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THE COMPILATION AND CORRELATION OF RETENTION DATA FOR ALKYL SILANES, GERMANES, DIGERMANES AND BORAZOLES AND SOME OF THEIR HYDROCARBON ANALOGUES

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Our studies in silicon, germanium and borazole chemistry have led to the preparation of a considerable number of alkyl derivatives of silane, germane, digermane and borazole, the latter both N- and B-substituted. Gas chromatographic retention data for these compounds will be presented in a condensed form in this paper; a large number of these values have not hitherto been published. It is also our object to show how there are patterns between log t_R values which enable retention times to be predicted. A comparison with analogous hydrocarbon systems is possible in all cases, and many of the carbon analogues have not yet been chromatographically characterised. Some of the rules formulated in this paper should be applicable to these uncharacterised hydrocarbons.

The samples were analysed in columns of 2–13 % squalane (and also on 13 % Carbowax 4000 for some of the borazoles) at 100° using flame ionisation detectors and oxygen-free hydrogen as carrier gas. The solid support was Embacel (May and Baker; 60–100 mesh; acid-washed), it was made inactive by treatment with hexamethyldisilazane[1]. Retention data of all the compounds were expressed as logarithms of their retention times relative to mesitylene (log t_R = 2). Results on different columns agreed to within ± 0.01.

SILICON AND GERMANIUM ALKYLS

The use of retention parameters for the calculation of retention times has been found to work well for silanes, germanes and silico-germanes[2], and also for tetraalkyl derivatives of silicon and germanium[3]. For the estimation of log t_R values of the latter, the alkyl group retention parameters are obtained by dividing the log t_R values of symmetrical SiR_4 and GeR_4 compounds by 4 to give:

Me 0.10; Et 0.45; Pr^n 0.69; Bu^n 0.93.

The methyl parameter used here was obtained from log t_R values of $SiMe_4$ and $GeMe_4$ found by graphical extrapolation from SiR_3Me, GeR_3Me and GeR_2Me_2 results. A value of 0.14 for methyl was used previously[3], this gives a closer overall average agreement between observed and calculated values for tetraalkyl derivatives. The parameters can be used to calculate the log t_R values of mixed tetraalkylsilanes direct; the same parameters can be used for tetraalkylgermanes when a constant of

0.14 is added to the results, and for CR_4 compounds when a constant of 0.09 is subtracted.

As examples of the use of retention parameters for the calculation of log t_R values, Table I shows some observed and calculated results for germanium allyl derivatives (allyl parameter 0.66 from $Ge(allyl)_4$) and hexaalkyldigermanes (Ge–Ge parameter 0.85 from Ge_2Me_6) none of which have been published previously.

TABLE I

OBSERVED AND CALCULATED log t_R VALUES OF SOME ALLYL AND ALKYL DERIVATIVES OF MONO- AND DI-GERMANE

Compound	*log t_R*		*Compound*	*log t_R*	
	(obs.)	*(calc.)*		*(obs.)*	*(calc.)*
$GeMe_3(allyl)$	1.14	1.10	Ge_2Me_6	(1.45)	(1.45)
$GeMe_2(allyl)_2$	1.69	1.66	Ge_2Me_5Et	1.80	1.80
$GeMe(allyl)_3$	2.25	2.22	$Ge_2Me_4Et_2$	2.15	2.15
$Ge(allyl)_4$	(2.79)	(2.78)	$Ge_2Me_3Et_3$	2.50	2.50
$GeEt_3(allyl)$	2.15	2.15	$Ge_2Me_2Et_4$	2.84	2.85
$GeEt_2(allyl)_2$	2.37	2.36	$Ge_2Me_5Pr^n$	2.05	2.03
$GeEt(allyl)_3$	2.59	2.57	$Ge_2Me_4Pr^n{}_2$	2.62	2.60
			$Ge_2Me_5(allyl)$	2.03	2.01
			$Ge_2Me_4(allyl)_2$	2.59	2.57

The numerical differences between observed and calculated log t_R values of a number of alkyl derivatives of carbon, silicon and germanium are listed in Table II. For mixed alkyls with only two alkyl radicals present, the results are grouped according to the carbon number difference between the radicals.

The results indicate that the more similar in size the alkyl groups in a mixed derivative, the more accurate is the calculation from the alkyl group parameters. In general, the observed differences from predicted values can be related to the symmetry in the molecule, for the more "unsymmetrical" the molecule the greater is the difference. When the alkyl groups in a mixed derivative differ by one carbon number there is an approximate difference of 0.00, when they differ by two carbon numbers it is +0.04 and three carbon numbers it is about +0.08.

Similar calculations for over fifty mixed trialkyl chlorine, bromine and hydrogen derivatives of silicon and germanium do not show as good an agreement with those observed as with the tetraalkyls, and no obvious relation to symmetry in the molecule can be observed[3]. Some preliminary studies with mixed trialkylphosphines, however, show that their retention behaviour resembles that of the corresponding tetraalkylsilanes and retention parameters can be used.

ALKYLBORAZOLES

Borazole is the inorganic analogue of benzene. The preparation of many of the alkylborazoles, whose retention times are discussed here, is described elsewhere. Their characterisation and identification by means of graphical plots of log t_R values against carbon content has been stressed[4–6].

TABLE II

DIFFERENCES BETWEEN OBSERVED AND CALCULATED log t_R VALUES OF SOME ALKYL DERIVATIVES OF CARBON, SILICON AND GERMANIUM

$\Delta C = 1$*	
−0.02	$SiMeEt_3$, $GeMeEt_3$
−0.01	$Ge_2Me_2Et_4$, $GePr^nBu^n_3$
0.00	$GeEt_3Pr^n$, $GePr^n_3Bu^n$, $SiPr^n_3Bu^n$, $CMeEt_3$, CEt_3Pr^n, $GeEt_3$(allyl), Ge_2Me_5Et, $Ge_2Me_4Et_2$, $Ge_2Me_3Et_3$
+0.01	$SiEt_3Pr^n$, $GeEt_2Pr^n_2$, $SiEtPr^n_3$, $GeEtPr^n_3$, $SiPr^n_2Bu^n_2$, $GePr^n_2Bu^n_2$, $SiPr^nBu^n_3$, $GeEt_2(allyl)_2$
+0.02	$SiEt_2Pr^n_2$, $GeEt(allyl)_3$
+0.03	CMe_2Et_2
$\Delta C = 2$	
+0.02	$GeEtBu^n_3$, $Ge_2Me_5Pr^n$, $Ge_2Me_4Pr^n_2$, Ge_2Me_5(allyl), $Ge_2Me_4(allyl)_2$
+0.03	$SiMe_2Pr^n_2$, $GeEt_3Bu^n$, $GeMe_2(allyl)_2$, $GeMe(allyl)_3$
+0.04	$SiMePr^n_3$, $SiEt_3Bu^n$, $SiEt_2Bu^n_2$, $GeMe_3$(allyl)
+0.05	$GeMePr^n_3$, $GeEt_2Bu^n_2$
+0.06	$GeMe_2Pr^n_2$, $SiEtBu^n_3$
$\Delta C = 3$	
+0.05	$GeMe_3Bu^n$
+0.06	$SiMe_3Bu^n$
+0.08	$SiMe_2Bu^n_2$
+0.10	$SiMeBu^n_3$
+0.11	$GeMe_2Bu^n_2$, $GeMeBu^n_3$
Mixed derivatives with more than two radicals	
+0.01	$SiMeEt_2Pr^n$, $GeMeEt_2Pr^n$
+0.02	$GeEt_2Pr^nBu^n$, $SiEtPr^n_2Bu^n$, $GeEtPr^n_2Bu^n$, $GeEtPr^nBu^n_2$
+0.03	$SiEtPr^nBu^n_2$, $SiMeEtPr^n_2$, $GeMeEtPr^n_2$, $SiMe_2EtBu^n$
+0.05	$GeMeEt_2Bu^n$, $GeMe_2EtBu^n$
+0.07	$GeMeEtPr^nBu^n$, $SiMePr^n_2Bu^n$
+0.08	$SiMe_2Pr^nBu^n$, $GeMe_2Pr^nBu^n$, $GeMePr^nBu^n_2$, $SiMeEtBu^n_2$
+0.09	$GeMeEtBu^n_2$
+0.10	$GeMePr^n_2Bu^n$

* ΔC is the carbon number difference between the radicals.

Retention parameters for individual alkyl groups were calculated from the log t_R values of the symmetrical 1,3,5-derivatives by subtracting the log t_R of borazole (0.54) and dividing by 3 (Table III). The corresponding values for alkyl groups attached to the benzene ring were found in a similar way (log t_R of benzene 0.85).

TABLE III

RETENTION PARAMETERS FOR ALKYL GROUPS

	H	*Me*	*Et*	*Pr*^{n}	*Pr*^{i}	*Bu*^{t}	*Bu*^{s}	*Bu*^{i}
Tri-N-alkylborazoles	0.00	0.33	0.50	0.77	0.65	0.85	0.88	0.94
Tri-B-alkylborazoles	0.00	0.32	0.65	0.90	0.77	—	—	—
Trialkylbenzenes	0.00	0.38	0.63	(0.87)	0.72	(0.95)*	(0.98)*	(1.04)*

* The trialkylbenzene Pr^n, Bu^t, Bu^s and Bu^i parameters were estimated from the tri-N-alkylborazole values.

In general, the retention parameters of B-alkylborazoles are closer to those of alkylbenzenes than to N-alkylborazole values. The difference between values for B- and N-alkylborazoles may be related to the free N–H groups (of polar character) in the former compounds, so that N-substituted values are about 0.13 less than B-substituted values or 0.10 less than the aromatic values. On this basis the methyl group parameters are anomalous, low for B-methyl- and high for N-methyl-borazoles. The high N-methyl value may be related to an effect observed by JAMES AND MARTIN[7], the unusual retardation of the tertiary amine, trimethylamine, in the ethereal column liquid "Lubrol MO". They attributed this retardation to "active" methyl groups of the amine forming hydrogen bridges with oxygen in the ether. This conclusion is supported by the unusually high boiling point of trimethylamine relative to other tertiary amines and alkyl analogues of both boron and carbon (Table IV).

The alkyl group retention parameters may be used to calculate the log t_R values of mixed 1,3,5-trialkyl derivatives. Table V lists the differences between some observed and calculated values.

As with the carbon, silicon and germanium tetraalkyls, the more similar in size the alkyl groups in the mixed derivative the more accurate is the calculation of retention times from the alkyl group parameters. Again, the mixed derivatives generally elute later than is predicted from the retention times of symmetrically derivatives, and the more "unsymmetrical" the molecule the greater is the difference. The average deviation between observed and calculated log t_R values for carbon number differences are as follows: 1 carbon number + 0.01; 2 carbon numbers + 0.07; 3 carbon numbers + 0.15(5); 4 carbon numbers + 0.21.

CHANG AND KARR have published retention data for a large number of aromatic hydrocarbons[8,9]. We applied our method of estimating log t_R values to their results which were measured on columns of 25 % Apiezon L on Firebrick at 150° relative to *n*-propylbenzene. We used a value of 1.30 for the log t_R of benzene; this was found by graphical extrapolation. Our alkylbenzene retention parameters had to be divided by 1.46 to give the CHANG AND KARR parameters. Table VI shows the differences between observed and calculated log t_R values for CHANG AND KARR's results. Their data give similar deviations to ours, and in keeping with the smaller (higher temperature) parameters the deviations are also somewhat smaller.

Parameters may also be used to calculate boiling points, when similar results are obtained. From 1,3,5-trimethyl- and 1,3,5-triethyl-benzene the boiling point parameters for methyl and ethyl groups are derived as 28.2° and 45.4° respectively. The deviations between observed and calculated boiling points for some 1,3,5-methyl- and ethyl-substituted benzenes are as follows: H_2Me + 2.3°; HMe_2 + 2.5°; Me_2Et + 1.9°; $MeEt_2$ + 1.6°; H_2Et + 10.7°; HEt_2 + 10.2°.

A plot of log t_R against boiling point shows that 10° corresponds to an approximate log t_R difference of 0.13. Thus the boiling point deviations are of greater magnitude than the log t_R deviations. This conclusion is in agreement with the observation that the general linear relation between log t_R and boiling point for alkylbenzenes is not exact. A similar observation has been made for alkylborazoles[4].

The log t_R values of methyl- and ethyl-borazoles, substituted on both boron and nitrogen atoms of the ring were calculated from retention parameters in the same way as the unsymmetrical trialkyl derivatives. Table VII lists the numerical differences in the observed and calculated log t_R values.

TABLE IV

BOILING POINTS OF TERTIARY AMINES AND ALKYL ANALOGUES OF BORON AND CARBON

Compound	*B.p.* (°C)	*Compound*	*B.p.* (°C)	*Compound*	*B.p.* (°C)
Me_3B	—21.8	Et_3B	95	$Pr^n{}_3B$	156
Me_3CH	—10.2	Et_3CH	93.3	$Pr^n{}_3CH$	162
Me_3N	3.5	Et_3N	89.5	$Pr^n{}_3N$	156

TABLE V

DIFFERENCES BETWEEN OBSERVED AND CALCULATED log t_R VALUES OF SOME 1,3,5-TRIALKYL DERIVATIVES OF BENZENE AND BORAZOLE

*ΔC = 1**

—0.02	B-Et_2Pr^n
—0.01	B-$EtPr^n{}_2$
+0.00	N-$Pr^iBu^t{}_2$
+0.01	N-H_2Me, N-HMe_2, B-HMe_2, N-$Pr^i{}_2Bu^t$, B-Me_2Et, B-$MeEt_2$, B-Et_2Pr^i, B-$EtPr^i{}_2$
+0.02	Me_2Et-benzene, $MeEt_2$-benzene, N-Et_2Pr^i, N-$EtPr^i{}_2$
+0.03	H_2Me-benzene, HMe_2-benzene, N-Me_2Et, N-$MeEt_2$

ΔC = 2

+0.03	B-Me_2Pr^n, B-$MePr^n{}_2$
+0.04	N-H_2Et
+0.05	N-Me_2Pr^i
+0.06	N-HEt_2, N-$MePr^i{}_2$
+0.07	B-H_2Et, B-HEt_2, N-Et_2Bu^t
+0.08	N-Me_2Pr^i, N-$MePr^i{}_2$
+0.09	N-$EtBu^t{}_2$, $MePr^i{}_2$-benzene
+0.10	Me_2Pr^i-benzene
+0.11	HEt_2-benzene
+0.12	H_2Et-benzene

ΔC = 3

+0.10	N-H_2Pr^n
+0.12	N-$HPr^n{}_2$
+0.14	N-H_2Pr^i
+0.15	N-$HPr^i{}_2$
+0.16	H_2Pr^n-benzene
+0.17	N-$MeBu^t{}_2$
+0.18	N-Me_2Bu^t
+0.22	H_2Pr^i-benzene

ΔC = 4

+0.16	N-H_2Bu^i, N-$HBu^i{}_2$
+0.20	H_2Bu^s-benzene
+0.21	N-H_2Bu^s, N-$HBu^s{}_2$
+0.22	H_2Bu^t-benzene
+0.25	N-$HBu^t{}_2$
+0.30	N-H_2Bu^t

Mixed derivatives with three radicals

+0.02	B-$MeEtPr^i$
+0.04	B-$MeEtPr^n$
+0.15	$HMePr^i$-benzene

* ΔC is the carbon number difference between the radicals.

TABLE VI

DIFFERENCES BETWEEN OBSERVED AND CALCULATED log t_R VALUES OF SOME 1,3,5-DERIVATIVES OF BENZENE BASED ON SOME RESULTS OF CHANG AND KARR[8]

$\Delta C = 1$*	
0.00	H_2Me-benzene
+0.01	HMe_2-benzene, Me_2Et-benzene, $MeEt_2$-benzene
$\Delta C = 2$	
+0.05	HEt_2-benzene
+0.06	H_2Et-benzene
$\Delta C = 3$	
+0.10	H_2Pr^n-benzene
+0.13	H_2Pr^i-benzene
$\Delta C = 4$	
+0.13	H_2Bu^i-benzene
+0.16	H_2Bu^s-benzene
Mixed derivatives with different groups 1,3,5	
+0.04	HMeEt-benzene
+0.07	$HMePr^n$-benzene
+0.09	$HMePr^i$-benzene

* ΔC is the carbon number difference between the radicals.

It can be seen that alkyl groups on adjacent atoms make retention times of these borazoles very different from those calculated from the simple alkyl group parameters. Thus hexaethylborazole has a retention time less than half that calculated, and hexamethylborazole a retention time twice that calculated. However, an underlying pattern is again apparent, indicating perhaps once again that alkyl groups attached to the borazole ring are affecting the retention times in a regular manner.

Similar calculations of the log t_R values of methylbenzenes measured by CHANG AND KARR give positive deviations of the same order of magnitude as found for methyl-borazoles (Table VIII); these deviations are again reflected in boiling point values.

The corresponding data for ethylbenzenes are not available, but a comparison with the ethylborazoles indicates that *negative* deviations might be expected for tetra-, penta- and hexa-ethyl-benzenes.

