds
Acetonitrile
Ethyl cyanoacetate
Diphenyl cyanarsine

Benzyl cyanide
Benzonitrile
p-Tolunitrile

Isothiocyanates and thiocyanates
Allyl *iso*thiocyanate
Phenyl *iso*thiocyanate

Methyl thiocyanate
Ethyl thiocyanate
iso-Propyl thiocyanate

Hydrazines
sym-Dimethylhydrazine, 2HCl
Dimethylketazine

Hydrazobenzene
Phenylhydrazine
α-Benzyl-α-phenylhydrazine
Tetraphenylhydrazine

Azo compounds
Azobenzene
Azoxybenzene
Benzene-azo-β-naphthol
Methylorange
Orange G
Scarlet red
Sudan III

Diazo compounds
Bis-(diazoacetic acid)
Ethyl diazoethanoate
Phenylbenzoyldiazomethane

p-Diazobenzenesulfonic acid
o-Diazobenzoic acid perbromide
2,6-Dimethoxy-benzenediazoniumsulfate

Triazo compounds
Triazobenzene
o-Triazobenzoic acid

Heterocyclic 5-member compounds
Pyrrole
N-Phenylpyrrole
2,4-Dimethylpyrrole
2,5-Dimethylpyrrole
2,4-Dimethyl-5-carbethoxypyrrole
2,4-Dimethyl-3,5-dicarbethoxypyrrole

Pyrrolidine

Indole
2-Methylindole
2-Phenylindole
Isatin
Indigotin
Carbazole

Imidazole
Benzimidazole

3,5-Dicarbetoxy-4-hydroxypyrazole
3-Methyl-1-phenyl-5-pyrazolone
Antipyrine

Benzotriazole
Chloracetylbenzotriazole
Phenylmethyl-1,2,3-triazole carboxylic acid
Phenylmethyl-1,2,3-triazole

Cardiazol

2-Methylbenzothiazole

Heterocyclic 6-member compounds
Pyridine
2-Picoline
2,6-Lutidine
2,4,6-Collidine
Picolinic acid
Nicotinic acid
iso-Nicotinic acid

α,α'-Dipyridyl
Quinoline
Quinaldine
6-Methylquinoline
Kynurenic acid
2-Phenyl-quinoline-4-carboxylic acid

Acridine

Uric acid
Uracil
Thymine
Trichloropurine

Morpholine
Piperazine
Dihydrodiphenylpiperazine
Cyanuric acid

Tetrazine dicarboxylic acid

Thiodiphenylamine

Mixed nitrogen compounds
Acetylcholine iodide
Alanine
Amygdalin
Arecoline bromide
Atropine sulfate
Bilirubin
Brucine
Coffeine
Cinchonine sulfate
Cocaine chloride
Codeine
L-Cysteine
Dihydrostreptomycine
3,5-Diiodotyrosine
Guanidine carbonate
Hematein
Heroin
Hexamethylene tetramine
Histidine
Hydroxyproline
Lactoflavin
Morphine
Narcotine
Penicillin G
Pilocarpine chloride
Spermidin phosphate
Sulfapyridine
Sulfathiazole
Synazur
Thacapzol

Chloro compounds
Methylene chloride
Chloroform
Carbon tetrachloride
Ethylene chloride
Trichloroethylene
Glycerol 1,3-dichlorohydrin

Acetyl chloride
Ethyl chlorocarbonate
Ethyl chloroacetate
Phenacyl chloride
Phosgene, 20 % in toluene
Diphosgene

Chlorobenzene
p-Chlorotoluene
Hexachlorobenzene

Chloranil
Chloramine T
Pyridiniun. chloride

Bromo compounds
Methyl bromide
Ethyl bromide
n-Propyl bromide
iso-Amyl bromide
Allyl bromide
Methylene bromide
Ethylene bromide
β-Butylene bromide
Bromoform

Bromoacetic acid
β-Bromopropionic acid
α,α'-Dibromoadipic acid
n-Propyl bromoacetate
n-Butyl bromoacetate
Bromobenzene

o-, *m*- and *p*-Bromotoluene
9,10-Dibromo anthracene
Eosin

Iodo compounds
Methyl iodide
Ethyl iodide
Iodoform

Iodobenzene
Iodobenzene dichloride
Iodosobenzene
Iodosobenzoic acid
Iodoxybenzene
p-Iodoaniline
2-Iodo-5-methylaniline

3,5-Diiodo-L-tyrosine
2,6-Diiodosozolic acid
Erythrosin
Synacyr
Tetramethylammonium iodide

Fluoro compounds
Dichloro difluoromethane
Trichlorofluoromethane
Trichlorotrifluoroethane
Trifluoroacetic acid

Fluorobenzene
p-Fluorobenzoic acid
Ethyl *p*-fluorobenzoate
m-Fluoronitrobenzene

DFP
Sarin

SUMMARY

A rapid scheme of micro elementary analysis by a modified Lassaigne method is presented. The scheme covers the elements As, Br, C, Cl, F, I, N, P, and S.

A quantity of 1—5 mg of the organic compound is combusted at 500—590° with sodium in a sealed ampoule of pyrex glass. The ampoules, containing a piece of sodium, and the reagents are made so durable and stable that an analysis can be started without noteworthy preparations.

Positive results were obtained for all the substances investigated, including 1) easily volatile compounds, 2) polynitro, azo and diazo compounds, and 3) heterocyclic compounds. Diazo compounds gave the weakest reactions.

Each element can be detected in the presence of all the other elements, with the exception of phosphorus, which cannot be detected by this scheme, when arsenic is present.

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Isolation and Properties of Crystalline Fe-Transferrin from Pig's Plasma

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A method for the isolation of crystalline β_1-metal-combining pseudoglobulin *(siderophilin, transferrin)* from blood plasma has been described by Koechlin [1], who used the plasma fractionation system of Cohn *et al.*[2,3]. The final product is obtained "in a mainly iron-free state" and is crystallisable.