TABLE VII

DIFFERENCES BETWEEN OBSERVED AND CALCULATED log t_R VALUES OF METHYL- AND ETHYL-BORAZOLES

	B-Me$_3$	*B-Me*$_2$	*B-Me*	*B-Et*	*B-Et*$_2$	*B-Et*$_3$
N-Me_3	+0.30	+0.21	+0.11	+0.05	+0.05	+0.02
N-Me_2	+0.18	—	—	—	+0.03	+0.04
N-Me	+0.08	—	—	—	—	+0.01
N-Et	+0.07	—	—	+0.10	+0.05	—0.03
N-Et_2	+0.10	+0.05	+0.07	+0.08	—0.04	—0.12
N-Et_3	+0.13	+0.07	+0.03	—0.07	—0.19	—0.35

TABLE VIII

DIFFERENCES BETWEEN OBSERVED AND CALCULATED log t_R VALUES AND BOILING POINTS OF METHYLBENZENES

	1,2,3,5-Tetramethylbenzene	*Pentamethylbenzene*	*Hexamethylbenzene*
log t_R, observed—calculated value	+0.12	+0.25	—
Boiling point, observed—calculated value (°C)	+3.1	+9.8	+14.7

The effect of alkyl groups on the retention times of tri-B-alkylborazoles on Carbowax 4000

The specific retardations of borazoles with free N–H groups on Carbowax 4000 have been discussed in the case of N-alkylborazoles[4], methylborazoles[5] and ethylborazoles[6]. It was suggested that hydrogen bonding occurred between N–H groups and oxygen atoms in the column liquid, and that "steric effects" of B-alkyl groups might influence the retardations of borazoles with free N–H groups. Such effects would correspond to those observed in the case of alkylpyrroles[10].

The retention behaviour of tri-B-alkylborazoles on Carbowax 4000 is of most interest in this respect, for with three N–H groups "sterically hindered" by a range of adjacent alkyl groups, they form a very convenient system for such a study. A useful method of comparing the behaviour of compounds in two different liquid phases is to plot the log t_R values against each other. For when this is done, the vertical distance between a compound and the paraffin line on the graph gives a measure of its specific retardation in the polar phase. Fig. 1 shows a plot for sixteen tri-B-alkylbora-

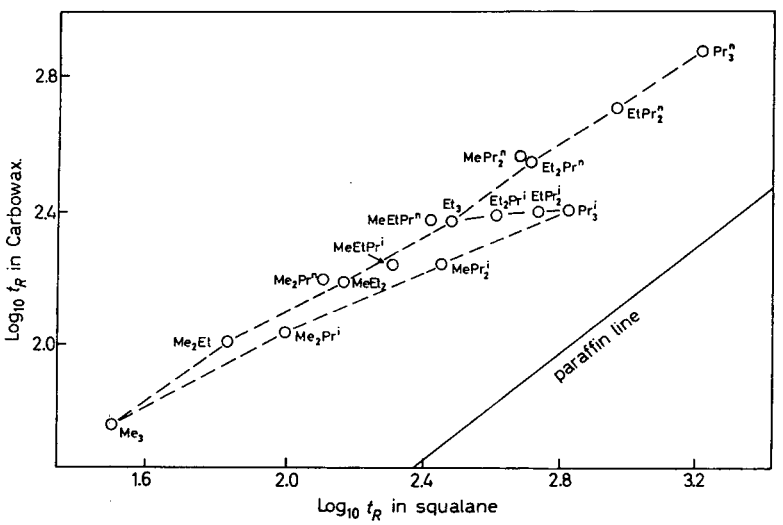

Fig. 1. Comparison of tri-B-alkylborazole retention times on Carbowax 4000 and on squalane.

zoles. The distances of the symmetrical tri-B-alkylborazoles from the paraffin line were divided by 3, to give four distances representing (if the theory is correct) the

effect of each alkyl group on the retardations due to the free N–H groups. In log t_R units these are:

Me 0.29; Et 0.22; Pr^n 0.19; Pr^i 0.14.

As expected the "steric effects" are in the order $Pr^i > Pr^n > Et > Me$. When the distance of mixed tri-B-alkylborazoles from the paraffin line are compared with those calculated by simply adding the individual alkyl group values, very good agreement is obtained (Table IX). Thus, if the log t_R values of tri-B-alkylborazoles are known in squalane, their retention times in Carbowax 4000 can be quite accurately predicted.

TABLE IX

RETARDATIONS OF TRI-B-ALKYLBORAZOLES ON CARBOWAX 4000

Tri-B-alkylborazole	*Distance (log t_R units)*		*Tri-B-alkylborazole*	*Distance (log t_R units)*	
	(obs.)	*(calc.)*		*(obs.)*	*(calc.)*
$EtPr^iPr^i$	0.49	0.50	$MePr^nPr^n$	0.68	0.67
$MePr^iPr^i$	0.56	0.57	$MeEtPr^n$	0.71	0.70
$EtEtPr^i$	0.57	0.58	$MeMePr^i$	0.71	0.72
$EtPr^nPr^n$	0.61	0.60	MeEtEt	0.74	0.73
$EtEtPr^n$	0.65	0.63	$MeMePr^n$	0.78	0.77
$MeEtPr^i$	0.66	0.65	MeMeEt	0.80	0.80

CONCLUSION

The simple correlations presented in this paper suggest that it should be possible to predict with reasonable accuracy the retention data of a very large number of other compounds. These will probably include (1) further alkyl-substituted silanes, germanes, digermanes and borazoles, (2) similar alkyl derivatives of methane, benzene, stannane, plumbane, and possibly other simple hydrides, and (3) alkyl derivatives of catenated hydrides. In connection with (2) it may be noted that many silane, germane and borazole derivatives can be more readily synthesised than their organic analogues. In connection with (3) we can report that we have some evidence for the existence of several alkyltrigermanes and for decamethyltetragermane with retention data as predicted.

Apart from the borazole–Carbowax interactions the retention patterns have been presented on an empirical basis. They must clearly, however, reflect subtle changes in intermolecular forces, for which there must be some basic structural explanation. So also the retention patterns provide us with a delicate method for examining subtle structural features in both inorganic and organic chemistry.

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SUMMARY

Gas chromatographic retention data for a very large number of alkyl-substituted silanes, germanes, digermanes and borazoles are presented in terms of a few retention parameters and compared with the retention and boiling point data for hydrocarbon analogues.

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VOLATILES FROM STRAWBERRIES

II. COMBINED MASS SPECTROMETRY AND GAS CHROMATOGRAPHY ON COMPLEX MIXTURES

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Extraction of hundreds of pounds, or even tons, of natural products for determination of the chemical constituents of odors and flavors generally yields only a few milligrams of complicated essence. The extracts will often contain more than 150 components having wide variations in chemical functionality. Such mixtures can usually be separated satisfactorily only by capillary gas chromatography, and the submicrogram quantities thus purified are not easily collected and identified.

The most promising method for obtaining analytical information on such mixtures appears to be the direct introduction of the chromatographic eluate into a fast-scan mass spectrometer. Recent developments in this field have demonstrated that sufficiently good mass spectra can be obtained even when scanning is limited to seconds and only submicrogram quantities enter the machine[1–3].

Research on a strawberry essence revealed three important features of this type of analysis. First, the extent of mass spectral interpretation was shown by the large number of compounds easily identified, and the limitation by the large number not identified. Second, the presence of two or more components in a single chromatographic peak was revealed by changes in the mass spectra of successive scans. Third, pump-out of the mass spectrometer is shown to be fast enough to cause no decrease in chromatographic efficiency for oxygenated materials eluted at 200°.

The identification of many of the strawberry oil components was, in itself, of considerable fundamental interest.

EXPERIMENTAL METHODS AND RESULTS

Part I of this series describes the method of concentrating to a few milliliters the ten tons of vapor condensate from strawberry jam pot stills[4]. The chromatographic equipment has been described[5], and several papers have outlined the techniques used to combine a capillary chromatograph with a fast-scan mass spectrometer[6–8]. In the present work, the simple method of direct introduction was employed, in which the capillary column operates with the exit at a pressure of about 10^{-3} torr and the inlet at a pressure reduced by one atmosphere from the usual inlet pressure. Comprehensive studies have shown that this method does not lead to any significant loss of chromato-

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

graphic efficiency[9]. The eluate was monitored by observing the mass spectral pattern on an oscilloscope and by recording the ionization due to mass 15 (CH_3^+ formed by electron impact fragmentation) as a chromatogram.

The chromatogram of the strawberry oil (Fig. 1) was obtained with a programmed-temperature capillary column, 200 ft. long and 0.01 in. I.D., coated with Tween 20 (Atlas Powder Co.)*. Normal exit pressure was 1 atm. A ^{90}Sr argon detector was used.

A chromatogram obtained during a mass spectral run using the ionization due to CH_3^+ is not presented here because different amounts of CH_3^+ were formed by different substances. Such a chromatogram is necessary, however, in order to match the mass spectral charts with the peaks on the standard chromatogram. Several such chromatograms have been shown in previous studies[4,10,11].

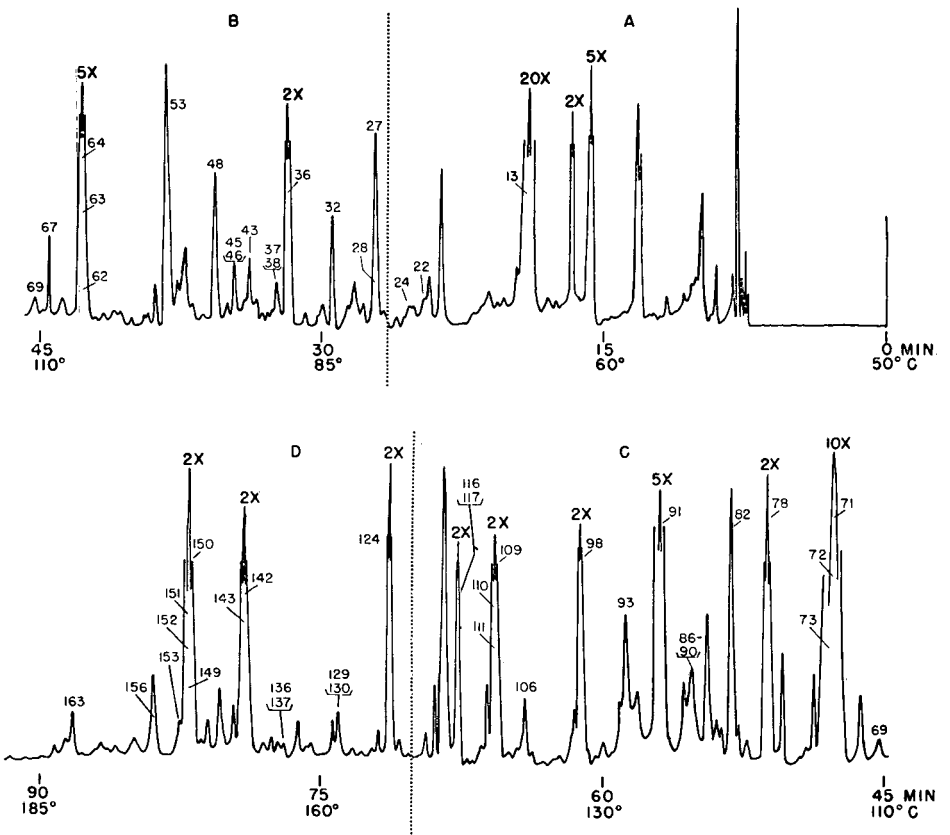

Fig. 1. Chromatogram of strawberry oil. Capillary column, 200 ft. long and 0.01 in. I.D., coated with Tween 20. The numbers identify the components of Table I.

Each component observed by gas chromatography (GC) and/or mass spectrometry was assigned a number. If a peak contained more than one component as revealed by mass spectrometry, each component was numbered. For a few of the

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

very dilute components, a chromatographic peak may have been observed but no mass spectral chart taken, or, alternately, a weak mass spectral chart may have been obtained but no chromatographic peak observed. These peaks also have generally been assigned numbers. (Zone A in Fig. 1 does not adhere to these rules. Because it was published earlier, the numbers used at that time are retained.)

Although all peak numbers are not printed in Fig. 1, it is easy to assign each peak its identification listed in Table I which presents the compounds not listed in the previous publication. (Peak 13 contains about 65 % diethoxyethane and 35 % ethanol. Mention of ethanol was accidentally omitted in Part I[4].) In addition to the components apparent in Fig. 1, about 15 minor components were eluted up to 230°. Those that were identified are also listed in Table I.

Three classes of identification are used to illustrate the certainty of analysis. The first is a good comparison of the unknown spectrum with the spectrum of an authentic compound, and also a match of the GC retention time of authentic compounds. Experience has shown that identification of even a simple mixture by its mass spectrum alone may be in error. A retention-time check is desirable whenever possible. In the work presented here, all retention times were obtained by enriching the strawberry oil with the authentic compound and running the sample through the entire temperature program. Thus, modest variations in experimental conditions

TABLE I

COMPOUNDS IDENTIFIED IN STRAWBERRY OIL*

Peak No.	*Mass spec. and GC retention*	*Mass spec. confirmation*	*Mass spec. indication*
13	Ethanol 1,1-Diethoxyethane		
22			1,1-Diethoxypropane 1,1-Methoxybutoxyethane
24	1,1-Ethoxypropoxyethane		
27	2-Methylbutane-2-ol		
28	Methyl α-methylbutanoate		
29			Isoamyl formate Amyl formate
31		1,1-Ethoxybutoxyethane	
32	Ethyl *n*-butyrate		
33		1,1-Methoxybutoxyethane	
36	Ethyl α-methylbutanoate		
37	*n*-Butyl acetate		
38			1,1-Diethoxybutane
42			2-Hydroxy-3-methylbutane
43			Ethyl isovalerate
45		1,1-Ethoxybutoxyethane	
46			1,1-Methoxypentoxyethane
47		1,1-Methoxypentoxyethane	
48			2-Hexyl acetate
49			2-Pentanol
50		1,1-Diethoxypentane	
51		*n*-Butanol	
52	Isoamyl acetate		
53		1,1-Ethoxypentoxyethane	
62		(3-Methyl-1-butanol)	
63		(2-Methyl-1-butanol)	

For footnote see p. 13. (*continued on p. 13*)

TABLE I (*continued*)

Peak No.	*Mass spec. and GC retention*	*Mass spec. confirmation*	*Mass spec. indication*
64	Methyl *n*-hexanoate		
67	2-Hexenal		
71	Ethyl *n*-hexanoate		
72			1,1-Methoxyhexoxyethane
73			Isopropyl hexanoate
75			1,1-Diethoxyhexane
77	*n*-Hexyl acetate		
78	1,1-Ethoxyhexoxyethane		
79		Ethyl heptanoate	
83		Ethyl heptanoate	
84		Hexenyl acetate	
85		1,1-Ethoxyhex-3-enoxyethane	
86	*n*-Hexanol		
88		Ethyl heptanoate	
89		3-Hexene-1-ol	
91		2-Hexene-1-ol	
92		Butyl hexanoate	
98	Furfural		
99	Methyl octanoate		
106	*n*-Butyl *n*-hexanoate		
107	*n*-Hexyl *n*-butyrate		
109			2-Acetylfuran
112	Ethyl octanoate		
113		Butyl α-methylbutyrate	
115	1,1-Diethoxypentane		
117	Benzaldehyde		
119		Pentyl hexanoate	
122			Methylfurfural
124	Linaloöl		
125	Pentyl hexanoate		
132			Pentenyl hexanoate
134			Pentenyl hexanoate
137	Acetophenone		
138	Methyl *n*-decanoate		
142	Ethyl benzoate		
143	*n*-Hexyl *n*-hexanoate		
145	1,1-Di-*n*-hexoxyethane		
146	*cis*-3-Hexen-1-yl caproate		
147	Ethyl *n*-decanoate		
149		*trans*-3-Hexen-1-yl caproate	
150	Benzyl acetate		
151	α-Terpineol		
152		2-Hexen-1-yl caproate	
153		Pentyl octanoate	
155			(Naphthalene)
157			1,1-Diethoxyoctane
162	β-Phenylethyl acetate		
163			1,1-Dihexenoxyethane
Others not on Fig. 1			
			2-Methylnaphthalene
			Hexyl octanoate
			1-Methylnaphthalene
			Ethyl dodecanoate
			Hexenyl octanoate
		cis-Ethyl cinnamate	
		trans-Methyl cinnamate	
		trans-Ethyl cinnamate	

* Duplicate listings are isomeric species not distinguished by available mass spectral data.

would not lead to erroneous results. This technique is desirable whenever a sufficient quantity of the sample is available.

The second class of identification is mass spectral comparison or correlation only. When the authentic compound is not available, a fairly certain identification can frequently be made by comparison with tabulated mass spectra[12–14] or by using correlations suggested by studies on series of compounds (for a list of these, see ref. 15). Thus, the identification of most of the compounds so classified is fairly certain. although in some cases a specific isomer cannot be suggested. For example, component 119 is certainly a pentyl hexanoate. The molecular ion, mass 186, is weak, but it is observed. The ions $C_5H_{10}^+$ and $C_6H_{11}O^+$ are strong, as expected. The characteristic ester rearrangement ions, $(CH_2COOC_5H_{11})H^+$ (mass 130) and $(C_5H_{11}COO)H_2^+$ (mass 117), are both present. However, from the mass spectral data, the only isomer information that can be accepted with confidence is that the acid moiety is not α-substituted.