The reactive groups responsible for the affinity of the transferrin for iron are fairly labile. The iron-binding capacity of the transferrin is therefore often reduced during the isolation. When transferrin forms an iron complex (Fe-transferrin), the reactive groups are protected and more stable. It was mainly this stabilisation that suggested the development of the method [4] for isolating iron-saturated transferrin (Fe-transferrin). This isolation procedure differs from that described by Koechlin, this difference being ascribable mainly to the fact that Fe-transferrin is more soluble than transferrin in strong salt solutions and in water-alcohol solutions of low ionic strength. It was later found that Fe-transferrin is fairly resistant to chloroform-alcohol solutions. This resistance has been utilised for eliminating those proteins which even in low concentration inhibit crystallisation of the Fe-transferrin. Satisfactory crystallisation is achievable only if at least 95 per cent of the final product consists of Fe-transferrin.

EXPERIMENTAL

Purification procedure*

I. Fe^{++} (Mohr's salt) is added to pig's serum (6 mg Fe/litre), the amount of iron being slightly in excess of the specific iron-binding capacity of the serum.

II. Fe-transferrin is precipitated with ammonium sulphate (60—72 % saturation).

III. The major part of the albumin is precipitated at pH 5.2 with 20 % by volume alcohol (low ionic strength, + 4°). In order to prevent partial dissociation of the Fe-transferrin, 1 mg of Fe^{++} (Mohr's salt) is added for every litre of protein solution. The Fe-transferrin is now in the supernatant after centrifugation.

IV. The concentration of alcohol in the supernatant layer is increased to 22 % by volume, and the temperature is lowered to −12° C. The precipitate is separated the following day. The precipitate, of which about 10 % consists of transferrin and the remainder of albumin, is dissolved in water and dialysed for 24 hours against running tap water. The dialysed solution is called fraction 1.

* Steps I—VI have been described in detail earlier [4].

V. The concentration of the alcohol in the supernatant is increased to 25 % by volume. The precipitate obtained after the mixture has been allowed to stand for 24 hours at −12° C is separated by centrifugation (−12° C). Some 30−50 % of the precipitate consists of Fe-transferrin. The precipitate is dissolved in water and dialysed for 24 hours against running tap water. The dialysed solution is called fraction 2.

VI. The concentration of the alcohol in the supernatant layer is increased to 28 % by volume. The precipitate consists of Fe-transferrin, a β_1-globulin with haemochromogen-like spectrum and a α_1-globulin. The precipitate is dissolved in water and dialysed for 24 hours against running tap water. The dialysed solution is called fraction 3.

VII. The Fe-transferrin can be isolated from fractions 1, 2 and 3 after precipitation with chloroform-alcohol in accordance with the procedure described below. The quantitatively and qualitatively best yield is obtained from fraction 2.

The pH of the dialysed protein solution is adjusted to 6.0 with 1 *M* acetate buffer (pH 5.5), and 50 μg Fe++ (Mohr's salt) per g protein is added. The solution is diluted with water until the concentration of the protein is about 4 %, and the solution is cooled down to 0° C.

250 ml chloroform-alcohol solution (1 part chloroform and 9 parts 95 % alcohol) is quickly added to each litre of the protein solution into which it is vigourously stirred. The mixture is centrifuged within 30 minutes at 0° C. Most of the transferrin is now in the supernatant layer. It is cooled to −5° C and a further quantity of 175 ml chloroform-alcohol per litre is gradually added, during which the solution is continuously stirred. The temperature of the solution must not rise above 0° C during the addition of the chloroform-alcohol. The mixture is centrifuged in a refrigerated angle centrifuge (−5° C). The major part of the transferrin is now in the precipitate, which is suspended in water and is immediately centrifuged for 10 minutes at about 20° C. The supernatant layer contains Fe-transferrin (fraction A).

After the last precipitation with the chloroform-alcohol the supernatant layer contains some Fe-transferrin and the main part of the abovementioned protein with haemochromogen-like spectrum. Both are precipitated on addition of 10 % solution of calcium chloride (10 ml/litre) to the supernatant. After the solution has been allowed to stand for 24 hours at −12° C the precipitate is collected by centrifugation at −10° C. The precipitate (fraction B) is suspended in distilled water.

Crystallisation of the Fe-transferrin from fraction A.

Crystalline Fe-transferrin can be obtained from fraction A if at least 90 % of the protein consists of Fe-transferrin. If Fe-transferrin is not present in such concentration, repetition of step VII will usually secure such purity. On careful addition of alcohol to a Fe-transferrin solution of sufficient strength (at least 5 %) and with a pH of 7.5 to 4.5 and low ionic strength at 0° C, Fe-transferrin will crystallise. Crystallisation occurs quickest at pH about 6.

Technique. Fraction A is dialysed for 18−24 hours against running tap water. The pH is adjusted to 6 by careful addition of a 2 % solution of acetic acid. The solution is then cooled to 0° C. Afterwards and during continous stirring 90 % alcohol (−10° C) is added dropwise until a slight cloudiness appears. The solution is then warmed to room temperature (20−22° C), at which the precipitate is re-dissolved. The solution is transferred to a closed vessel and allowed to stand at 4° C. Inspection under a loupe (× 10) will often reveal the formation of crystals within an hour, though sometimes not until the following day. Formation of an amorphous precipitate indicates that too much alcohol had been added or that the fraction was not sufficiently pure. The use of too much alcohol can be remedied by the addition of a little water to the solution. The more homogeneous the preparation the quicker do crystals form.

The yield of crystals increases during the first few days. Crystallisation can be accelerated by increasing the concentration of the alcohol by a couple of per cent after 24 hours. On re-crystallisation the crystals assume a macroscopically visible size within an hour.

On re-crystallisation of Fe-transferrin from more concentrated solutions (at least 10 %) the crystals formed measure several millimetres (Fig. 1). The lower the concentration of the alcohol the slower the crystallisation, but the larger the crystals in the end product.

Fig. 1. Fe-transferrin crystallised in a 0.1 cm thick cuvette at pH 6 from an ethanol-water mixture.

Analysis of re-crystallised Fe-transferrin

The determinations given below refer to analyses of preparations re-crystallised four to six times. The degree of purity of each preparation was checked by paper electrophoresis, performed at pH 8.6 at which each of the preparations showed only a spot corresponding to the position of β-globulins. No other proteins were present in measurable concentration.

The dry weight determinations (drying to constant weight at 105° C) were used as a standard for the calculation of the constants given below.