The third class of identification consists of structures suggested by mass spectra, but not certain. Various reasons may exist for this uncertainty. The spectrum may be too weak for confident interpretation; the compound may have been eluted with another so that the spectrum is partially obscured; or the suggestion may be based on comparison with only one or two similar compounds.

Three components listed in Table I have been placed in parentheses. Components 62 and 63 gave a good mass spectral confirmation but the retention times of the authentic samples did not give a good check (about 45 sec late). Component 155 was indicated by the mass spectrum but the retention time of authentic naphthalene was 1 min early.

Two additional classes of identification represent the components not listed in Table I. The fourth includes cases where the mass spectra give some definitive information but not enough to assign a structure. Thus, a component may appear to be a terpene, a secondary alcohol, a possibly unsaturated ester, etc., but without structure assignment. The fifth class is that in which the mass spectrum does not yield any suggestion, even tentatively, as to the identity of the component.

INTERPRETATION OF DATA

The nature of the data obtainable and the general interpretation are illustrated by Fig. 2, which shows the mass spectral charts taken during the latter part of the chromatographic run, after 80–85 min and at about 175°.

The first chart in Fig. 2 was taken towards the top of the chromatographic peak and shows the mass spectral pattern of component 142, ethyl benzoate (the mass peaks shown at higher intensity), and a small amount of component 143. The identification of ethyl benzoate can be made quite readily by comparing the ratios of the mass peaks obtained with those of ethyl benzoate as listed in the mass spectral catalogues. Other compounds of parent mass 150 would have different ratios and, in many cases, some peaks would be absent and others present. The next chart was taken a few seconds later but shows very little ethyl benzoate. Component 143 is readily identified as hexyl caproate by means of the established mass spectral correlations already discussed[16]. Again, the only isomer information obtainable from the mass spectrum is that the acid moiety is not α-methyl pentanoate.

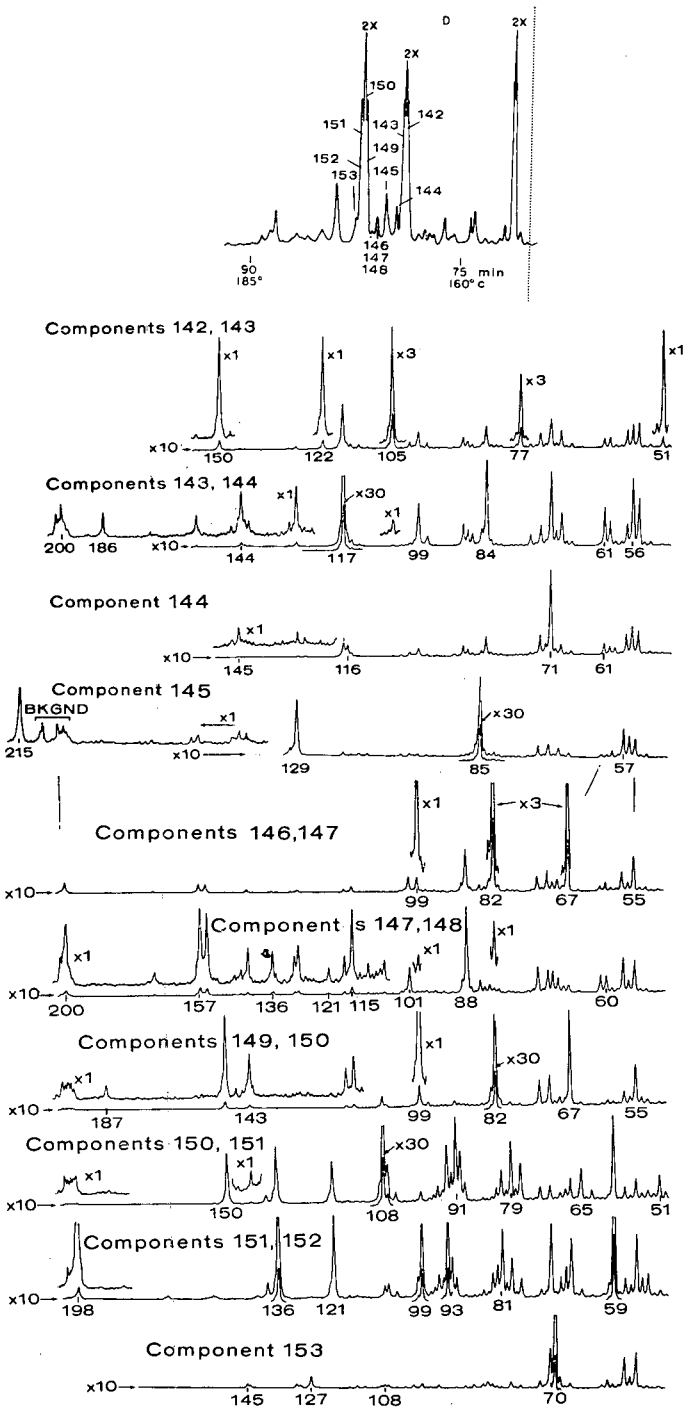

Fig. 2. Mass spectra obtained during latter part of run.

The mass spectrum obtained on the small adjacent peak is weaker and not too clear. The persistence of the mass 71 ionization is not easily explained since the ionization at masses 84, 99 and 117 has dropped as a result of effective elution of the hexyl caproate. Ionization at masses 56, 116 and 145, which might also be present in the previous mass spectral chart, may indicate a butyl octanoate. However, the spectrum does not clearly define these features so that listing this even in the third classification does not seem warranted.

The next chromatographic peak appears to be the single component dihexoxyethane. This compound is quite easily identified tentatively by the ion peaks at mass 215 [$CH(OC_6H_{13})_2^+$] and at mass 129 [$CH_3CHOC_6H_{13}^+$]. Comparison with known acetal spectra[17] confirms this identification but there is not enough mass spectral data in the literature to permit any isomer assignment.

The next two mass spectral charts were taken on the two small peaks preceding the last major peak. The first chart shows mass spectral features of both 3-hexen-1-yl caproate (146) and ethyl decanoate (147). Although, in the mixture, 3-hexen-1-yl caproate shows only three characteristic peaks (masses 99, 82 and 67), their ratios match the spectrum of the authentic compound well enough to give firm identification. In the next chart, the spectrum of component 146 is essentially absent and that of ethyl decanoate predominates. Note also the trace component, 148, evidenced at masses 136 and 121. These mass peaks are definite indications of a C_{10} terpene (presumably an alcohol or aldehyde) but further structural identification is not feasible.

The next mass spectral chart was taken at the start of the larger chromatographic peak. Two components are observed. First, the features of 3-hexen-1-yl caproate are again evidenced. It is concluded that this must be *trans* and the previous one *cis*. A chromatographic retention-time check with *cis*-3-hexen-1-yl caproate confirmed this. The other component is identified as benzyl acetate by comparison of this spectrum with other mass 150 compounds catalogued in the mass spectral literature. (The features of this spectrum give an interesting contrast with those of ethyl benzoate, component 142.) A few seconds later, 3-hexen-1-yl caproate was absent, but another component, 151, was observed with the benzyl acetate. The mass peaks at 136, 121 and 93 immediately suggest a C_{10} terpene and the peak at mass 59 [the ion fragment $(CH_3)_2COH^+$] indicates possible α-terpineol. Thus, identification by comparison with the mass spectrum of authentic α-terpineol and by GC was confirmed. Still on this same peak, the next mass spectral chart showed no benzyl acetate; α-terpineol is still present but in addition, another component, identified as 2-hexen-1-yl caproate, is recorded. Unlike the 3-hexen-1-yl caproates (components 146 and 149), this isomer gives significant parent ionization at mass 198 so that the identification is simplified.

As a final illustration, the chart taken on the small shoulder peak (component 153) is shown. The α-terpineol and 2-hexen-1-yl caproate are essentially absent and a reasonably clear spectrum corresponding to a pentyl octanoate is obtained. This is quickly identified by the characteristic ester peaks ($C_5H_{10}^+$ from the pentyl, $C_7H_{10}CO^+$ from the octanoate, etc.) but isomer information is again not given.

This section of the chromatogram was chosen for illustration for two reasons. First, it shows that even components of relatively high boiling points can be quickly pumped from a mass spectrometer. Second, the peaks in this section have several components which, fortuitously, can be easily identified. It is noted, however, that

only about one-half the components of the total oil have been reasonably identified but, as would be expected, almost all the major peaks are determined.

Curiously, all the more abundant peaks that have not been identified are in section C, notably components 82, 93, 110, 111, 116 and 118. Even though the mass spectra of these unknowns are fairly intense, only crude speculation is possible regarding their identity. Some firm conclusions can still be made, however. For example, it can be stated that they are not aliphatic esters, acetals, ketones, common aromatic esters, etc. In two cases (components 111 and 116), nearly identical mass spectra were obtained, so it may be concluded that these are *cis* and *trans* isomers. The further speculation that they are methyl ethers of terpene alcohols requires substantiation before they could be listed in Table I.

Identification of the unknown components may become possible as the number of compounds catalogued in the mass spectral literature increases. Separation by use of several different packed columns would be extremely difficult. Possibly, the identity of some will be clearer if high resolution mass spectra can be obtained, thus revealing the true elemental composition of each ion fragment. The technique for obtaining such data has been recently demonstrated[18].

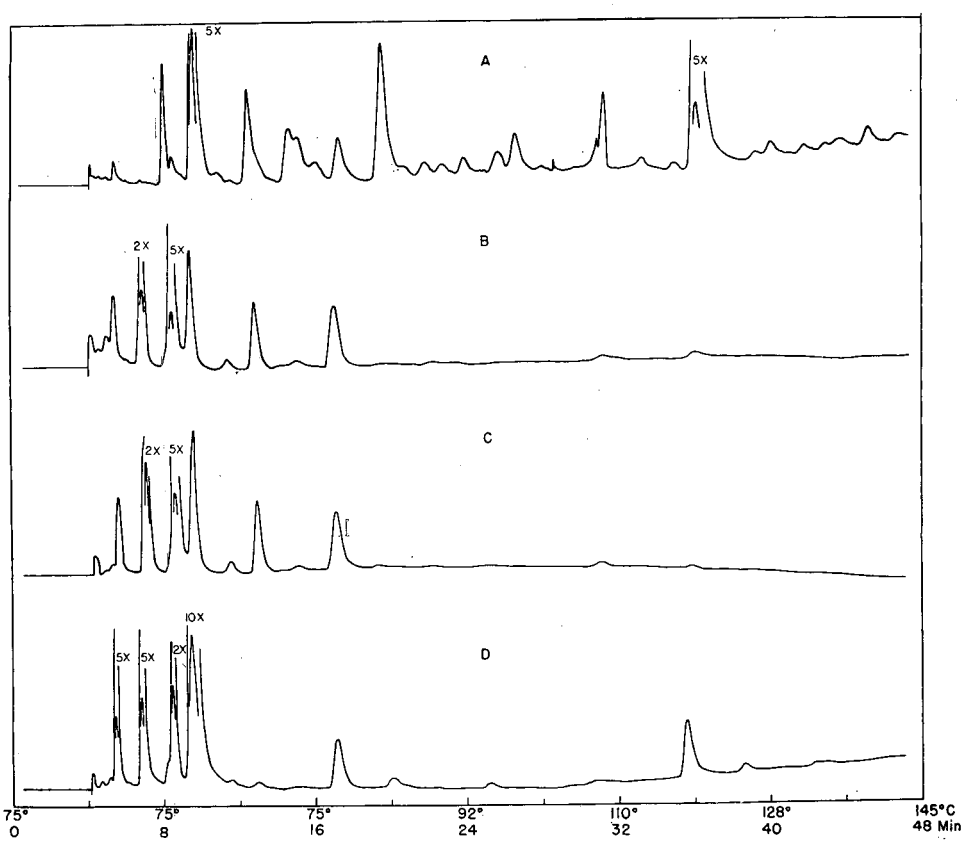

Fig. 3. Chromatograms of strawberry vapors (aromagrams): A, vapor of oil used for mass spectral analysis; B and C, duplicate samples of vapor above whole strawberry; D, vapor above a whole strawberry of different variety.

Many of the identifications made in this work have been previously reported[19–23]. However, determination of the many ester isomers had not been previously possible. The existence of acetals was not realized prior to Part I[4] of this series. Some of the aromatic compounds had been suggested but the confirmation here by mass spectrometry seems desirable. Furfural had been reported but the furfural derivatives are new identifications. Of the terpenes, α-terpineol had been previously established but linaloöl had not, even though it is one of the more prominent components (component 124). Borneol, reported in previous work, was not detected and, from mass spectral evidence, it could not be any of the three or four trace terpenes reported here. However, such differences could be due to use of different strawberry varieties.

The present work demonstrates significant progress in the investigation of volatiles from fruits and vegetables. The ability to analyze microgram quantities existing in complicated mixtures facilitates the correlation of chemical structure with organoleptic evaluation, and will make direct vapor analysis by gas chromatography (aromagrams[24]) more meaningful and permit chemical identification of aroma quality.

The four aromagrams shown in Fig. 3 illustrate how this study will be useful in flavor and odor studies of fresh fruits. Chromatogram A is a vapor sample from the strawberry oil used in this study. Chromatograms B and C are from duplicate vapor samples from a whole strawberry of one variety and chromatogram D is from a whole strawberry of another variety. Although more components are observed in the vapor from the strawberry oil, it is possible to correlate the components of the vapors from a fresh berry with the constituents identified in the isolated oil. Also, it can be seen that differences between fruits of different varieties can be chemically determined as is indicated by the differences between D and the duplicates B and C. Differences due to different storage conditions have also been reported[24].

Correlations of this type will be easier and better as columns are developed to separate the components of vapor samples as efficiently as the small-bore capillaries can separate liquid samples.

SUMMARY

The technique of combined mass spectrometry and gas chromatography has been applied to the analysis of a complex oil of strawberry volatiles. Capillary gas chromatography indicated over 150 components. Most of the major components have been identified. These include alcohols, esters, acetals, aldehydes, furfural, methyl furfural, aromatic aldehydes, ketones, and esters, a few terpenes, and a few aromatic hydrocarbons. The strength of this technique is shown by the determination of several compounds in one sharp chromatographic peak. On the other hand certain prominent species could not be identified by the low resolution (resolution about 200) mass spectral data obtained.

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THE QUANTITATIVE DETERMINATION OF LINOLENIC ACID BY MEANS OF THE LOVELOCK IONISATION DETECTOR*

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Over the past few years there have been several reports on parallel quantitative determinations of fatty acid mixtures containing linolenic acid by gas–liquid chromatography with a LOVELOCK ionisation detector[1] and by other chromatographic and non-chromatographic methods. It has been shown that in some cases linolenic acid determined by means of an ionisation detector gives abnormally high values[2–4]. It seemed desirable therefore to investigate this technique more closely in order to obtain more accurate results during the study of the fatty acid composition of linseed oil and other fatty acid mixtures rich in linolenic acid.

EXPERIMENTAL AND RESULTS

Linseed oil triglycerides were separated by reversed-phase partition chromatography as described earlier[5,6]; the system acetone–glacial acetic acid (1:1)/dodecane was used. Total triglycerides and separate triglyceride fractions were converted into fatty acid methyl esters[7]. The ester composition was determined by gas–liquid chromatography[5,**]. Peak area was measured by multiplying peak height by its width at mid-height, and the fatty acid concentration was expressed as weight or moles per cent. Detector voltages used in the various experiments were 750, 1000, 1250 or 1500 V. Detector sensitivity at 750 V decreased considerably; the use of "$\times 3$" amplifier sensitivity at this voltage instead of lower "$\times 10$" sensitivity setting does not affect the accuracy of results.

Fatty acids were separated by reversed-phase chromatography and quantitatively estimated by densitometry[8,9]. The iodine value of the oil was determined by the HANUS method[10].

The determination of the methyl palmitate: methyl linolenate ratio in a model mixture (1:1 by weight) at various detector voltages gave the results shown in Table I.

It can be seen that the true proportion between the esters was only obtained at 750 V. If higher voltages were applied, the detector response for methyl linolenate became abnormally high. It seems that this ester is more easily ionised by metastable argon atoms than methyl palmitate.

* Abbreviations and conventions: *P*, *P-O*, *S*, *O*, *L*, *Le* = palmitic, palmitoleic, stearic, oleic, linoleic, linolenic acids respectively, and acyls of these fatty acids in the triglyceride. The sequence of *P*, *S*, ... and other symbols does not represent the actual structure of the triglyceride molecule. [*P*], [*S*], [*OLeLe*], etc. indicate fatty acid and triglyceride concentrations (moles %).

** Argon Chromatograph, W. G. Pye & Co. Ltd., Cambridge, England.

TABLE I

RATIOS OF PALMITATE:LINOLENATE IN A 1:1 MIXTURE FOUND AT VARIOUS DETECTOR VOLTAGES

Detector voltage	*Palmitate:linolenate (peak area proportion)*
750	1:1.00
1000	1:1.10
1250	1:1.23
1500	1:1.74

TABLE II

DEPENDENCE OF THE RELATIVE DETECTOR RESPONSE FOR FATTY ACID METHYL ESTERS ON THE DETECTOR VOLTAGE*

Fatty acid	*Voltage*			
	750	*1000*	*1250*	*1500*
P	1	1	1	1
S	0.61	0.65	0.65	0.63
O	2.31	2.42	2.39	2.31
L	2.93	2.98	2.96	2.96
Le	8.72	11.11	12.55	13.47

* The response for methyl palmitate is arbitrarily equal to 1 at each voltage.