The nitrogen content, as determined by the method of Hiller, Plazin, & van Slyke [5], was 16.3 %.

The carbohydrate content, as determined by Friedmann's modification [6] of the method described by Sörensen *et al.*, was 1.4 %.

The level of the total lipids was so low that it lay below the range of sensitivity of the method [7] used. The preparations were practically lipid-free, at least less than 0.4 %.

The iron content of seven preparations studied ranged between 0.120 and 0.136 % (average 0.126 %).

The extinction coefficients (E) were determined at those wave lengths at which the maximum absorption was obtained in the visible as well as in the ultraviolet part of the spectrum. All values correspond to the extinction of a 1 % protein solution in a 1 cm cuvette at 20° C.

E (470 mμ, pH 7.0) varied between 0.48 and 0.55; E (278 mμ, 0.1 N HCl) was 11.0; E (280 mμ, pH 7.0) was 13.8 and E (290 mμ, 0.1 N NaOH) was 12.7 immediately after addition of sodium hydroxide and reached a relatively stable level at 13.3 60 minutes later.

DISCUSSION

The method described above is useful for the isolation of a product from which Fe-transferrin can readily be crystallised. The crystals (Fig. 1) may grow to 2—4 mm in length.

The iron content of the final product varied between 1.20 and 1.36 μg Fe/mg protein. The iron content, as calculated on theoretical grounds, was 1.27 (1.24) μg/mg protein, if the molecular weight be taken as 88 000 (90 000) and if two atoms of iron are linked to every molecule of protein. The range of variation in the experimental determinations may be explained by the addition of iron during the isolation procedure in order to prevent dissociation of the

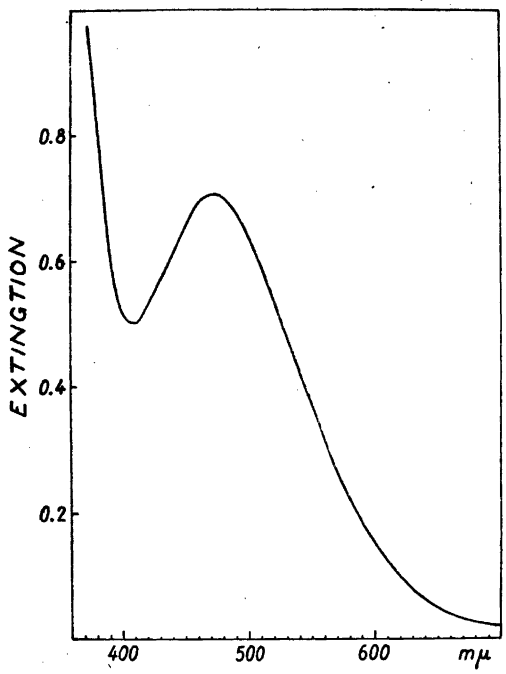

Fig. 2. Light absorption of Fe-transferrin (1.29 %) between 380 and 700 mμ at pH 7.0 (phosphate M/15) (1 cm cuvette).

complex when the pH of the solution fell below 6. Transferrin, just as well as other proteins, is capable of taking up iron in a loose non-specific bond. Unlike the specific Fe-transferrin complex with maximum absorption at 470 mμ, these types of non-specific iron complexes do not absorb any appreciable amount of light in the visible part of the spectrum. The loosely bound iron in one of the preparations was eliminated from the final product by passing the preparation through a column with a cation exchange resin (IRA 120) at pH 7.5. The iron content of the preparation decreased to 1.25 μg per mg protein without any accompanying change in light absorption per mg protein measured at 470 mμ ($E = 0.55$). It may be assumed that the loosely bound non-specific iron was, roughly speaking, quantitatively eliminated by the passage of the solution through the exchange resin. The extinction value at 470 mμ and recalculated per μg iron /E(1μg Fe/ml, pH 7.5) = 0.44/ was slightly higher than that of any of the other preparations. Extinction at 470 mμ per unit of concentration of the iron is the best determination for deciding whether any iron present is specifically bound.

The E-values can be used for checking the purity and integrity of the final product. Values below 0.55 indicate either that the protein is not homogeneous or that the iron-binding groups have been damaged during the isolation procedure, provided the value cannot be increased to 0.55 by the addition of a ferrisalt to the preparation.

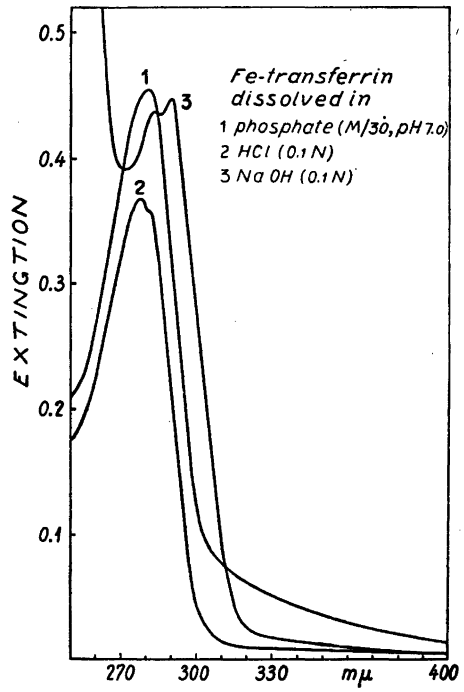

Fig. 3. U.V. light absorption of Fe-transferrin (0.0243 %) in neutral, alcaline and acid medium (1 cm cuvette).

After the Fe-transferrin has been crystallised once it is, as judged by electrophoretic analysis, practically homogeneous, but examination of the light absorption of the preparation in a wave range of 400—500 mμ, will show that such homogeneity is only apparent. Before crystallisation the preparations contain a haemi-globulin presumably formed during the isolation procedure. As far as solubility is concerned, this haemi-globulin resembles Fe-transferrin, but it contains about 10 % carbohydrate. Its electrophoretic mobility lies between that of α_2 and $\beta_1$globulins. It is characterised by very strong absorption of light at 408 mμ and a weaker absorption at 533 mμ. The strongest band falls within a wave range in which the absorption of Fe-transferrin is least (Fig. 2). The purity of the Fe-transferrin, as measured by its absorption spectrum, can thus be checked by the ratio between the absorption at 470 mμ and 408 mμ. Before crystallisation this ratio is usually less than 1, but after the preparation has been crystallized four or five times the ratio is usually 1.40—1.41 and cannot be increased by further crystallisation.

Earlier [8–10] spectra of Fe-transferrin have been illustrated simply as difference spectra between the irony and the iron-free protein, this probably being dictated by the difficulties presented by the quantitative elimination of the haemi-muco-globulin. By using difference spectra the influence of the haemi-muco-globulin is eliminated.

According to Surgenor *et al.* the maximum light absorption by Fe-transferrin is found at 465 mμ and according to Schade [10] at 460 mμ. Both found

E to be 0.57. These values, which were found for transferrin isolated from human serum, agree rather well with those found for pig's serum in the present study (E at 470 mμ = 0.55).

The extinction coefficients for the light absorption of Fe-transferrin with respective maxima in the ultraviolet part of the spectrum are summarised on p. 1409 (Fig. 3).

The absorption at 290 mμ increases during the first hour after the addition of 0.1 N NaOH, after which it reaches a fairly steady level, at which it persists for the few following hours. The light absorption of Fe-transferrin (pH about 13, 280—290 mμ) suggests that the molar ratio between the tyrosine and tryptophane in this protein is 2 : 1.

Comparison of the absorption curves (Fig. 3) distinctly showed that the tyrosine peak was about 25 % higher in neutral environments than in acidic medium. As indicated by the disappearance of the peak at 470 mμ the bond between the iron and the transferrin breaks down in the presence of 0.1 N HCl. The difference between the level of the tyrosine peak in neutral and acid environments may possibly suggest the inclusion of tyrosine in the groups responsible for the specific bond between the iron and the transferrin. In neutral medium the light absorption lies in the region of 300—350 mμ thus likewise strikingly high. This was observed in all of the preparations. Centrifugation for 1 hour at 16 000 r.p.m. produced no change in the shape of the absorption curve. Light absorption at 300—350 mμ is probably related to the specific iron bond of the Fe-transferrin because the absorption values decrease on release of iron from Fe-transferrin both in acid and alkaline medium.

SUMMARY

1. A procedure is described for the isolation of Fe-transferrin from pig's serum in a crystalline state.

2. Data are given about the composition and light absorption of re-crystallised Fe-transferrin.

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Separation of Conjugated Bile Acids by Partition Chromatography. Bile Acids and Steroids 6

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A method for obtaining a quantitative separation of some conjugated bile acids was described by Ahrens and Craig [1]. They applied counter-current distribution to the extraction of bile acids from other components of bile and to the subsequent separation of mixtures of various conjugated and free bile acids into individual components. This method is, however, too time-consuming for routine analyses. More convenient for this purpose is the reversed phase partition chromatography of Howard and Martin [2], used by Bergström and Sjövall [3,4] for separation of free bile acids. Some results with this technique for the separation of conjugated acids have been reported recently by Bergström and Norman [6].

In this paper a more systematic study is described of the separation of the taurine and glycine conjugates of the common bile acids from each other and from free bile acids.

EXPERIMENTAL

The samples of conjugated bile acids used have been synthesized according to a method recently described by Bergström and Norman [5]. Cholic acid-24-^{14}C was material prepared by Bergström, Rottenberg and Voltz [7].

For details of the chromatographic procedure see Bergström and Sjövall [3] and Sjövall [4].

Preparation of columns for reversed phase partition chromatography

The columns were prepared essentially as described by Howard and Martin [2]. 4 ml of the stationary phase and 4.5 g hydrophobic kieselguhr was stirred until homogeneous. Moving phase was added to obtain a slurry that could be poured into the chromatographic tube, int. diam. 12 mm, height about 300 mm. After homogenization with a perforated plunger the kieselguhr was left to settle under weak suction. When all the material was packed, a solution of the bile acids to be analyzed in the smallest possible volume of moving phase was added.

Preparation of columns for ordinary partition chromatography

For separation of the most hydrophilic taurine conjugates ordinary partition chromatography has been used with amyl alcohol as moving phase and water as stationary phase. When the stationary phase was added to the kieselguhr, homogenized and suspended in the moving phase the material was very lumpy. Even after careful homogenization in the chromatographic tube with a plunger the kieselguhr had a marked tendency to form inhomogeneous columns. The best results were obtained when the columns were prepared in the following way:

9 g of hydrochloric acid washed, ordinary kieselguhr and enough stationary phase to make a slurry was poured into a chromatographic tube, int. diam. 17 mm and height about 400 mm. The slurry was homogenized with a perforated plunger and allowed to settle by gravity. The stationary phase is allowed to drain and at the moment when the solvent meniscus disappears in the kieselguhr the moving phase is added. The excess of the stationary phase was then removed by adding the moving phase until no stationary phase could be detected in the effluent, generally after 24 hours in a thermostat room (23°). The solution of the bile acids to be run were then introduced on to the top of the column.

The chromatographic procedure

All chromatographic work was done at a constant temperature of 23° C.

The effluent was collected in test tubes by using an automatic fraction collector, each fraction containing 1.5—2 ml (5—10 minutes). The eluted bile acids were determined by micro titration under N_2 with 0.02 *N* KOH in methanol using bromothymol blue as indicator. When ^{14}C-labelled acids were used, each fraction was brought to dryness after titration and then transferred to copper planchets for determination of the isotop content.

Phase systems for reversed phase partition chromatography

	Moving phase.*	Stationary phase.
A.	180 ml of methanol 120 ml of distilled water	45 ml of chloroform 5 ml of heptane
C.	150 ml of methanol 150 ml of distilled water	15 ml of *iso*-octanol 15 ml of chloroform
D.	300 ml of distilled water	100 ml of *n*-butanol
F.	165 ml of methanol 135 ml of distilled water	45 ml of chloroform 5 ml of heptane
G.	255 ml of methanol 45 ml of distilled water	50 ml of heptane

* For the preparation of a certain phase system the volymes of solvents indicated were mixed in a separatory funnel and equilibrated when the mixture had reached the temperature of the room (23°).