TABLE III

COMPARISON OF THE DETERMINATION OF LINSEED OIL FATTY ACID COMPOSITION BY GAS–LIQUID CHROMATOGRAPHY AT VARIOUS DETECTOR VOLTAGES AND BY PARTITION CHROMATOGRAPHY (MOLES %)

Fatty acid	*PC**	*Voltage*			
		750	*1000*	*1250*	*1500*
P	7.1	7.6	6.7	6.1	6.0
P-O	—	0.3	0.2	0.2	0.2
S	6.6	5.5	5.2	4.9	4.7
O	20.4	21.6	20.6	19.4	18.7
L	16.3	15.2	13.6	12.6	12.1
Le	49.6	49.7	53.7	56.7	58.3
Iodine value calculated	177.1	176.8	183.4	188.6	191.3
Found	177.2				

* PC = quantitative determination by reversed-phase partition chromatography and densitometry.

TABLE IV

DETERMINATION OF THE FATTY ACID COMPOSITION OF SEPARATE TRIGLYCERIDE FRACTIONS AT VARIOUS DETECTOR VOLTAGES (MOLES %)

Fatty acid		*P*				*S*				*O*				*L*				*Le*			
Detector voltage		*750*		*1500*		*750*		*1500*		*750*		*1500*		*750*		*1500*		*750*		*1500*	
K_2	R_2	*c**	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*
55	0.32	7.7	7.5	—	7.1	9.5	8.8	—	9.0	17.1	16.9	—	14.6	24.4	23.5	—	21.0	42.0	43.3	39.7	48.3
57	0.42	7.7	8 8	—	7.5	—	—	—	—	19.3	20.4	—	17.2	5.2	9.7	—	8.2	63.3	61.0	53.5	67.0
59	0.63	—	—	—	—	—	—	—	—	—	—	—	—	33.3	33.4	—	27.5	66.8	66.6	55.0	72.5

* c = Calculated from other fatty acids concentration according to equations (1)–(4); f = found at this voltage. The data obtained at 1500 volts were only used for calculations of the linolenic acid concentration.

It was therefore of interest to determine whether methyl esters of other fatty acids changed their ionisation pattern depending on detector voltage. The data of Table II show that the relative detector response for stearic, oleic, and linoleic acid esters remains practically unchanged at all voltages studied. In the same range the response for methyl linolenate grows abnormally.

In order to investigate the practical implications of the relationship found we carried out a parallel determination of the fatty acid composition of linseed oil with a known iodine value by reversed-phase partition chromatography and by gas–liquid chromatography at 750–1500 V.

As shown in Table III reliable quantitative data can be obtained by densitometry and by gas–liquid chromatography at 750 V. At higher voltages the detector response for methyl linolenate becomes too high.

After determining the fatty acid composition of the total linseed oil triglycerides we analyzed separate fractions of glycerides of the same polarity. Each fraction was characterized by the polarity constant

$$K_2 = 100 - m + 2e,$$

where m is the number of carbon atoms and e the number of double bonds. Each polarity constant value K_2 corresponds to a different R_2 value (ratio of R_F of triglyceride to the R_F value of butyl hexabromostearate[6]). The $K_2 = 55$ fraction is a mixture of *PLLe*, *SLeLe*, and *OLLe* and the $K_2 = 57$ fraction is a mixture of *PLeLe*, *LLLe*, and *OLeLe*. The $K_2 = 59$ fraction contains only linoleodilinolenin. Thus, the molar relationships between the fatty acids in different fractions can be expressed as follows:

$$[Le] = 2[S] + [O] + [P] \quad (1)$$

and

$$[L] = [O] + [P] \quad (2)$$

for the $K_2 = 55$ fraction;

$$[Le] = 2[O] + 2[P] + {}^1/_2[L] \quad (3)$$

for the $K_2 = 57$ fraction, and

$$[Le] = 2[L] \quad (4)$$

for the $K_2 = 59$ fraction.

The fatty acid composition of separate triglyceride fractions determined by gas–liquid chromatography at 750 V and 1500 V, as well as the composition of these fractions calculated from equations (1)–(4), are shown in Table IV. These data demonstrate that the values found agree with the calculated ones only at 750 V.

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SUMMARY

The fatty acid composition of total linseed oil triglycerides and of different triglyceride fractions separated in a reversed-phase system have been determined by gas–liquid chromatography with a LOVELOCK argon-ionisation detector. At all detector voltages in the range of 750–1500 V the detector response was directly proportional to a given mass of substance for methyl esters of all fatty acids, with the exception of linolenic acid. The mass-response proportionality for methyl linolenate was observed only at 750 V.

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THEORY OF PARTITION CHROMATOGRAPHY

II. NUMERICAL CALCULATIONS

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The theoretical treatment of partition chromatography, advanced in an earlier article[1], has now been supplemented by numerical calculations performed on a digital computer. In the present calculations the treatment in ref. 1 has been closely followed and therefore only a short summary of the method is given here.

In the model used in the theory, the chromatographic column is divided into cells of equal width and the operation of the column is assumed to take place in discontinuous steps. During a step the solution in the mobile phase of a cell is allowed to exchange solute with the stationary phase of that cell (lateral diffusion) and with solutions of the mobile phase in neighbouring cells (longitudinal diffusion). At the end of the step the solutions of the mobile phase in each cell are instantaneously shifted one step to the adjacent cells and the equilibration procedure is repeated. The solute concentration in the mobile phase of a particular cell is designated $f_{i\,j}$, where i is the number of the cell and j the time (with the duration of a step being taken as the time unit). Thus $f_{i\,j}$ defines a matrix where the ith row gives the solute concentration in the ith cell at different times and the jth column gives the distribution of the solute concentration in the mobile phase over the entire chromatographic column at the time j. The elements of the matrix $f_{i\,j}$ are calculated from the characteristic parameters and the initial conditions of the column operation according to Eqns. (23) and (24) in ref. 1.

In order to simplify the treatment the last term in Eqn. (24) is neglected, *i.e.* longitudinal diffusion in the stationary phase is not taken into account. This approximation is of minor significance for the theory, but it makes possible the use of a single recursion formula for the determination of $f_{i\,j}$. It takes the form:

$$f_{i+1,\,j} = {}^1/_2\,\alpha_1\,f_{i+1,\,j-1} + (1 - \alpha_1 - \eta)\,f_{i,\,j-1} + {}^1/_2\,\alpha_1\,f_{i-1,\,j-1} + \\ + \eta(1-\xi)\,f_{i,\,j-2} + \eta(1-\xi)\,\xi\,f_{i,\,j-3} + \cdots + \eta(1-\xi)\,\xi^{j-3}\,f_{i,1} \qquad (1)$$

where:

$$\alpha_1 = \frac{2D_1}{rv^2}$$

$$\xi = \frac{\gamma V_2}{V_1 + \gamma V_2}\left(1 + \frac{V_1}{\gamma V_2}\,e^{-mr}\right)$$

$$\eta = \frac{\gamma V_2}{V_1 + \gamma V_2}\,(1 - e^{-mr})$$

with:

$$m = \frac{2D_2}{V_2}\left(\frac{\gamma}{V_1} + \frac{1}{V_2}\right)^{\star}$$

The following parameters characterize the column operation:

V_1, V_2 = volumes per interphase area of mobile and stationary phase respectively
D_1, D_2 = diffusion coefficients in mobile and stationary phase respectively
γ = partition coefficient
v = translational velocity of the mobile phase.

The initial conditions are given by the elements $f_{0\,j}$ and $f_{1\,j}$ in Eqn. (1), and were in all cases chosen to represent a rectangular concentration peak in the solution entering the chromatographic column.

The results were abstracted from the computor in the form of a few selected columns of a matrix, representing the concentration distribution in the mobile phase of the chromatographic column at different times. For a detailed characterization of the distributions their zeroth, first and second moments, with respect to the origin and with the cell width as unit of length, were also calculated. For the jth column they are defined in the following way:

$$A_0 = \sum_i f_{i\,j} \tag{2}$$

$$A_1 = \sum_i i\, f_{i\,j} \tag{3}$$

$$A_2 = \sum_i i^2 f_{i\,j} \tag{4}$$

It was found during the course of the calculations that the zeroth moment A_0 (representing the amount of solute in the mobile phase) in a given matrix generally very rapidly converges to a constant value. Hence a normalization on a common basis of the distributions represented by the different matrix columns is possible. The normalized distributions may then be characterized by the mean μ and the second moment around the mean μ_2. They are defined as follows:

$$\mu = A_1/A_0 \tag{5}$$

$$\mu_2 = A_2/A_0 - \mu^2 \tag{6}$$

In addition the mode M, defined as the location of the maximum of the smoothed distribution curve, was also determined.

Both μ and M are measures of location of the distribution, whereas μ_2 is a measure of dispersion. Further, as a measure of skewness Pearson's measure S is used. It is defined in the following way:

$$S = \frac{\mu - M}{\sqrt{\mu_2}} \tag{7}$$

In the following the results will in general be given in terms of the parameters A_0, M, μ, μ_2 and S.

* Here the dependence of m on the partition coefficient γ is taken different from that in ref. 1. It takes into account the assumption that diffusion in the stationary phase is the rate determining step in lateral diffusion. It simply states that the volume term of the mobile phase (where the concentration is kept uniform by convection) is changed in proportion to γ.

The present calculations were carried out with the Facit digital computer and matrixes of the order 200 were evaluated. The effective time for the evaluation of a matrix was about one hour.

RESULTS

In the calculations the above mentioned characteristic parameters were varied in order to determine their effect on the column operation. The parameters occur in the coefficients of Eqn. (1) only in form of the combinations $D_2\tau/V_2^2$, $V_1/\gamma V_2$ and $D_1/v^2\tau$. Therefore, not all of the parameters need be varied independently, and in the present calculations thus only the parameters D_1, D_2 and γ were varied. The others were kept fixed and had the values:

$$v = 0.01 \ cm \cdot sec^{-1} \tag{8}$$

$$V_1/V_2 = 0.35 \tag{9}$$

$$V_2 = 0.01 \ cm \tag{10}$$

Relation (9) refers to a column filling consisting of tight-packed spherical beads. According to (10) the radius of a bead is then 0.03 cm.

Convergence of the numerical solutions

In the numerical calculations the column operation occurs in discontinuous steps. As the exact solution is approached only in the limit when the number of steps tends to infinity, it is necessary to examine the convergence of the numerical solutions. For this reason calculations were carried out using alternatively 10, 20, 50 and 200 cells for the same length of a given column. The corresponding matrixes are numbered 1, 2, 3 and 4, respectively. In all cases the characteristic parameters have the same values, namely $D_1 = 10^{-5}$ cm$^2\cdot$sec^{-1}, $D_2 = 5\cdot10^{-6}$ cm$^2\cdot$sec^{-1} and $\gamma = 1$. The initial conditions are:

$$f_{1j} = \begin{cases} 1 \text{ for } j = 1,\cdots, n \\ 0 \text{ for } j = n+1,\cdots, 200 \end{cases} \tag{11}$$

$$f_{0j} = \begin{cases} 1 \text{ for } j = 1,\cdots, n-1 \\ 0 \text{ for } j = n,\cdots, 199 \end{cases} \tag{12}$$

$$f_{i1} = 0 \text{ for } i = 2,\cdots, 200 \tag{13}$$

In order to represent the same initial concentration peak the value of n in (11) and (12) is 1, 2, 5 and 20 for the matrixes 1, 2, 3 and 4, respectively. The cell width in the corresponding column models is 0.1, 0.05, 0.02 and 0.005 cm and the equilibration time τ has the values 10, 5, 2 and 0.5 sec, respectively. The results are recorded in Table I in the form of μ and μ_2 values for the 10th, 20th, 50th and 200th columns of the respective matrixes, representing the situation at the same nominal time instances. In Fig. 1 plots of μ and μ_2 against $1/n$ are shown. These curves indicate the way of convergence to the solutions of continuous column operation, represented by the extrapolated values on the ordinate axis.

TABLE I

MATRIX NOS. 1, 2, 3 AND 4

Matrix No.	*1*	*2*	*3*	*4*
Column No.	*10*	*20*	*50*	*200*
μ	4.1086	3.4014	3.0498	2.9028
μ_2	1.6891	1.211	1.037	1.006

Influence of diffusion and partition coefficients

The influence of both the lateral and longitudinal diffusion coefficients and the partition coefficient is established by separately changing these parameters. In all these calculations the value $n = 5$, in the initial conditions (11) and (12), is used and the value of τ is 2 sec giving a cell width of 0.02 cm. The conditions are then reasonably close to those of continuous column operation.

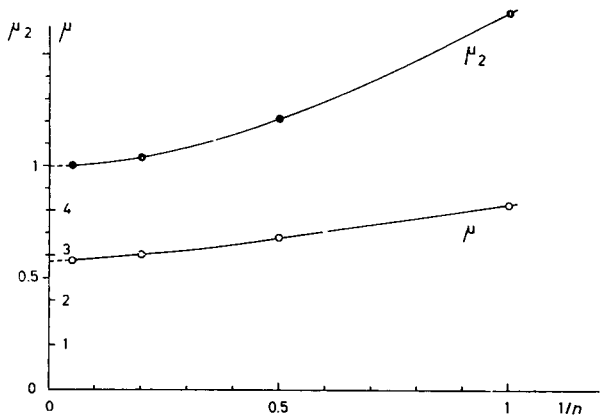

Fig. 1. Convergence of solutions from discontinuous column operation.

In the matrixes numbered 5, 6 and 7 different longitudinal diffusion coefficients are used, D_1 having the values 0, 10^{-5} and $5 \cdot 10^{-5}$ cm$^2 \cdot$sec^{-1}, respectively. (The results are recorded in Table II.) In the matrixes numbered 8, 9 and 10, different lateral diffusion coefficients are used, the values of D_2 being ∞, 10^{-6} and 10^{-7} cm$^2 \cdot$sec^{-1}, respectively. (The results are recorded in Table III). To this group belongs also matrix No. 5 in Table II, for which $D_2 = 5 \cdot 10^{-6}$ cm$^2 \cdot$sec^{-1}. Finally, in the group of matrixes numbered 11, 12, 13, 14, 15 and 16 the partition coefficient is varied, the values of γ being 0, 0.1, 0.2, 0.5, 2 and 5, respectively. (The results are shown in Table IV). To this group belongs also matrix No. 6 in Table II, for which $\gamma = 1$.

DISCUSSION

From the results in Tables II–IV it is seen that in all cases, except matrix No. 10, steady state conditions are established in the chromatographic column. The steady state is characterized by constancy in the value of total solute concentration in the

TABLE II

MATRIX NOS. 5, 6 AND 7

D_1 = 0, 10^{-5} and $5 \cdot 10^{-5}$ $cm^2 \cdot sec^{-1}$, respectively
D_2 = $5 \cdot 10^{-6}$ $cm^2 \cdot sec^{-1}$
γ = 1

Matrix No.	*Parameter*	*Column number*				
		25	*50*	*100*	*150*	*200*
5	A_0	1.2963	1.2963	1.2963	1.2963	1.2963
	μ	8.7187	15.201	28.165	41.129	54.092
	M	7.841	14.285	27.237	40.197	53.159
	μ_2	11.35	24.40	50.53	76.54	102.7
	S	0.261	0.185	0.131	0.107	0.092
6	A_0	1.2834	1.2834	1.2834	1.2834	1.2834
	μ	8.7666	15.249	28.212	41.174	54.137
	M	7.731	14.182	27.134	40.094	53.055
	μ_2	12.24	25.92	53.31	80.84	108.3
	S	0.296	0.210	0.148	0.120	0.104
7	A_0	1.2315	1.2315	1.2315	1.2315	1.2315
	μ	8.9704	15.452	28.416	41.380	54.343
	M	7.430	13.901	26.853	39.812	52.773
	μ_2	15.75	32.05	64.64	97.20	129.8
	S	0.388	0.274	0.194	0.159	0.138

TABLE III

MATRIX NOS. 8, 9 AND 10

D_2 = ∞, 10^{-6} and 10^{-7} $cm^2 \cdot sec^{-1}$, respectively
D_1 = 0
γ = 1

Matrix No.	*Parameter*	*Column number*				
		25	*50*	*100*	*150*	*200*
8	A_0	1.2963	1.2963	1.2963	1.2963	1.2963
	μ	7.4445	13.926	26.389	39.852	52.815
	M	7.180	13.681	26.646	39.609	52.572
	μ_2	4.166	8.97	18.57	28.19	37.78
	S	0.130	0.082	0.056	0.046	0.040
9	A_0	1.4236	1.2990	1.2963	1.2963	1.2963
	μ	16.090	22.826	35.770	48.734	61.698
	M	13.453	19.027	31.405	44.318	57.211
	μ_2	39.81	114.48	239.6	364.3	488.9
	S	0.418	0.355	0.282	0.231	0.203
10	A_0	3.9352	3.0909	2.1259	1.6794	1.4727
	μ	22.891	47.080	89.825	121.74	143.97

TABLE IV

MATRIX NOS. 11, 12, 13, 14, 15 AND 16
$D_1 = 10^{-5}$ cm^2·sec^{-1}
$D_2 = 5 \cdot 10^{-6}$ cm^2·sec^{-1}
γ = 0, 0.1, 0.2, 0.5, 2 and 5, respectively

Matrix No.	*Parameter*	*Column number*				
		25	*50*	*100*	*150*	*200*
11	A_0	4.9500	4.9500	4.9500	4.9500	3.4664
	μ	23.071	48.071	98.071	148.071	
	M	22.950	48.009	98.039	148.050	
	μ_2	4.13	6.55	11.6	16.7	
	S	0.059	0.023	0.009	0.005	
12	A_0	3.8540	3.8500	3.8500	3.8500	3.8489
	μ	19.892	39.358	78.247	117.135	156.013
	M	21.878	43.777	81.422	120.212	159.044
	μ_2	18.94	54.27	125.8	197.1	267.7
	S	—0.457	—0.600	—0.283	—0.219	—0.186
13	A_0	3.1519	3.1500	3.1500	3.1500	3.1500
	μ	17.385	33.302	65.121	96.937	128.756
	M	20.329	34.641	66.309	98.087	129.887
	μ_2	23.72	62.37	139.6	217.3	294.3
	S	—0.604	—0.170	—0.101	—0.078	—0.066
14	A_0	2.0383	2.0382	2.0382	2.0382	2.0382
	μ	12.581	22.875	43.463	64.052	84.638
	M	12.026	22.234	42.790	63.366	83.951
	μ_2	20.56	47.04	99.89	152.8	205.6
	S	0.122	0.093	0.067	0.056	0.048
15	A_0	0.73724	0.73724	0.73724	0.73724	0.73724
	μ	5.7741	9.4976	16.944	24.391	31.838
	M	4.842	8.789	15.986	23.424	30.876
	μ_2	5.602	11.38	22.95	34.52	46.10
	S	0.394	0.210	0.200	0.165	0.142
16	A_0	0.32386	0.32386	0.32386	0.32386	0.32386
	μ	3.5086	5.1442	8.4154	11.687	14.958
	M	2.913	4.404	7.695	10.966	14.228
	μ_2	1.874	3.717	7.403	11.10	14.78
	S	0.436	0.384	0.265	0.216	0.190

mobile (and stationary) phase of the chromatographic column. This value is independent of the diffusion coefficients D_1 and D_2 and, for a column of given geometry, depends only on the partition coefficient γ. The minor differences found in Tables II and III are due to end effects (diffusion out through the column ends). Under steady state conditions very simple rules exist concerning the translational velocity (peak velocity) and spreading of a concentration peak.