Phase system for ordinary partition chromatography

E. Moving phase: 510 ml of *iso*-amyl alcohol and 90 ml of chloroform. Stationary phase: 200 ml of distilled water.

RESULTS

A. Separation of the tauroconjugates from the glycine conjugated and free bile acids

The separation of the taurine-conjugated acids from other bile acids offers no difficulties since the taurine-conjugates are more hydrophilic than all other bile acids. The solvent system used for this purpose has recently been described by Bergström and Norman [6].

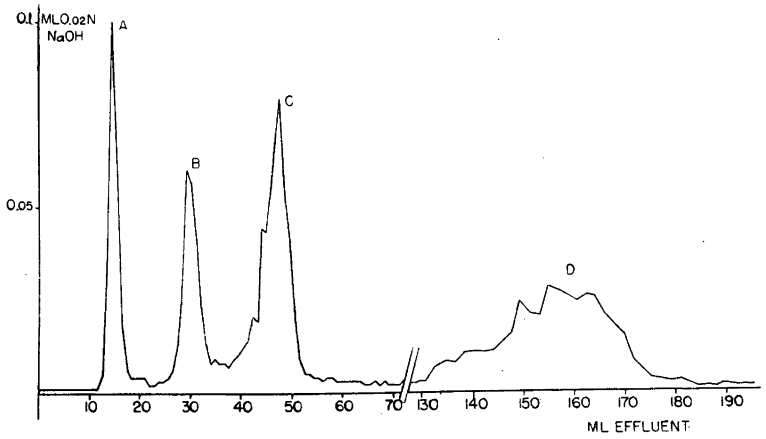

Fig. 1. Separation of taurocholic acid (A. 4.1 mg), taurocholanic acid (B. 4.4 mg), glycocholic acid (C. 6.2 mg) and cholic acid (D. 5.9 mg). Moving phase: 46 % methanol. Stationary phase: 50 % chloroform and 50 % iso-octanol. Cf. Table 1.

Table 1. Rate of elution of bile acids in relation to the composition of the moving phase. Stationary phase: isooctanol / chloroform 1 : 1.

Moving phase:	50% MeOH	47.5 % MeOH	46 % MeOH	40% MeOH	30% MeOH	25%MeOH
Cholic acid	80—98—112	110—140—170	130—160—175			
Glycocholic acid	32—37—42	38—44—50	40—48—54			
Taurocholic acid	12—14—16	12—14—16	12—14—16	12—14—16	14—17—20	14—17—20
Taurodesoxycholic acid	12—14—16	12—14—16	12—14—16			
Taurolithocholic acid	16—22—28				20—25—30	30—38—44
Taurocholanic acid	22—27—34	24—29—36	26—30—36	30—35—40	48—56—70	60—70—90

Column: 4.5 g hydrophobic Supercel with 4 ml stationary phase. The phases have been prepared by mixing 15 ml chloroform, 15 ml *iso*octanol and 300 ml aqueous methanol of the composition indicated (v/v). The figures give the beginning, peak and end of each bile acid band as the total volume (ml) of effluent at these points.

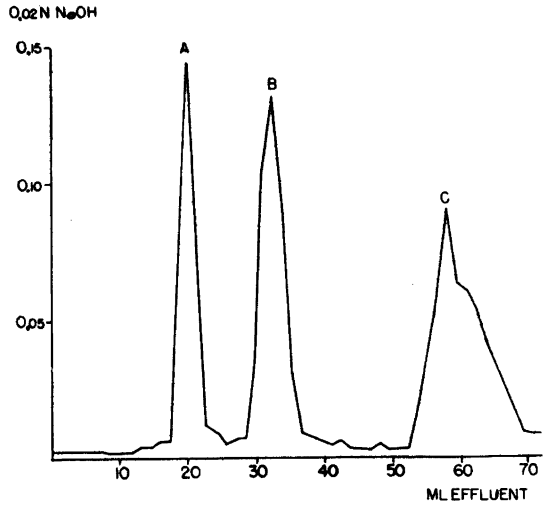

Fig. 2. Separation of taurocholic acid (A. 4.5 mg), taurodesoxycholic acid (B. 5.6 mg) and taurolithocholic acid (C. 6.5 mg). Phases: Type D, see p. 1414.

The influence of the taurine group is so pronounced that even the taurine conjugate of unsubstituted cholanic acid can be eluted from column before glycocholic acid using phase system C. This is best illustrated in a chromatography with 46 % methanol as moving phase and chloroform-*iso*octanol 1 : 1 as stationary phase (Fig. 1), where taurocholic, taurocholanic and glycocholic acid are clearly separated from each other. It is of interest to point out the great influence on the rate of elution of cholic acid caused by lowering the methanol concentration in the moving phase from 50 to 46 %. By comparison, the elution of taurolithocholic acid is not delayed more than 20 ml when the methanol concentration in the moving phase is lowered from 50 to 25 %, cf. Table 1.

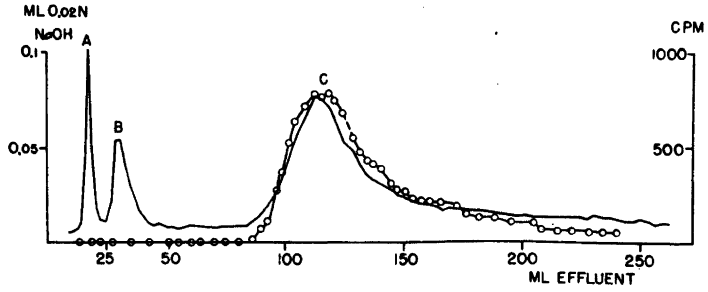

Fig. 3. Separation of taurolithocholic acid (A. 2.5 mg), taurodesoxycholic acid (B. 3.5 mg) and taurocholic acid (C. 15 mg) containing a sample of taurocholic acid-24-^{14}C. Phases: E see p. 1414. The solid lines in the figure connect the titration values and the open circles connected by a broken line show the c.p.m. of each sample.