Peak velocity

The absolute peak velocity may be defined as the translational velocity of the center of mass of a concentration peak. A more convenient quantity is the relative

peak velocity, which will be designated by ν and is defined as the ratio between the absolute peak velocity and the velocity of the mobile phase. It is obtained directly as the absolute velocity of the peak if the width of a cell and the equilibration time τ are used as length and time units respectively, as then the velocity of the mobile phase becomes unity.

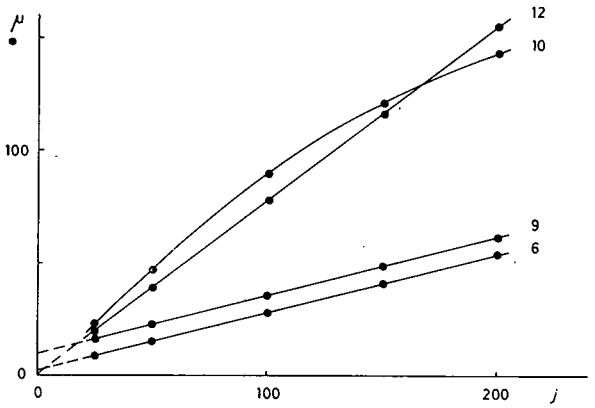

Fig. 2. Peak location as functions of time. Numbers in the figure indicate matrix numbers. For matrix No. 10 steady state is not established.

From the data in Tables II, III and IV it follows that under steady state conditions the peak velocity is constant. This is illustrated for some representative cases in Fig. 2, where plots of μ against time are shown. The calculated values of ν are recorded in Table V. It is seen to be independent of the diffusion coefficients D_1 and D_2, but strongly dependent on the partition coefficient. The latter is illustrated in Fig. 3, where a plot of ν against γ is shown.

Peak spreading

The breadth of a concentration peak is determined by its second moment around the mean μ_2. Under steady state conditions this is found to be a linear function of

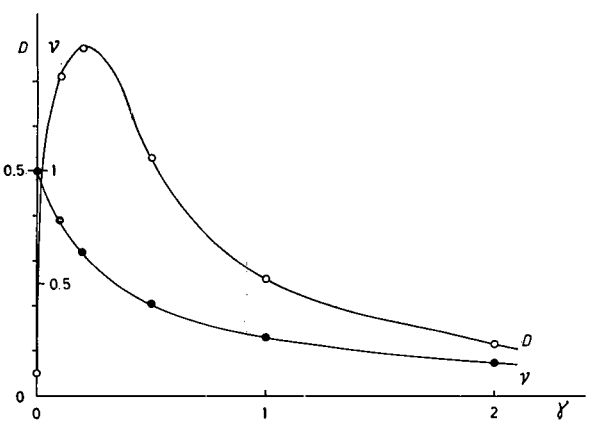

Fig. 3. Peak velocity and spreading coefficient as function of partition coefficient.

TABLE V

VALUES OF RELATIVE PEAK VELOCITIES AND SPREADING COEFFICIENTS

Matrix No.	v	D *(local units)*	$D \cdot 10^5$ *(c.g.s. units)*
5	0.2593	0.2610	5.220
6	0.2593	0.2745	5.489
7	0.2593	0.3260	6.519
8	0.2593	0.09605	1.921
9	0.2593	1.248	24.96
11	1.000	0.0503	1.006
12	0.7778	0.711	14.22
13	0.6364	0.773	15.46
14	0.4118	0.529	10.57
15	0.1489	0.1157	2.314
16	0.06543	0.03686	0.7372

time. This is seen from Fig. 4, where some representative plots of μ_2 against time are shown. It is thus possible to characterize the peak spreading by a spreading coefficient D, defined by:

$$\mu_2 = 2Dt + C \tag{14}$$

where C is a constant, which takes care of the "end effect".

There is a close analogy between the present treatment of peak spreading and the spreading of concentration gradient curves in diffusion experiments[2]. Thus, the definition of the spreading coefficient is analogous to the definition of the diffusion coefficient in free diffusion experiments and the factor 2 in Eqn. (14) is included to make the correspondence complete. In the case of matrix No. 11, where the peak spreading is due only to longitudinal diffusion the value of D is $1.006 \cdot 10^{-5}$ cm$^2 \cdot$sec^{-1}, which is in good agreement with the longitudinal diffusion coefficient $D_1 = 10^{-5}$ cm$^2 \cdot$ sec^{-1}, used in these calculations. It is obvious that even other methods, *e.g.*, the area method may be used to determine the spreading coefficients.

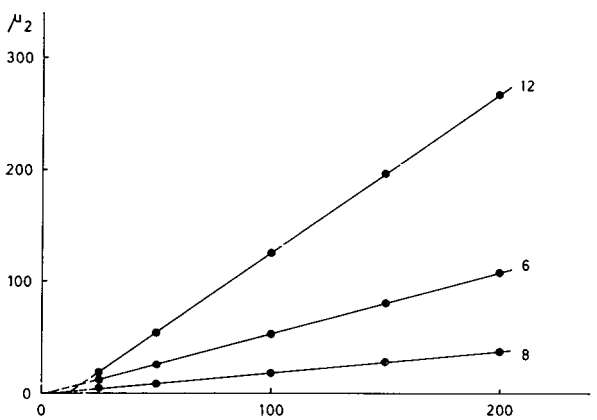

Fig. 4. Second moment as function of time. Numbers in the figure indicate matrix numbers.

The calculated values of D are listed in Table V. It is seen that longitudinal diffusion is of relatively minor importance as a cause for peak spreading. The major cause is the partition process, and its effect is considerable even at instantaneous equilibration ($D_2 = \infty$), and is greatly accentuated by local non-equilibrium. The influence of the partition coefficient is somewhat complicated, and is illustrated in Fig. 3. For $\gamma = 0$ the spreading coefficient equals the longitudinal diffusion coefficient; it rises steeply with increasing γ, passes through a maximum and then decreases monotonously with increasing γ.

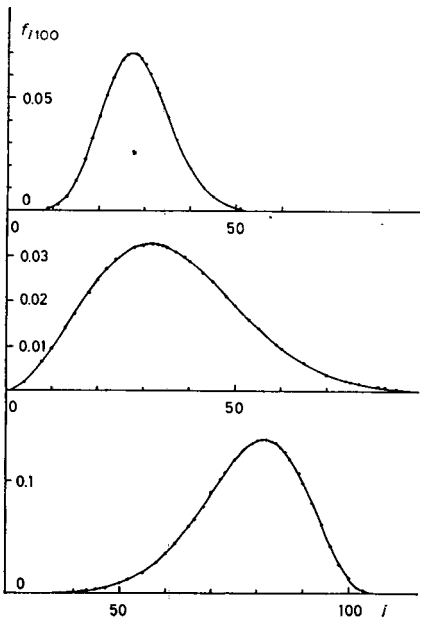

Fig. 5. Distribution curves for some peaks. Columns $j = 100$ for matrix Nos. 6, 9 and 12 from top to bottom.

Peak asymmetry

Besides the peak velocity and peak spreading, the asymmetry of a concentration peak is of fundamental importance in characterizing the chromatographic process. In the present treatment the asymmetry of the concentration distribution is described by the measure of skewness S, defined by Eqn. (7). As S admittedly gives a poor characterization of the form of a concentration peak, some typical peaks are reproduced in Fig. 5 for illustration purposes. It is seen from the S values in Tables II, III and IV that the peaks may exhibit both positive and negative skewness. It is also seen that the skewness invariably decreases with time. This indicates that the asymmetry is an "end effect", which is introduced into the distribution when the peak enters the chromatographic columns.

Peak exit from column

Hitherto the solute concentration distribution inside a chromatographic column has been considered. It is also of interest to examine the behaviour of a con-

centration peak at the exit from the column. From rather qualitative reasoning it can be induced that at exit a considerable spreading of the peak occurs. The reason for this is that the part of the peak which already has left the column has the same velocity as the mobile phase, whereas that part of the peaks which still is inside the column has a velocity very close to the peak velocity. Thus, the distortion is due to the difference in velocities of the peak and the mobile phase and is accentuated by low relative peak velocities. An illustration of the process is given in Fig. 6.

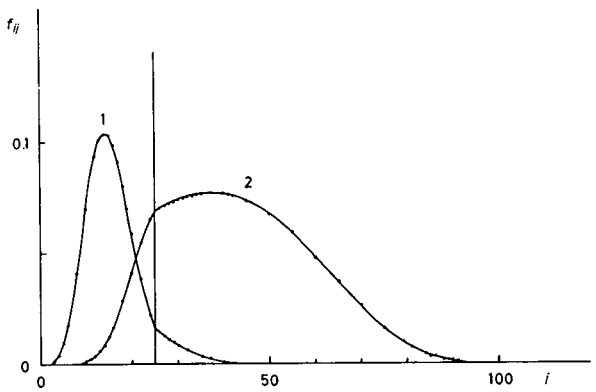

Fig. 6. Peak exit from column. Column end at cell No. 25. The data were obtained from matrix No. 5. Curve 1 refers to time $j = 50$, curve 2 to $j = 100$.

The present calculations represent "experiments" with the model column and, within this frame, constitute a complete study of the process of partition chromatography. They cover wide ranges of operational conditions and include all practical situations as to the magnitudes of the diffusion coefficients, the partition coefficient, etc. Although the model represents an idealization of a real chromatographic column it is felt that it brings out the pertinent features of the chromatographic process and, due to the high accuracy obtainable in the calculations, the significance of such characteristic quantities as peak velocity and the spreading coefficient of a peak are established. It thus makes a better understanding of the chromatographic process possible.

It should be mentioned here that, independent of the present work, the process of partition chromatography has been studied by LAURENT AND LAURENT[3] with the help of an electrical analogy computer. A comparison of the results will therefore be of interest.

ACKNOWLEDGEMENTS

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SUMMARY

The operation of a chromatographic column has been simulated by numerical calculations on a digital computer. The calculations cover wide ranges of operational conditions for a column and give a detailed characterization of the chromatographic process.

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A COLUMN DESIGN FOR REVERSE-FLOW SEPHADEX GEL PERMEATION CHROMATOGRAPHY*,**

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INTRODUCTION

The use of cross-linked dextran gels (Sephadex***) for molecular sieve fractionation has achieved wide popularity. The sieve properties of a particular gel are a function of the porosity of the gel particle, which in turn is determined by the degree of cross-linking between dextran chains. Of the commercially available materials, Sephadex G-200 has the largest pore size. This material allows the separation of spherical particles with molecular weights above 200,000 or asymmetric particles of equivalent Stokes radii[1]. As discussed by FLODIN[2], for a given gel particle size, separation is a direct function of the number of "theoretical plates". The number of "theoretical plates", N, is given by the formula, $N = (L/\mathrm{EHTP})$, where L is the column length and EHTP is the equivalent height of a theoretical plate, which, for a given particle size, can be decreased by reducing the flow rate.

Because of the great compressibility of the large pore-sized Sephadex G-200 particles, increasing the bed height can result in flow rates so slow as to be impractical. Moreover, many workers have observed the reduction of flow rate with repeated use of G-200 columns. In the course of studies on the separation of various proteins by G-200, we[3] began using the technique of reverse-flow (solvent flow up the column) in order to minimize or even avoid the compaction of the gel material. Independently, PORATH AND BENNICH[4], also dealing with the problem of gel compaction, described the use of reverse-flow in conjunction with the technique of recycling chromatography. At that time we adopted their column design, but subsequent experience led to major modifications resulting in the design reported in the present paper.

The reverse-flow technique has several distinct advantages. It enables one to use long columns of G-200 repeatedly while maintaining the same flow rate. Furthermore, since solvent flow is directed against the field of gravity, formation and maintenance of sharp sample bands can readily be achieved.

This paper presents a description of the reverse-flow column in use at present in this laboratory, the method of handling the G-200 in the formation of the column bed and the technique of sample application.

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** See MOORE[5] for a discussion of the term "gel permeation".

*** Obtained from Pharmacia Fine Chemical Co., New York 17, N.Y.

COLUMN DESIGN

Fig. 1 presents a diagram of the column. It consists of a lucite cylinder fitted at each end with a plunger, stem, and handle made of polyvinylchloride (PVC), which is much easier to machine than lucite. The face of the plunger has a funnel-shaped recess which directs the flow of liquid into the outlet channel. A disc of hydrophilic porous polyethylene* is inserted into a Teflon gasket and this assembly, in turn, is inserted into the recess in the face of the plunger. The Teflon gasket is employed in order to prevent leakage around the edges of the porous disc, which was

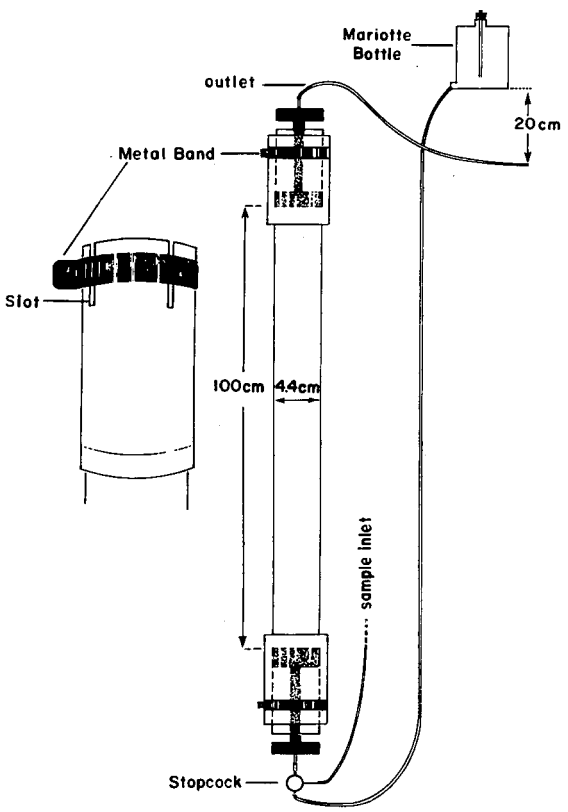

Fig. 1. Diagram of reverse-flow column. Left: Diagram of slotted column end with clamp.

observed when the porous polyethylene was directly fitted into the plunger. Hydrophilic porous polyethylene was chosen over other porous plastics because of its more rapid water flow rate. A lucite collar is threaded onto the stem of the plunger and acts as a guide upon insertion of the plunger into the column. The walls of the column are slotted at both ends. This permits the plunger assembly (Fig. 2) to be secured by compressing the column walls against the lucite collar by means of an adjustable metal band. The portions of the column into which the plunger assemblies are in-

* Obtained from Porex Materials Corp., Fairburn, Ga.

serted are double-walled in order to withstand the strains resulting from the tightening of the metal band. Prior to use, the porous filter is cleaned with a detergent and then thoroughly washed by passage of water through the plunger assembly channel. Boiled latex tubing, $^1/_{16}$-in. I.D. and $^1/_{16}$-in. wall thickness, is slipped over the tips. The use of latex tubing is preferable because the flow rate is faster with rubber, which is wettable, than with any of the plastics, which are not.

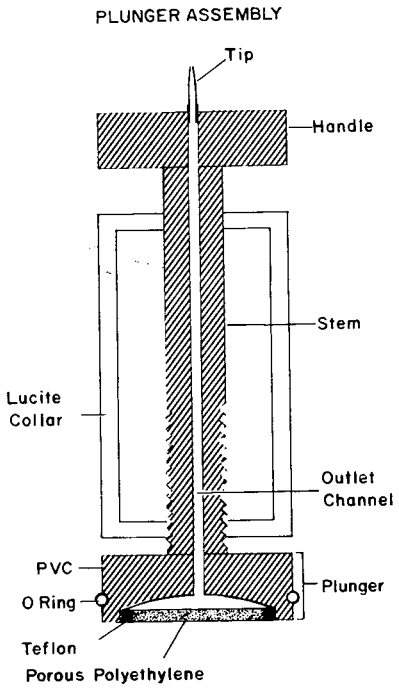

Fig. 2. Diagram of plunger assembly.

FORMATION OF THE COLUMN BED

Sephadex G-200 is passed through a set of U.S. Standard sieves. The 140–200 mesh (100–74 μ) fraction* is equilibrated at room temperature with gentle stirring for at least 72 h in the buffer to be used. Filling of the column is done at room temperature. A plunger assembly is inserted into the bottom of the column and the metal band is tightened. The filling vessel (Fig. 3) is put into the top of the column, which is then filled with buffer warmed to 40–50°. A small amount of coarse Sephadex G-25 (50 mesh, 300 μ) is added to form a $^1/_4$ cm layer on top of the filter, in order to prevent any of the finer G-200 particles from clogging the pores of the filter. After the G-25 has settled, about $^1/_3$ of the buffer in the column is allowed to flow out, so as to improve the packing of the layer of G-25. The outlet tubing is then closed and the column refilled with warmed buffer.

The suspension of G-200 is poured into the filling vessel and stirred with a

* For finer resolution the 200–270 mesh fraction should be used.

"serpentine" or paddle stirrer which reaches into the column. In agreement with BELING[6], it is essential for uniform packing of the bed material that the liquid in the entire column should be agitated by alternating the direction of rotation of the stirrer. This is accomplished by attaching the stirrer to a reversing motor programmed* to change the direction of rotation at 2-min intervals. Agitation of this type reduces the tendency of the gel particles to pile up along the column walls. The gel particles are allowed to settle until a 3–4 cm layer is formed. The outlet tubing is raised so that the distance between the buffer level in the filling vessel and the end of the

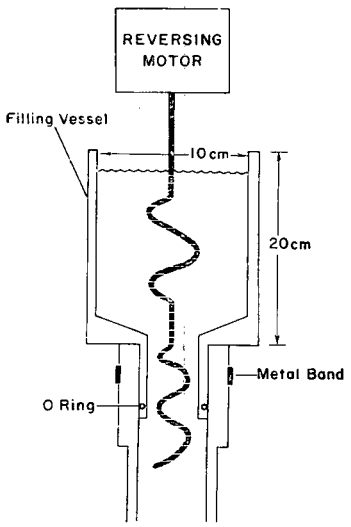

Fig. 3. Diagram of filling vessel with stirrers.

outlet tube is approximately 20 cm. The fluid is now allowed to flow at the rate of 35–40 ml/h under a 20 cm hydrostatic head. After the column is packed to the necessary level, it is moved into the cold room (+4°) where the column bed contracts slightly with temperature equilibration. Buffer is then run through the column for a period of 12–18 h, maintaining the same hydrostatic head. A $^1/_4$ cm layer of coarse G-25 (50 mesh, 300 μ) is then applied to the top of the bed of G-200. The flow is stopped and the column is allowed to equilibrate overnight. During this equilibration period the column bed expands. This last point is extremely important, for if the expansion is not allowed to occur, compaction of the bed will result with time. With a layer of buffer on top of the bed surface, the plunger assembly is carefully inserted to avoid trapping air bubbles and then pushed down to a level just above the bed surface. The plunger is then brought into contact with the bed surface by one or two turns of the handle on the threaded stem.

SAMPLE APPLICATION

The application of the sample from below has the distinct advantage of allowing the formation of a well-defined sample band. This condition is accomplished by es-

* Available from Technical Services, Cambridge 41, Mass.

tablishing density shelves at the upper and lower boundaries of the sample band, resulting in boundary stabilization. In general, the sample is denser than the elution buffer (if not, it can be made so by the addition of sucrose), so that the upper boundary of the sample zone is stabilized by the density shelf. In order to stabilize the lower boundary of the sample zone, the sample is followed by a denser solution made of buffer and sucrose or an inert material of high molecular weight, such as Ficoll or Dextran 10*. The polymer is used rather than sucrose in those cases where one wishes to avoid the "droplet effect" reported and discussed by SVENSSON *et al.*[7,8]. The choice of Dextran 10 or Ficoll is determined by the molecular size of the material under investigation. Ficoll (mol. wt. 400,000) is employed when materials which are not excluded from the gel particles are being studied. Dextran 10 (mol. wt. 10,000) is used when materials of greater molecular size are under investigation.

Application of the sample to the bottom of the column bed is facilitated by the use of a plastic three-way micro-stopcock. As shown in Fig. 1, the micro-stopcock is placed immediately below the bottom of the column in order to avoid mixing of the sample solution with the buffer of lower density. Care must be exercised to prevent the entrance of air into the tubing which leads into the column. Prior to application, the sample is filtered in order to remove any particulate matter which might clog the porous filter. After filtration, the sample is slowly fed into the column by means of gravity or by a motor-driven syringe. The stop-cock is closed and the "sample tubing" disconnected. New tubing is connected, and 10–15 ml of the high-density solution is slowly fed into the column. The stop-cock is then set so that the elution buffer enters the column from a Mariotte bottle so positioned that there is a hydrostatic head of approximately 20 cm across the column. The flow rate is regulated by means of a screw clamp on the exit tubing.

DISCUSSION

The modifications of the column design of PORATH AND BENNICH[4] described above facilitate assembly of the column. The presence of the lucite collar permits the guided insertion of the plunger assembly, eliminating the disturbance of the gel bed surface occasionally observed with the other column design. The method of securing the plunger assemblies (slots and clamps) eliminates the problems of machining and maintenance of lucite/lucite threads. The threading of the PVC stem through the lucite collar presents no problem. Furthermore, the accurate machining necessary for the leak-proof fitting of the filter disc is readily obtainable with PVC as opposed to lucite. The use of hydrophilic porous polyethylene contributes greatly to the reduction of the resistance to flow and in this regard is superior to other porous plastics.

Employing the column and techniques described in this report, a 4.4 cm × 100 cm column of Sephadex G-200 has been in continuous use for over 4 months with no change in flow rate (25 ml/h). The systems subjected to gel permeation chromatography have included human serum, crude collagenase preparations, vitreous and crude actin preparations. The column effluents have been monitored by continuous flow instruments which measure U.V. absorption, U.V. fluorescence and

* Obtained from Pharmacia Fine Chemical Co., New York 17, N.Y.

relative refractive index. When such equipment is placed in the flow line, the Mariotte bottle must be raised in order to compensate for the additional resistance of the tubing added to the system.

The separation patterns obtained are similar to those reported by other workers[2,9]. The patterns obtained with the continuous flow refractometer* are of interest and will be reported in a later paper. Fluorescence was measured by a Turner continuous flow fluorometer, Model 111, which was modified so as to automatically attenuate the instrument sensitivity. This modification** will be described in a later paper.

In a very recent article, BROMAN AND KJELLIN[10] described a plunger somewhat similar to ours for use in a reverse-flow column of Sephadex G-100.

The column described in this paper is commercially available from Future Plastics, Inc., 152 Columbia Street, Cambridge 39, Mass., U.S.A.

ACKNOWLEDGEMENTS

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SUMMARY

The design of a column for reverse-flow chromatography has been presented. The column has been employed for gel permeation chromatography with Sephadex G-200. Techniques for the formation of a column bed of G-200 are described as well as a method for applying the sample to the column. The techniques described allow for the continuous use of the G-200 column without changes in the flow rate with extended use.

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* Model R4, available from Waters Associates, Framingham, Mass.
** Available from Technical Services, Cambridge 41, Mass.

EXTRUSION COLUMN CHROMATOGRAPHY ON CELLULOSE

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INTRODUCTION

Extrusion column chromatography has long been successfully applied to adsorption systems[1]. The qualitative correlation of thin-layer techniques with extrusion column chromatography has been effectively applied in this laboratory for the adsorbent Magnesol[2]. An improved thin-layer partition chromatography on microcrystalline cellulose was recently reported[3]. This paper describes the extension of thin-layer chromatography on microcrystalline cellulose (Avirin*) to a cellulose extrusive technique which can be correlated qualitatively with the thin-layer technique.

Extrusion column chromatography offers an analytical as well as preparative tool which is both compact and rapid, provided the proper conditions can be found for separating compounds and locating zones. The extrusive technique reported herein is a modification of that reviewed by THOMPSON[1]. It was the purpose of the present work to apply the extrusive technique to a representative range of organic and inorganic compounds containing polar functional groups which permit good partitioning effects.

EXPERIMENTAL

Adsorbent

Avirin is a microcrystalline cellulose obtained from the Avicel Sales Division of American Viscose Division, FMC Corp., Marcus Hook, Pa. Avicel is the pharmaceutical grade of the same material. We found that this grade possessed no advantage over our samples of Avirin, but either grade is satisfactory.

Column preparation

The ZECHMEISTER[1,4] type column was packed by pouring, onto a cotton filter pad, a blended suspension of cellulose (Avirin) in the development solvent (1:4, w/v) and permitting the solvent to filter through the cotton pad with about one-third of an atmosphere pressure (provided by bleeding air into the trap of a water aspirator with stopcock open). This pressure was maintained during washing, elution, and drying of the column to assure an even partitioning surface inside the column.

When the desired amount of developing solvent (determined by experimentation) had passed through the column and free dripping from the bottom of the

* Now designated Avicel-Technical Grade.

column had ceased, the cellulose was assumed dry enough for extrusion and was extruded in the usual manner. The surface of the extruded column was dried with a stream of warm air in preparation for the location of the zones.

Sugars were located by the silver nitrate–sodium hydroxide method[5]. A strip 0.5 cm wide was formed by spraying through a slit in a plastic shield, equal in length to the column, followed by spraying with *M* sodium thiosulfate. Sugars were also detected by streaking with a solution containing four parts of 2 % aqueous sodium metaperiodate and one part of 1 % potassium permanganate in 2 % aqueous sodium carbonate[6]. Compounds containing the primary amino group were located by spraying through the shield with 0.2 % ninhydrin in ethanol. The colored iron complexes were easily visible during all phases of the separation.

Procedure

A typical separation was that effected between D-galactose and D-xylose. A column (34 × 145 mm) was prepared as described above by blending together cellulose (60 g) and 1-butanol–ethanol–water (8:1:1, 240 ml, developing solvent). After washing for 1 h with about 200 ml of the same solvent, the solvent level above the column was permitted to come to a point where the top of the cellulose was just uncovered. D-Galactose (80 mg) and D-xylose (80 mg) dissolved in 1-butanol–ethanol–water (4:1:1, 5 ml) were poured on the column top. When the solution of sugars had settled into the column, a filter paper was placed on the column top and the developing solvent, 1-butanol–ethanol–water (8:1:1, 200 ml) was poured through the column over a 2-h period under the same vacuum conditions as used for packing the column. When no more solvent dripped from the bottom of the column, the column was extruded, partially dried with a stream of warm air, and streaked with permanganate–periodate reagent[6] as described above. Two zones appeared (see Table I), one zone 25 mm from the top of the column and 25 mm wide, the second zone 75 mm from the top of the column and 35 mm wide. The zones were excised, eluted three times with 30 ml of water, centrifuged, and fractions concentrated to about 5 ml, and filtered through a 4-mm Celite pad on a sintered glass funnel. Thin-layer chromatography[3] on cellulose showed chromatographic homogeneity for the fractions on developing with 1-butanol–ethanol–water (4:1:1). The fractions were concentrated to sirups, taken up in hot methanol, and filtered to remove insoluble residues. Crystals formed on concentrating both solutions; yield, 65 mg (81 %) of D-xylose and 62 mg (78 %) of D-galactose, m.p. identical with literature values.

Other separations are recorded in Table I.

The colored iron compounds constitute the crude mixture of complexes formed by self-condensation of *o*-aminobenzaldehyde in the presence of iron(II) salts[7]. These are the subject of continuing investigations. Elution of the excised zones containing the complexes was most effectively carried out by blending the zone into a slurry of the developing solvent and pouring into a column which was then eluted further with several more volumes of the developing solvent.

DISCUSSION OF RESULTS

A great asset of this extrusion technique is that it apparently can be applied to compounds usually separable by paper chromatography (see range of compounds in

TABLE I

EXTRUSION COLUMN ZONE MOBILITIES

Expt.	*Compounds*	*Mg applied*	*Column (mm)*	*Developer*	*Solvent (ml)*	*Time (h)*	*Indicator**	*Zone dimensions (mm)***	
1	D-Galactose	80	34 × 145	BuOH–EtOH–H_2O (8:1:1)	200	2.0	A	25	25
	D-Xylose	80						75	35
2	(+)-Tartaric acid	100	34 × 145	BuOH–HOAc–H_2O (4:1:1)	50	0.5	A	50	30
	Oxalic acid	100						83	20
3	Gentiobiose	20	40 × 200	BuOH–EtOH–H_2O (4:1.2:1.2)	300	2.8	A, B	31	28
	Maltose	50						31	28
	D-Glucose	50						108	62
4	Gentiobiose	20	40 × 200	BuOH–EtOH–H_2O (8:1:1)	1000	9.0	A, B	12	66
	Maltose	50						25	31
	D-Glucose	50						108	63
5	D,L-Valine	10	34 × 145	BuOH–HOAc–H_2O (8:1:1)	200	2.0	C	90	10
	L-Leucine	80						105	20
6	2-Amino-2-deoxy-D-galactose·HCl		40 × 184	EtOAc–Pyr–H_2O–HOAc (5:5:3:1) (Ref. 8)	1000	8.0	B, C	107	65
	D-Glucose							effluent	
7	Condensation products of *o*-amino-benzaldehyde and iron(II) salts		40 × 200	BuOH–EtOH–H_2O (4:1.2:1.2)	400	3.0	Colored complexes		
	Orange baseline***							0	20
	Orange-yellow							45	25
	Green							121	31
	Brown							197	12
	Yellow-brown							effluent	

* Reagent A: $KMnO_4$–$NaIO_4$; reagent B: $AgNO_3$–NaOH–$Na_2S_2O_3$; reagent C: ninhydrin.

** Distance in mm from top of column (1st column of numerals) and width of zone (2nd column).

*** Zone color.

Table I). The microcrystalline cellulose needs no filter-aid and permits easy packing of the ZECHMEISTER[1,4] column.

Applying the mixture of compounds to be separated to the top of the column is done effectively and evenly on this medium. The relative proportions of polar components in solvent mixtures and the amount of developing solvent necessary were effectively patterned on the known solvent systems and developing times for thin-layer partition chromatography on cellulose (see Table I).

After development, the column was permitted to drip solvent with the same vacuum as during elution until a relatively solid mass formed. The column was then easily extruded intact. This is in contrast to the Magnesol–Celite column which requires thorough drying before extrusion. After some surface solvent was removed from the column core with the aid of a warm air stream, no further drying was necessary before streaking to locate zones, excising the zones, and eluting the separated products from the cellulose.

It would appear that a less polar solvent which moves the zones quite slowly on a cellulose thin-layer plate brings about a more efficient partitioning effect between the cellulose, solvent, and compounds being separated at the flow rate of the developing solvent employed. Thus, comparing Table I, in Expt. 3 and Expt. 4, D-glucose moved the same distance on the column with both solvents, but in one case the mobility noted was effected in 2 h and in the other in 8 h. However, maltose and gentiobiose failed to separate in Expt. 3, whereas with the longer elution time in the less polar solvent (Expt. 4), effective separation occurred.

When the developed column was dried rapidly with vacuum instead of dripping until enough solvent was removed to permit extrusion, the zones moved quickly from their location at the end of the desired development time and smeared badly over the cellulose. This was avoided by the suggested technique.

The colored iron complexes[7] provided an interesting system with which to study development time. These possessed mobilities similar to mono- and disaccharide derivatives with butanol–ethanol–water solvent systems. The elution of the excised zones from the iron complexes was best carried out by placing the zone in a Waring blender with the same solvent used on the column and pouring this slurry into a column fitted with a cotton filter pad and stopcock and then passing several column volumes of the developing solvent through the filter. The iron complexes evidently adhere to the cellulose more tenaciously than do the sugars and amino acids, which were easily eluted by simpler washings of the cellulose.

Although a more complex mixture of amino acids (Expt. 5) was not investigated, the results obtained here indicated that solvent systems and developing conditions for a wide range of amino acids could be found.

ACKNOWLEDGEMENTS

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SUMMARY

Sugars, amino acids and transition metal complexes were shown to be separable

by cellulose extrusion column chromatography. The technique may be correlated with cellulose thin-layer chromatography, as well as with paper methods.