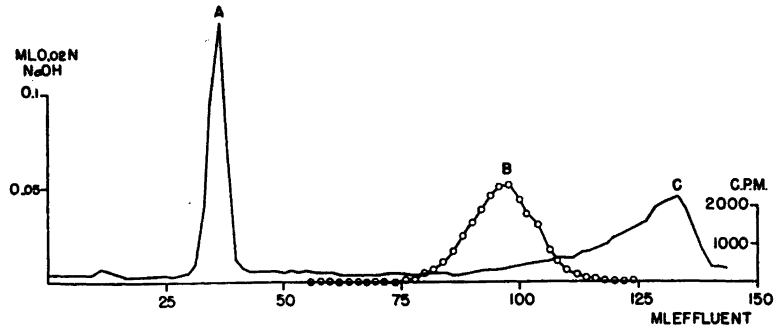

Fig. 4. Separation of glycocholic acid (A. 6.5 mg), cholic acid-24-^{14}C (B. tracerdose) and glycodesoxycholic acid (C. 7.1 mg). Phases: Type C. *See p. 1414.*

B. Separation of the tauroconjugates

For separation of the tauroconjugates from each other it is necessary to use an aqueous system because of the strong hydrophilic properties of these compounds. The best results were obtained with *n*-butanol as stationary phase and water as moving phase. Fig. 2 shows a separation of the taurocompounds of cholic, desoxycholic and lithocholic acid. The more hydrophobic taurocompounds *viz.* taurolithocholic and taurocholanic acid can be separated using 25 % MeOH as moving phase and chloroform-*iso*octanol 1 : 1 as stationary phase, *cf.* Table 1.

As taurocholic acid is the most commonly occurring compound in bile it was found necessary to have another system for separating this compound from other substances leaving the column with, or just after, the front in phase system C. Various systems with acetic acid/water as stationary phase and chloroform, amyl alcohol, and heptane as moving phase have been tried but very bad tailing of the eluted bile acids was observed. Using water as stationary phase and amyl alcohol as moving phase tailing of the eluted taurocholic acid (the peak at 100 ml) was minimised. The elution can be delayed by addition of chloroform to the moving phase. The peak appears at 110 ml with 15 % chloroform and at 220 ml with 20 % chloroform in the moving phase. Fig. 3 shows a separation of the taurine conjugates of cholic, desoxycholic and lithocholic acid by ordinary partition chromatography.

3. Separation of a glycine conjugated bile acid from free bile acids

The influence of conjugation with glycine is not so pronounced as the taurine group, so that a separation of the glycine conjugated bile acids as a group from the free acids is not possible. Conjugation with glycine of a bile acid changes its partition behaviour roughly corresponding to the introduction of a hydroxylgroup. Phase system C offers a possibility for separating the glycoconjugates and free forms of cholic and desoxycholic acid (Fig. 4). Gly-

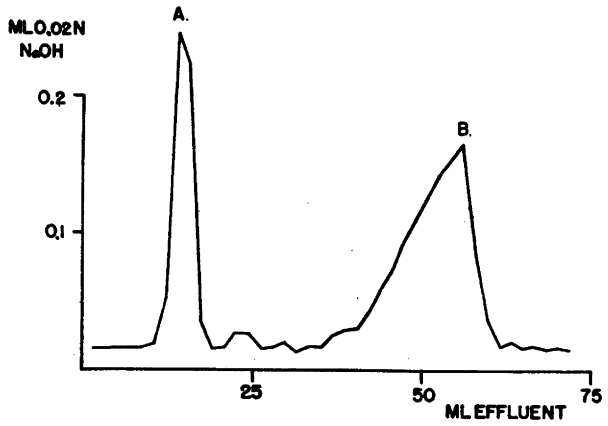

Fig. 5. Separation of glycocholanic acid (A. 4.0 mg) and cholanic acid (B. 5.5 mg). Phases: Type G. See p. 1414.

cocholic acid is clearly separated from cholic acid. Between cholic acid and glycodesoxycholic acid there is, however, some overlapping, whereas free desoxycholic aad chenodesoxycholic acid stay in the column. There exists a difference in the rate of elution of glycochenodesoxycholic and glycodesoxycholic acid in this phase system (*cf.* Table 2). This is, however, too small for a complete separation of these acids.

By using a system described by Bergström and Sjövall (phase system A) one can separate the glycine conjugate and the free form of lithocholic acid, *cf.* Table 2. Separation of glycocholanic acid from cholanic acid requires a more hydrophobic system (Fig. 5).

Table 2. Appearance of free and glycine conjugated bile acids with different phase systems.

Acid	Phase system C	Phase system G	Phase system A
Cholic acid	80—98—112	10—13—18	10—13—18
Desoxycholic acid }	> 250	48—60—66	24—30—36
Chenodesoxycholic acid }			
Lithocholic acid	> 250	> 250	70—78—93
Glycocholic acid	32—37—42	14—16—20	10—13—18
Glycodesoxycholic acid	100—132—148	18—24—28	10—13—18
Glycochenodesoxycholic acid	95—120—132	18—24—28	10—13—18
Glycolithocholic acid	> 250	60—80—90	32—40—45
Glycocholanic acid	> 250	> 250	> 250

Column: 4.5 g hydrophobic Supercel with 4 ml stationary phase.
The figures give the beginning, peak and end of each bile acid band as the total volume (ml) of effluent at these points.

SUMMARY

Partition chromatography on kieselguhr columns has been used for the separation of the taurine and glycine conjugates of cholic, desoxycholic, lithocholic and cholanic acid. Solvent systems are presented for (1) separation of the taurine conjugates from other bile acids, (2) individual separation of the taurine conjugates and (3) separation of the glycine conjugate from the corresponding free acids.

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Microdetermination of Zinc in Soil Extracts by Paper Chromatography

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Small amounts of zinc are separated from other metals, except mercury, that form coloured complexes with dithizone. The isolated zinc zone on the chromatogram is cut out, and the zinc is eluted and determined colourimetrically with dithizone.

In an investigation on the zinc content of danish soils, the problem of finding a rapid analytical method suitable for routine work arose [1]. In the absence of a good specific colourimetrical reagent for zinc, it was desirable to develop a separation method for those small zinc concentrations found in soil extracts.

According to two papers by Venturello *et al.*[2,3] Zn^{++} can be separated from Cu^{++}, Fe^{++}, Fe^{+++}, Ni^{++}, Pb^{++}, Mn^{++}, Mg^{++} and Al^{+++} by means of horizontal circular chromatography. The solvent used was *n*-butanol in equilibrium with an equal amount of 2 *N* HCl. This solvent gives an R_F value, defined by Le Rosen [4] as the ratio of the distance traversed by a zone to the distance traversed by the developing solvent, of 0.87 for zinc, while the corresponding value for the other metals are about 0.4.