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CHROMATOGRAPHIC BEHAVIOUR OF ORGANIC ACIDS ON DOWEX 1-×10

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In a previous communication[1] a method was described for the paper chromatography of organic acids, in which the indicator was incorporated in the solvent mixture, and a list was later presented[2] of the R_F values of more than 120 acids. The method was used successfully to monitor the separation by chromatography on anion exchange resins of mixtures of organic acids obtained by oxidative degradation of large molecules (humic acids derived from coal), and the list of R_F values allowed tentative identification of the component acids to be made. The acids were eluted with formic acid from a column of Dowex 1 (Dow Co. Inc.) in the formate form, and it was noted that the molarity of formic acid required to elute a particular acid was characteristic of that compound.

The elution of organic acids from columns of ion exchange resins is a complex procedure. In general acids of similar constitution are eluted in order of increasing ionisation constant, the weakest acid emerging first[3]. However, aromatic acids are generally eluted after aliphatic acids and the presence of a long aliphatic chain can lead to elution of a weak acid after a stronger acid having a short carbon chain.

LAWSON AND PURDIE[4] examined the elution of several aliphatic carboxylic acids from the anion exchange resin Dowex 1, and found no difference in the order of elution when using Dowex 1-×2 or the more highly cross-linked Dowex 1-×10. In the present investigation 94 organic acids were eluted from Dowex 1-×10 with formic acid of gradually increasing concentration, under standard conditions; the results are shown in Table I, which includes the molarity of formic acid over which each acid was eluted, and also the recovery obtained. Table II shows the R_F values of those organic acids used in the present work which were not included in the previous communication[2]; with the exception of oxamic acid these acids showed R_F values consistent with their basicities.

The molarity of formic acid at which a particular acid began to be eluted was considered to be the significant value. The degree of tailing, measured by the range of molarity over which an acid was eluted, probably depended on the solubility of the organic acid in the formic acid and hence on the amount used, and also possibly on the slope of the elution gradient employed.

It is evident from the results that structure and acid strength play important roles in the elution pattern. There seems to be no clearly defined general relationship between the molarity of formic acid required to elute a particular acid and its pKa_1

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TABLE I

ORDER OF ELUTION OF ORGANIC ACIDS FROM DOWEX 1-X 10

Acid	pKa_1[5-9]	*Formic acid molarity range*	*Weight*	
			applied (mg)	*recovered (mg)*
Picolinic	6.39	0.0–0.8	116	109
Nicotinic	4.82	0.05–1.6	103	103
Glycollic	3.83	0.05–1.8	214	212
Isonicotinic	4.84	0.1–0.8	66	62
1,3,4,5-Tetrahydroxycyclohexanecarboxylic (quinic)	—	0.3–1.0	88	86
Succinic	4.13	0.5–2.1	123	113
Glutaric	4.34	0.5–2.2	102	96
Glyoxylic	3.3	0.8–1.7	109	82
DL-Malic	—	0.9–2.5	103	102
Ethylsuccinic	4.08	0.9–3.5	101	99
Methylsuccinic	—	1.1–2.2	67	84
Adipic	4.41	1.1–2.7	117	115
cis-Dimethylsuccinic	—	1.1–3.2	84	85
Itaconic	—	1.2–2.8	138	138
Pimelic	4.48	1.4–3.1	113	108
Propane-1,2,3-tricarboxylic	—	1.5–4.0	132	131
meso-Tartaric	3.11	1.6–2.8	110	113
2-Aminobenzoic	2.05	1.6–3.6	79	87
cis-Methylethylsuccinic	—	1.7–4.3	120	125
trans-Methylethylsuccinic	—	1.7–6.5	89	88
Diaminoethanetetraacetic	—	1.8–3.2	102	96
Butane-1,2,4-tricarboxylic	—	2.0–3.3	99	114
Diglycollic	—	2.1–3.6	95	96
Oxamic	—	2.3–4.6	103	84
Suberic	4.51	2.3–4.8	59	55
Pyridine-3,4-dicarboxylic	2.64	2.5–4.0	107	97
Citric	3.13	2.6–4.3	149	150
Sulphanilic	—	2.9–4.2	91	77
Malonic	2.75	2.9–4.8	121	123
Pyrrole-2-carboxylic	—	2.9–5.0	84	38
Pyridine-2,3-dicarboxylic (quinolinic)	2.44	3.1–4.7	85	85
DL-Tartaric	2.96	3.1–5.2	111	111
Pyridine-2,5-dicarboxylic	—	3.3–4.8	96	95
Mandelic	3.41	3.5–5.4	104	103
Pyridine-3,5-dicarboxylic	—	3.6–5.3	92	90
Fumaric	3.02	3.6–5.6	105	106
Azelaic	4.55	3.9–6.4	125	120
Butane-1,2,3,4-tetracarboxylic	—	4.2–5.9	85	83
Tetrahydroxysuccinic	—	4.2–5.9	88	15
Cholic	—	4.3–8.7	85	89
Phenylacetic	4.32	4.5–7.0	94	74
Ethylmalonic	2.96	4.8–5.4	87	79
Tartronic	—	4.8–6.3	80	79
2-Oxoglutaric	—	4.9–6.7	91	87
Nitrilotriacetic	—	4.9–6.7	85	80
Pyridine-2,6-dicarboxylic	—	5.2–6.8	106	106
Benzoic	4.17	5.2–7.6	126	64
Homophthalic	3.72	5.4–8.0	70	66
3-Phenylpropionic	4.66	5.5–8.4	85	57
3,4-Dihydroxybenzoic	—	5.5–8.8	124	125
Pyrazole-3,5-dicarboxylic	—	6.0–7.9	97	104
Methylmaleic	2.42	6.1–7.1	91	81

(*continued on p. 49*)

TABLE I (*continued*)

Acid	pKa_1[5–9]	*Formic acid molarity range*	*Weight*	
			applied (mg)	*recovered (mg)*
p-Toluic	4.37	6.3–10.2	77	52
o-Toluic	3.91	6.4– 9.0	70	23
Mesoxalic	—	6.5– 9.4	97	100
m-Toluic	4.27	6.9–10.9	117	75
4-Nitrobenzoic	3.44	7.3–11.1	105	104
Isophthalic	3.28	8.0–13.0	72	69
Pyrazine-2,3-dicarboxylic	2.21	8.1–11.0	77	74
Phthalic	3.00	8.4–11.2	101	98
4-Hydroxycinnamic	—	8.5–11.4	92	94
2,3,4-Trihydroxybenzoic	—	8.5–12.0	119	119
2,5-Dihydroxybenzoic	—	8.6–12.0	105	101
Cinnamic	4.43	9.1–12.5	86	87
2,4,6-Trimethylbenzoic	3.44	9.1–22.3	105	104
2,4-Dihydroxybenzoic	3.22	9.2–13.0	111	112
Diphenic	3.5	9.3–11.4	106	101
Benzene-1,2,3-tricarboxylic	2.82	9.3–11.9	102	98
Oxalic	1.27	9.4–14.1	108	106
2-Hydroxybenzoic	2.90	9.5–12.5	122	114
3,5-Dinitrobenzoic	2.82	9.6–12.0	80	75
Benzene-1,3,5-tricarboxylic	2.52	10.3–13.4	105	106
2-Hydroxycinnamic	—	11.2–13.9	114	110
4-Methylhydrocinnamic	—	11.2–14.3	30	23
4-Methylcinnamic	4.56	11.5–15.7	93	89
Benzene-1,2,4-tricarboxylic	3.12	11.7–13.7	83	82
Naphthalene-1-acetic	4.24	11.8–14.9	81	83
2-Hydroxy-3-methylbenzoic	—	12.0–15.2	82	78
4-Benzoylbenzoic	—	12.1–15.2	61	53
4-Hydroxyisophthalic	—	12.2–17.3	100	101
Phthalonic	—	13.0–15.7	81	77
2,6-Dicarboxyphenylglyoxylic	—	13.1–15.6	88	87
α-Naphthoic	3.7	13.9–16.2	68	65
β-Naphthoic	4.15	14.1–18.7	111	102
Benzene-1,2,3,5-tetracarboxylic	2.38	14.3–17.5	98	89
2-Hydroxyisophthalic	—	15.0–17.8	96	95
3-Hydroxy-2-naphthoic	—	17.0–20.7	96	94
2,4,6-Trimethylphenylglyoxylic	—	17.4–20.2	125	119
Benzene-1,2,3,4-tetracarboxylic	2.06	17.4–20.6	81	69
Benzene-1,2,4,5-tetracarboxylic	1.92	19.4–23.0	95	105
Phenanthrene-9-carboxylic	—	21.5–23.5	64	66
Picric	0.8	25.0	111	113
Benzenepentacarboxylic	1.80	0–2 *M* sodium formate	61	60
Mellitic	1.40	0–2 *M* sodium formate	121	101

value, where this is known; this latter value is, however, useful in predicting the order of elution of structurally similar acids.

The results enable accurate predictions to be made of the ease with which any mixture of acids included in Table I may be separated. Also some information may be obtained concerning the class of compound to which a particular acid belongs; for example, none of the simple benzenecarboxylic acids is eluted with formic acid of strength less than 5.2 *M*. The difference between the strengths of formic acid required

TABLE II

R_F VALUES OF ACIDS NOT PREVIOUSLY REPORTED

Acid	*Source*[a]	R_F *value* (× 100)[b]					
		Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*	*Solvent 5*	*Solvent 6*
Oxamic	BDH	66–75	74–84	51–61	41–51	35–45	59–67
Pyrrole-2-carboxylic	Light	92–97	94–98	92–98	52–62	55–65	66–75
Mandelic	HW	95–98	96–100	92–97	62–71	70–77	75–82
4-Nitrobenzoic	BDH	97–100	97–100	96–100	65–72	72–80	76–84
4-Methylcinnamic	LH	99–100	99–100	94–100	74–82	76–85	82–89
Pyrazole-3,5-dicarboxylic[c]	Light	60–70	64–77	33–52	22–37	11–23	41–54
Pyrazine-2,3-dicarboxylic	Light	72–82	79–88	33–38[d] 51–69	27–41	16–25	50–61
2-Hydroxyisophthalic	Light	89–95	93–97	75–90	41–49	33–43	58–66
4-Hydroxyisophthalic	Light	89–95	92–98	89–97	45–54	40–51	63–71
Butane-1,2,4-tricarboxylic[c]	Light	73–81	81–88	72–82	19–23[d] 25–36	7–19	51–61
Butane-1,2,3,4-tetracarboxylic	Light	53–65	68–79	58–69	6–19	0–6	29–43
Methylsuccinic	LH	88–95	92–98	83–92	40–50	31–44	60–73

[a] BDH: The British Drug Houses Ltd., Poole, Dorset, Great Britain; HW: Hopkin and Williams Ltd., Chadwell Heath, Essex, Great Britain; Light: L.Light and Co. Ltd., Colnbrook. Bucks., Great Britain; LH: courtesy of Dr. L. HORTON. All acids were of laboratory reagent quality.

[b] Solvent 1: Ethyl formate–98% formic acid–water (12:5:3 v/v) containing bromophenol blue (0.015% w/v) and sodium formate (0.05% w/v). Solvent 2: Ethyl formate–98% formic acid–water (2:1:1 v/v) containing bromophenol blue (0.015% w/v) and sodium formate (0.05% w/v). Solvent 3: Ethyl acetate–glacial acetic acid–water (2:1:1 v/v) containing bromophenol blue (0.015% w/v) and sodium acetate (0.05% w/v). Solvent 4: Ethanol–water–0.880 ammonia (35:13:2 v/v) containing thymol blue (0.03% w/v). Solvent 5: Ethanol–buffer (7:3 v/v) containing chlorophenol red (0.03% w/v). Solvent 6: Ethanol–buffer (1:1 v/v) containing chlorophenol red (0.03% w/v). Buffer: An aqueous solution of ammonia and ammonium carbonate, 1.5 *N* with respect to each.

[c] Acid applied as ammonium salt.

[d] –– between numbers indicates a spot of low intensity.

to elute different acids determines the shallowness of elution gradient required to achieve complete or partial separation; the lower limit of shallowness will depend upon the sensitivity of the paper chromatographic monitoring technique[3]. Experience with the complex mixtures of acids obtained by oxidative degradation of coal has shown that it is possible to achieve at least partial separations of acids eluted by similar strengths of formic acid; these include DL-malic from *meso*-tartaric, DL-tartaric from quinolinic, and succinic from adipic acid. These separations were achieved by means of a carefully selected elution gradient after tentative identification of the acids by paper chromatography.

METHOD

A glass column (50 cm × 0.7 cm diam.) was filled with Dowex 1-×10 anion exchange resin (100–200 mesh, 10 g) and converted to the formate form by eluting with 2 *M* sodium formate solution. The column was then washed with 25 *M* formic acid to remove any soluble impurities, and finally washed with water. Most of the acids were put on to the column individually as aqueous solutions; those sparingly soluble in water were put on as their sodium or ammonium salts. The purity and

sources of the acids not listed in Table II were as given previously[2]. All the acids were thoroughly dried *in vacuo* over phosphorus pentoxide before use. In order to speed up the investigation some acids which from previous experience were known to separate efficiently were put on to the resin column in pairs.

The acids were eluted by gradient elution with formic acid of strength increasing gradually from 0 to 25 *M*, using a method similar to that described by BOCK AND LING[10]. In each separation approximately 92–95 10 ml fractions were collected with the aid of an automatic fraction collector, and were monitored by paper chromatography, using the method and one of the acidic solvents described previously[1]. The range of molarity over which each acid was eluted was determined by titration to phenolphthalein with *N* or *N*/10 sodium hydroxide of samples (1.00 ml) of the fractions immediately preceding and following those in which the eluted acid was detected. The latter were combined and concentrated by distillation *in vacuo* from a water bath maintained at 45° ± 2°. The concentrates were then evaporated to dryness *in vacuo* in a desiccator over silica gel, and the residues were finally dried over phosphorus pentoxide. Neither benzenepentacarboxylic acid nor mellitic acid could be eluted from the resin with 25 *M* formic acid, but were removed by gradient elution with 0–2 *M* sodium formate solution, which provided a greater concentration of formate ions. Because of the high concentrations of cations in the eluate fractions it was not possible to monitor the elutions by paper chromatography or to determine readily the range of formate ion concentration over which each acid was eluted. To determine the recoveries each total eluate was passed down a column of cation exchange resin (Zeo-Karb 225; Permutit Ltd.) to remove sodium ions before being concentrated as described above.

The recovery of the acids by these techniques (see Table I) was very good in most cases. Low recovery (*e.g.* with benzoic acid) was attributed to the volatility of the acid in steam which resulted in some loss during the concentration stage. Some recoveries were apparently greater than the theoretical amount; the increase in weight was due to the tenacious retention by some of the acids of the formic acid eluant, which could not be removed completely by the standard drying conditions employed. Low recoveries were not thought to arise by incomplete elution of the acids from the anion exchange resin, nor high yields by incomplete separation; although the same column of resin was used repeatedly for successive experiments with different acids, paper chromatographic examination showed no evidence of contamination of any eluted acid with another.

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SUMMARY

The molarity of formic acid has been determined that is required to elute each of 94 organic acids under standard conditions from the anion exchange resin Dowex 1-×10. The R_F values in six solvent mixtures are presented of several organic acids not included in a previous communication.

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SEPARATION OF VITAMINS A_1 AND A_2 AND ALLIED COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY

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Within the last few years, thin-layer chromatography has made very rapid strides in the separation of fat-soluble vitamins and particularly of a few derivatives of vitamin A, kieselgel and alumina being employed as adsorbents[1–7]. A recent report[8] also indicates distinct separation of the isomers of vitamins A_1 and A_2 on kieselgel plates. The present communication describes a simple, rapid and reproducible method for the resolution of vitamin A_1 and A_2 compounds, including the epoxy-derivatives of vitamin A with established structures[9].

EXPERIMENTAL

The modified procedure for preparing thin-layer plates described below is essentially similar to that of STAHL[10,11]. Kieselgel (25.5 g, E. Merck) mixed with plaster of Paris (4.5 g, 300 mesh) in the ratio 85:15 (w/w) is well slurried with 60.0 ml of distilled water. The slurry is then applied on five glass plates (20 × 20 cm) with the help of a special applicator to give a fine thin layer of the adsorbent with a uniform thickness of 0.25 mm. The plates are dried at 120° for one h and stored in big desiccators until used.

Vitamins A_1 and A_2 and the allied compounds under examination are applied with the aid of a 0.1 ml micropipette, either individually or in mixtures, each spot containing about 5–30 μg of the substance. The substances are applied along a straight line on the plate, about 2.5 cm from the bottom, and the spots are spaced at a distance of 3 cm from one another. The chromatogram is developed by the ascending method for about 2 h in a special closed rectangular all-glass chamber, with 200 ml of any one of the following three solvent systems: (a) 6% (v/v) acetone in light petroleum (40–60°), (b) 15 % (v/v) diethyl ether in light petroleum and (c) 3 % (v/v) acetone in iso-octane.