On basis of these results, the present author has tried to include the ordinary metals, which give colour reactions with dithizone (Co^{++}, Ag^{+}, Bi^{+++}, Cd^{++} and Hg^{++}). In the method outlined below, it was possible to separate zinc from all these metals except from mercury, and the R_F values for the various metals found under these conditions, are shown in Table 1.

Table 1. R_F values for the different metals.

Metal	Cu	Ni	Co	Pb	Bi	Ag	Zn	Cd	Hg
R_F	0,13	0,06	0,08	0,11	0,12	0,36	0,84	0,68	0,87

EXPERIMENTAL

The solutions were all made with distilled water from which traces of metals had been removed by passage through an ion exchange column (Amberlite Resin IR—100 (H)).

Standard Zn-solution, 100 mg Zn/l. 0.4400 g $ZnSO_4$, 7 aq. (Merck p.a.) is dissolved in 1 liter of purified water containing 10 ml 10 % H_2SO_4.

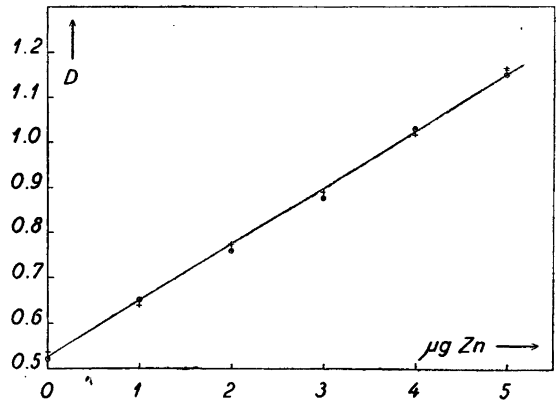

Fig. 1. Standard curve for the zinc-dithizon complexes. D = optical density measured in 1.0 cm cells at 540 mμ. The ⊙ points mark the D values of a solution which only contains Zn, while the + points indicate the D values of a solution, containing Cu, Ni, Co, Pb, Bi, Ag and Cd in addition to Zn.

Dithizone solution. 0.04 g dithizone (di-phenylthiocarbazone, Merck p.a.) is dissolved in 200 ml freshly distilled CCl_4. This solution is filtered, care being taken to exclude all trace metals from the filter, into a dark bottle and stored at 0 °C. Each day 10 ml of this stock solution is diluted with CCl_4 to make 100 ml. — All CCl_4 residues containing dithizone can be purified by a simple distillation in an allglass apparatus.

Ammonium citrate buffer solution. 113 g Ammoniumcitrate $((NH_4)_2C_6H_6O_7$, Merck p.a.) is dissolved in 500 ml purified water, and 150 ml 25 % NH_3 is added. This solution is repeatedly shaken with 50 ml portions of the dithizone-stock-solution, which are discarded, until the colour of the dithizone solution remains green. The water phase is now yellow from dissolved dithizone, and must be shaken several times with 50 ml portions of CCl_4, until both phases remain colourless. The solution is then made up to 1 liter with purified water.

2 N Hydrochloric acid solution, was made from constant boiling HCl.

n-Butanol-HCl-solvent. 1 liter of freshly distilled *n*-butanol is shaken for one hour with 1 liter of the 2 *N* hydrochloric acid solution, and the water phase is discarded.

All these stock solutions must be stored in Pyrex bottles with glass stoppers to avoid contamination by trace metals.

Whatman filter paper no. 1 contains appreciable amounts of metals giving a colour reaction with dithizone and must therefore be eluted before use. This is done by using the following technique. One sheet is cut into 6 smaller sheets of 14 × 23 cm, and each sheet is placed with its long side against the bottom of an aquarium jar 20 cm high and 30 × 30 cm base. A grooved glass-plate is placed on the bottom of the jar, and to keep the sheets upright a grate of parallel glass tubes, 1.5 cm apart, is placed 11 cm above the

Table 2. D values of the zinc-dithizon complexes in a pure Zn solution (A) and in a metal mixture solution (B).

	D	
μg Zn	*A*	*B*
0	0.522	0.541
1	0.655	0.640
2	0.760	0.772
3	0.880	0.890
4	1.029	1.015
5	1.147	1.157

bottom. The glass tubes are held together by a wooden frame, supported by four vertical glass tubes.

900 ml of the butanol-HCl-solvent is poured into the jar, one sheet of paper is placed between each glass tube, and the jar is closed with a tight cover to prevent evaporation. By standing overnight the sheets are completely irrigated by the vertical ascending movement of the solvent, and all the impurities of metals contained in the filterpaper like zinc are found in the upper edge of the sheets as indicated by a faint yellow colouration. The sheets are withdrawn from the jar, air-dried and neutralized for one hour in gaseous NH_3. (On acid papers the Cd and Zn zones are not distinctly separated.) It is important to handle their upper edges only. With a micropipette a known amount of the metal mixture solution to be analysed is applied in a horizontal stripe about 2 cm from the lower edge and extending almost halfway across the sheet to get two analyses on each sheet. After drying, the sheets are replaced in the jar and left there for about two hours until the solvent front has reached the upper edge of the sheets.

In order to identify the zinc zone, one of the sheets, upon which a pure zinc solution has been applied, is dipped in a solution of dithizone in CCl_4. After drying the zinc zone will then be indicated by a reddish colour. By comparison with this sheet, the zinc zone of the other sheets, representing a strip of about 1 × 11.5 cm can be located and cut out.

These strips are cut in 5 pieces and placed in 100 ml Erlenmeyer flasks. The zinc is eluted by pouring a mixture of 1 ml 2 *N* HCl solution and 15 ml purified water in the flasks and boiling for two minutes. When cold, 3 ml ammonium citrate buffer solution is added together with 10 ml dithizone reagent. The solution is shaken vigorously which makes the CCl_4-phase turn red to a degree depending on the zinc content, while the filterpaper fibres remain in the water phase. After 5 minutes the optical density, *D*, of the red colour is measured on a Beckman DU spectrophotometer in 1.0 cm Corex cells at 540 mμ. The reference cell contained pure CCl_4.

RESULTS

To test the reliability of this method, two different solutions were analysed; one containing only Zn, 100 mg/l, and another containing Cu, Ni, Co, Pb, Bi, Ag and Cd in addition to Zn; all 100 mg/l. From these solutions 0, 10, 20, 30, 40 and 50 μl, corresponding to 0, 1, 2, 3, 4 and 5 μg zinc were pipetted onto the paper sheets. The optical densities, *D*, were found as previously described, and the results are shown in Table 2 and Fig. 1. There was a good agreement between the zinc concentrations found in the pure zinc solution and in the metal mixture solution, indicating a complete separation of zinc from the other metals.

In this experiment the sulphates, chlorides and nitrates of the metals were used. In case of complexes of the metals stable in the *n*-butanol — HCl solution, however, the R_F values may vary radically. But in practice the actual metal complexes with ligands from the soil are likely to be so unstable that they will not disturb the analysis.

A report on the application of this method in soil analysis will later appear in *Acta Agriculturæ Scandinavica*.

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Short Communication

Formation of Homoserine in Germinating Pea Seeds

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In this laboratory homoserine was recently [1] found for the first time in green plants. Growing peas contained appreciable amounts of free homoserine.

We have now investigated the appearance of homoserine in pea seeds more closely, and its formation during germination of the seeds, and have found the following:

1. Homoserine does not occur either in a free state or bound in pea seeds. In the alcohol extract of finely ground seeds homoserine was not to be found paper-chromatographically (Fig. 1 a). Glutamic acid was quantitatively the most important amino acid in this extract; arginine, glycine, alanine, asparagine, aspartic acid, and serine appeared in smaller amounts. γ-aminobutyric acid, and some other amino acids were also present in small amounts. After total hydrolysis of the seeds homoserine was not to be found either.

2. In the alcohol extract obtained 24 hours after the seeds had been moistened (germination always taking place in the dark at about 20° C) a small amount of homoserine had already appeared (Fig. 1b). Glutamic acid was still, as in nongerminating seeds, the most abundantly appearing free amino acid. The amount of nearly all amino acids had increased very considerably. Glutamine also appeared in considerable amounts.

3. 48 hours after the seeds had been moistened, when the root already had a length of about 1 cm, the amount of homoserine had increased greatly. Glutamic acid, the amount of which had further increased, as had that of many other amino acids too, was still superior in quantity, and next to it was serine.

4. After 72 hours of germination the amount of homoserine had increased even more (Fig. 1c). This amino acid was now the most abundantly appearing free amino acid. The amount of glutamic acid had decreased, taking second place after homoserine. The amount of serine was much the same as after 48 hours, and that of alanine and arginine considerably decreased. On the chromatogram there appeared two new spots "X" and "Y". Their nature has not yet been investigated.

5. After 5 days the amount of homoserine had still increased, and was now by far the most abundantly appearing free amino acid (Fig. 1d). Glutamic acid as well as serine had still decreased. The amount of the unknown "X" had increased considerably, whereas that of "Y" had decreased.

When the root and the young stem (epicotyl) were removed from the seed, homoserine was to be found in far greater amounts in the stem and the root than in the seed. The unknown "X" appeared mainly in the young stem.

6. After 10 days of germination the amount of homoserine was much the same as after 5 days, but after continual decreasing in the amount of the other free amino acids, the proportional amount of homoserine had still increased. The amount of serine had decreased very greatly, as had that of glutamic acid. There was no longer any trace of the unknown "Y".

7. After 15 days of germination the situation was generally much the same as after 10 days. Asparagine, however, had increased considerably occupying now second place after homoserine. The spot of the unknown "X" was about the same as after 3 and 5 days.

At present, it is not known from which precursor, or how, homoserine is formed during germination of the pea seed. Similarly, it is unknown what function homoserine has in plants. It can be the precur-

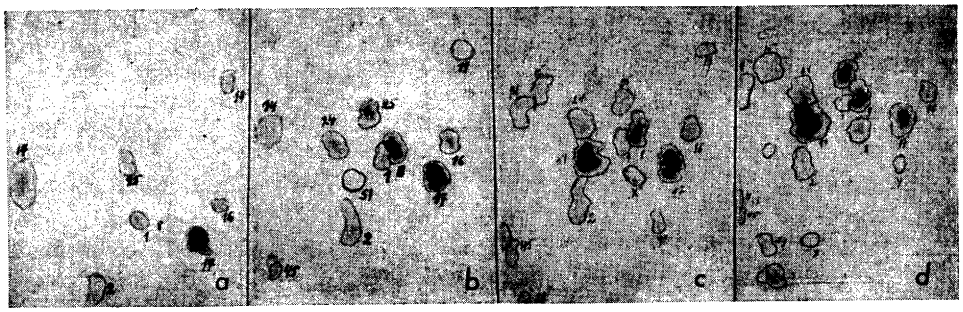

Fig. 1. Chromatograms prepared from alcohol extract of pea seeds before and after germination. Twodimensional chromatograms with solvents: 1. Butanol-acetic acid, 2. Phenol in NH_3-atmosphere. 1 = gly, 2 = ala, 7 = tyr, 8 = ser, 11 = pro, 14 = arg, 15 = lys, 16 = asp, 17 = glu, 19 = glutathione, 24 = glu NH_2, 25 = asp NH_2, 29 = γ-amino-butyric acid, 45 = ethanolamine, 51 = homoserine, "X" and "Y" = unknown spots. Between 14 and 15 some histidine.

a. Nongerminating pea seeds
b. After 24 hours' germination
c. » 72 » »
d. » 5 days »

90 μg N was used for all chromatograms. The amino acids have travelled various distances on different chromatograms, because of the differences in time and temperature.

sor of threonine as well as of methionine, but the composition of the fraction of the free amino acids does not give any proof of this conception.

The same results were obtained from germination experiments performed in the light over a period of two days, and a similar experiment, performed in the dark. After 5 days, the amount of approximately all free amino acids in peas germinated in light was notably smaller than in those germinated in the dark, depending probably on CO_2-assimilation and protein synthesis. In addition the alcohol extract of the peas grown in light gives a new unknown spot between those of homoserine and arginine.

The work is still in progress, and will be published fully in *Physiologica Plantarum*.

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