When the solvent front reaches a distance of about 16–18 cm from the point of application of the compounds, the plate is removed from the chamber and allowed to dry at room temperature for a few seconds. Immediate examination of the plate, under ultraviolet light (wavelength 366 mμ) reveals the distinct separation of the various compounds which either fluoresce or absorb, depending on their characteristic property. The boundary of each concentric spot can be very easily marked with the help of a pin and further conclusive characterisation of the various spots is carried out by spraying the chromatogram with $SbCl_3$ reagent (25 % w/v) when the various compounds of the vitamin A_1 and A_2 group give characteristic blue, violet, pink and yellow spots. Alternatively, the various spots located under ultraviolet light can also be

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TABLE I

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF VITAMIN A_1 AND A_2 COMPOUNDS

Substance	*Band as observed in U.V. light**	*R_F values***			*$\lambda_{max.}$ in light petrol ($m\mu$)*	*$SbCl_3$ colour-test $\lambda_{max.}$ ($m\mu$)*
		6% (v/v) acetone in light petrol	*15% (v/v) diethyl ether in light petrol*	*3% acetone in iso-octane*		
Anhydrovitamin A_1	Bright yellow fluor.	0.93 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	350, 370, 390	620 (blue)
Vitamin A_1 palmitate	Blue fluor.	0.91 ± 0.01	0.76 ± 0.02	0.79 ± 0.01	325	620 (blue)
Vitamin A_1 acetate	Blue fluor.	0.70 ± 0.02	0.47 ± 0.01	0.49 ± 0.01	325	620 (blue)
Vitamin A_1 aldehyde	Dull black absorb.	0.53 ± 0.01	0.26 ± 0.01	0.21 ± 0.02	370	664 (blue)
Vitamin A_1 alcohol	Blue fluor.	0.20 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	325	620 (blue)
Vitamin A_1 acid	Dull black absorb.	0.00	0.00	0.00	350	573 (red)
Anhydrovitamin A_2	Bright yellow fluor.	0.87 ± 0.02	0.69 ± 0.02	0.61 ± 0.01	350, 370, 390	693 (blue)
Vitamin A_2 acetate	Green fluor.	0.69 ± 0.01	0.45 ± 0.01	0.47 ± 0.01	350	693 (blue)
Vitamin A_2 aldehyde	Dull black absorb.	0.50 ± 0.01	0.33 ± 0.01	0.20 ± 0.01	385	735 (greenish blue)
Vitamin A_2 alcohol	Green fluor.	0.17 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	350	693 (blue)
Vitamin A_2 acid	Dull black fluor.	0.00	0.00	0.00	370	—
5,6-Monoepoxy-vitamin A palmitate	Faint blue fluor.	0.84 ± 0.01	—	—	310, 325	460 (yellow)
5,6-Monoepoxy-vitamin A acetate	Faint blue fluor.	0.68 ± 0.01	—	—	310, 325	460 (yellow)
5,6-Monoepoxy-vitamin A aldehyde	Dull black absorb.	0.44 ± 0.02	—	—	352	440 (yellow)
5,6-Monoepoxy-vitamin A alcohol	Faint blue fluor.	0.07 ± 0.01	—	—	310, 325	465 (yellow)
5,8-Monoepoxy-vitamin A palmitate	Faint blue fluor.	0.81 ± 0.01	—	—	280	460 (yellow)
5,8-Monoepoxy-vitamin A acetate	Faint blue fluor.	0.65 ± 0.01	—	—	280	460 (yellow)
5,8-Monoepoxy-vitamin A aldehyde	Dull black absorb.	0.42 ± 0.01	—	—	317	440 (yellow)
5,8-Monoepoxy-vitamin A alcohol	Faint blue fluor.	0.07 ± 0.01	—	—	280	465 (yellow)
β-Carotene	Orange spot	1.00	1.00	1.00	430, 450, 470	555 (blue)

* Fluor. = fluorescence; absorb. = absorbance.
** Mean values of eight observations.

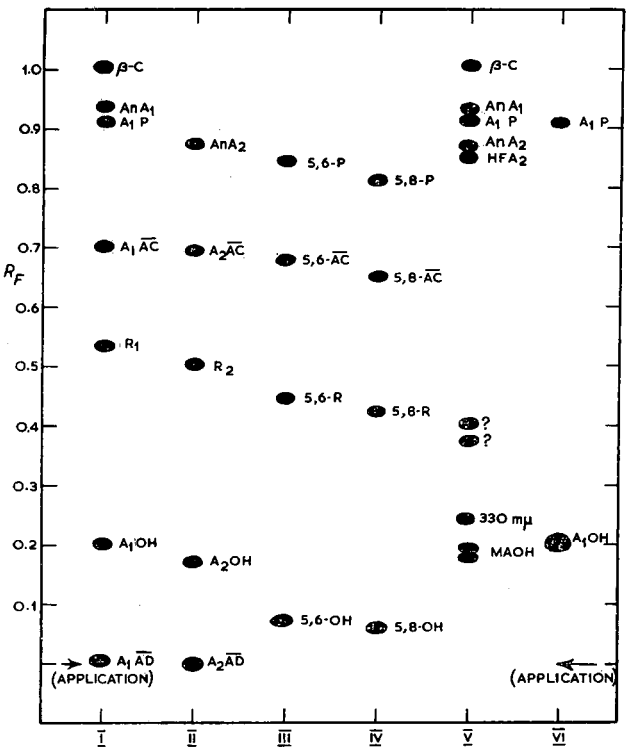

Fig. 1. Schematic representation of the separation of vitamins A_1 and A_2 and allied compounds by thin-layer chromatography, using kieselgel.

Mixture I: β-C = β-carotene; AnA_1 = anhydrovitamin A_1; A_1P = vitamin A_1 palmitate; $A_1\overline{AC}$ = vitamin A_1 acetate; R_1 = vitamin A_1 aldehyde; A_1OH = vitamin A_1 alcohol; $A_1\overline{AD}$ = vitamin A_1 acid.

Mixture II: AnA_2 = anhydrovitamin A_2; $A_2\overline{AC}$ = vitamin A_2 acetate; R_2 = vitamin A_2 aldehyde; A_2OH = vitamin A_2 alcohol; $A_2\overline{AD}$ = vitamin A_2 acid.

Mixture III: 5,6-P = 5,6-monoepoxy-vitamin A palmitate; 5,6-$\overline{AC}$ = 5,6-monoepoxy-vitamin A acetate; 5,6-R = 5,6-monoepoxy-vitamin A aldehyde; 5,6-OH = 5,6-monoepoxy-vitamin A alcohol.

Mixture IV: 5,8-P = 5,8-monoepoxy-vitamin A palmitate; 5,8-$\overline{AC}$ = 5,8-monoepoxy-vitamin A acetate; 5,8-R = 5,8-monoepoxy-vitamin A aldehyde; 5,8-OH = 5,8-monoepoxy-vitamin A alcohol.

V. Wallago Attu liver oil: β-C = β-carotene; AnA_1 = anhydrovitamin A_1; A_1P = vitamin A_1 palmitate; AnA_2 = anhydrovitamin A_2; HFA_2 = higher fatty acid esters of vitamin A_2; 330 mμ = the 330 mμ compound; MAOH = mixture of vitamins A_1 and A_2 alcohols.

VI. Rat-liver oil: A_1P = vitamin A_1 palmitate; A_1OH = vitamin A_1 alcohol.

scraped out and eluted with a diethyl ether–light petroleum mixture (1:1 v/v).

After removal of the solvent under reduced pressure, the absorption spectra of the various compounds in light petroleum were recorded in a Beckman DU spectrophotometer. In addition, to confirm the authenticity of the respective compounds, the $SbCl_3$ colour-test absorption maxima were also recorded in the spectrophotometer as described by CAMA, COLLINS AND MORTON[12].

RESULTS AND DISCUSSION

The R_F values of the various compounds analysed as well as some of their properties are given in Table I. Fig. 1 shows a typical schematic representation of the separation of compounds in a 6 % (v/v) acetone in light petroleum system.

This technique has been successfully applied to the separation of constituents of fish-liver oils and rat-liver extracts. Confirming the separation achieved by column chromatography[13] and reverse phase paper chromatography[14], freshwater fish-liver oil of *Wallago attu* gave nine distinct bands corresponding to β-carotene, anhydro-vitamins A_1 and A_2, vitamin A_1 palmitate, higher fatty acid esters of vitamin A_2, two unidentified substances, the uncharacterised compound with $\lambda_{max.}$ at 330 mμ and a mixture of vitamins A_1 and A_2 alcohols. The rat-liver unsaponifiable fraction gave a faint band of vitamin A_1 palmitate and a prominent band of vitamin A_1 alcohol. The presence of trace amounts of vitamin A palmitate in the rat-liver unsaponifiable fraction may well be due to incomplete saponification. Furthermore, the 5,6- and 5,8-monoepoxy-vitamin A group of compounds also gave distinct separations agreeing very well with respect to the palmitate, acetate, aldehyde and alcohol forms.

Thus, thin-layer chromatography as applied to the vitamin A group of compounds has the advantage of clear and complete resolution of individual components from a mixture in micro and macro quantities without any trailing whatsoever. Further, as it is much less time-consuming, it has the added advantage that the decomposition of the generally extremely unstable and labile vitamin A_1 and A_2 group of compounds is prevented. Attempts to apply the above method quantitatively were, however, unsuccessful.

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SUMMARY

A simple and rapid method for the separation of vitamins A_1 and A_2 and allied compounds by thin-layer chromatography using kieselgel has been described. The method, however, cannot be applied for quantitative estimation.

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FRACTIONATION AND DETERMINATION OF THE LIPID AND STEROID CONSTITUENTS OF THE ADRENAL GLANDS OF RATS BY MEANS OF THIN-LAYER CHROMATOGRAPHY

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The determination of very small quantities of steroids in the tissues is often difficult owing to the presence of lipids. AXELROD AND HERNANDEZ[1] and GOLDZIEHER, BAKER AND RIHA[2] have recently described the use of silicic acid columns for the extraction and purification, in the presence of lipids, of steroids obtained from tissues and blood.

Particularly for the determination of corticosteroids in the adrenal glands of small animals or human and in the incubation media of the adrenals a micro-technique is required.

Paper-chromatographic techniques have been used for the determination of corticoids in the plasma of rats[3] and in the incubates of the adrenal glands of rats[4].

DYRENFURTH[5] made use of the same technique for the determination of corticoids in the incubates of human adrenal glands. A silica gel column followed by paper chromatography has been used by DAVIGNON[6] for the study of steroids in adrenal tumours in man, and by DE ROOS[7] for the study of the *in vitro* production of chicken adrenocorticoids.

The aim of the present work was to investigate the lipids and steroids of the adrenal glands of normal rats and of rats suffering from a deficiency of pantothenic acid, and to study the basic constituents of these glands or of their incubates by subsequent tests under various conditions.

In the course of previous investigations of the *in vitro* synthesis of the adrenocorticoids of normal rats and of those deficient in pantothenic acid, either in the presence or in the absence of ACTH, one of us had identified corticosterone amongst the steroids in the incubate. However, owing to the difficulties encountered in the purification of the extracts, paper chromatography using BUSH's toluene–methanol–water system did not permit a reliable identification of other adrenocorticoids[10].

The recent developments in the use of thin-layer chromatography for the analysis of lipids by MALINS AND MANGOLD[11] and the use of this technique by CAVINA[8,9] in the analysis of steroids in adrenocortical extracts led us to try a micro-technique for the fractionation and purification of the corticoids present in quantities of adrenal tissue, as small as 20 adrenal glands of rats, or the incubates of these glands.

This method, which also permits the isolation of the lipid components of the extract, involves the following steps:

(1) Preparation of the extract of the organ using the method described by FOLCH, LEES AND SLOANE STANLEY[12].

(2) Thin-layer chromatography on silica gel G (Merck). The thickness of the layer varied between 0.250 and 1.0 mm, according to the quantity of lipids used (20–60 mg). The solvent systems was benzene–ether–acetic acid (70:30:1), and the dimensions of the plates were 20 × 20 cm. The mixture was resolved into the following zones; cholesterol esters, triglycerides, fatty acids, cholesterol, and phospholipids. The corticoids remained in the phospholipid fraction, which was the least mobile.

(3) Colorimetric determination of the fractions obtained by elution of the various zones with suitable solvents. For free cholesterol and cholesterol esters[13] zinc chloride and acetyl chloride were used. Triglycerides were determined as glycerol by oxidation and reaction with chromotropic acid[14], fatty acids by the titrimetric method described by DOLE[15], phospholipids as phosphorus[16], corticoids by U.V. spectrophotometry and colorimetrically with tetrazole blue[17].

(4) Thin-layer chromatography for the purification and analysis of the corticosteroids. Plates measuring 20 × 20 cm coated with a 0.250 mm layer of fluorescent silica gel G were used for the first purification. The solvents used was chloroform containing 0.2 % ethanol. The zone between the start and the deoxycorticosterone introduced as a standard was removed and eluted. The eluate was fractionated on 20 × 40 cm plates using chloroform–methanol–water (90:10:0.25) as the solvent. Corticosterone, aldosterone and small quantities of 11-dehydrocorticosterone and 11-deoxycorticosterone were thus separated and identified. The first two steroids could be determined directly in the eluates by U.V. spectrophotometry and by colorimetry using tetrazole blue.

EXPERIMENTAL*

Animals

Male Wistar rats with a mean initial weight of 65 g were fed on the following purified diet[10] (with or without pantothenic acid): 67 % sucrose, 18 % casein without water-soluble components, 9 % butter, 4 % Osborne-Mendel saline mixture, 2 % cod-liver oil. The following quantities of vitamins were incorporated per kg of the diet: 10 mg thiamine hydrochloride, 20 mg riboflavin, 10 mg pyridoxine, 100 mg nicotinic acid, 100 mg *p*-aminobenzoic acid, 100 mg ascorbic acid, 200 mg choline hydrochloride, 200 mg inositol, 50 mg menadione sodium bisulphite, 100 mg α-tocopherol acetate, 10 g linoleic acid, and 100 mg calcium pantothenate (only for the controls).

After about 60 days on this diet, when a state of deficiency had set in, as shown by the cessation of growth and by cutaneous symptoms, rats of the "deficient" group together with normal rats of the "control" group were decapitated after a 12 h fast. The adrenal glands were quickly removed, freed from the surrounding fat, and weighed on a torsion balance.

*Extraction of the adrenal lipids***

The adrenal glands (10 to 45) were ground with a little quartz sand (0.22 to 0.36 mm diameter), which had been washed and degreased with ether. A chloroform–methanol mixture (2:1) was used as the extraction solvent (15 ml/100 mg of fresh

* The experimental work was carried out in collaboration with A. MOLLICA.

** Extraction was by a slightly modified version of the method of FOLCH, LEES AND SLOANE STANLEY[12].

tissue), the whole of the solvent being divided into 4 equal portions. The adrenal tissue was triturated with each of these for a few minutes, and the samples were then allowed to stand for about 10 min. The solvent was carefully removed with a Pasteur pipette and transferrred to a cylinder through a G 3 filter. Twenty per cent water was added and the solution was agitated and allowed to stand for a few hours, after which the top layer of the two phases was removed. The bottom layer was washed thrice with chloroform–methanol–water (3:48:47) solvent for the top layer and about 1 ml of methanol was finally added to clarify the solution. The lower chloroform layer was transferred to a flask and evaporated in a rotary evaporator at about 40° and then evaporated to dryness in an atmosphere of nitrogen. The residue was redissolved in a few ml of chloroform–methanol (2:1) and transferred into small weighing tubes (tare 6–7 g). The solution was then evaporated again in a current of nitrogen and the residue was kept under vacuum in a desiccator until constant weight was reached, the desiccator having previously been flushed several times with nitrogen to remove all oxygen. The samples was finally redissolved in chloroform–methanol (2:1), placed in small test tubes fitted with ground stoppers, and stored in the refrigerator.

Chromatographic separation of the lipid constituents of the extract

The lipids were fractionated by thin-layer chromatography on silica gel G (Merck) which had previously been washed with chloroform. The thickness of the layer varied between 0.25 and 1.0 mm according to the quantity of lipids used (20–60 mg). Plates measuring 20 × 20 cm were used, with benzene–ether–acetic acid (70:30:1) as solvent. Zones corresponding to the following constituents were resolved, from front to start: cholesterol esters, triglycerides, fatty acids, free cholesterol (which migrates together with the diglycerides); the corticosteroids remained in the least mobile fraction, namely the phospholipids. The zone nearest to the start was covered, and the remainder of the plate was sprayed with dichlorofluorescein, Na salt, 0.05 % in ethanol and viewed under a U.V. lamp.

Each lipid fraction was scraped off, transferred to a Soxhlet thimble, and extracted for 3 h with peroxide-free ether in a Kumagawa type extractor (in which the substance to be extracted can be kept at the same temperature as the solvent). The resulting extracts were evaporated to dryness and redissolved in 10 ml of chloroform. The following colorimetric determinations were carried out on these solutions: free cholesterol and cholesterol esters were determined with zinc chloride and acetyl chloride by the method of HANEL AND DAM[13], triglycerides as glycerol by VAN HANDEL AND ZILVERSMIT's method [14] and fatty acids by DOLE's[15] titrimetric method. The zone containing the phospholipids and the corticosteroids was marked (Fig. 1) and sucked into a suitable percolator through which 10 ml of anhydrous acetone were passed. The corticosteroids passed into solution, while the phospholipids remained practically insoluble. The total crude corticosteroid content was determined on aliquot portions (about $^1/_{10}$) of this solution, using the colorimetric method with tetrazole blue[17,9]. The phospholipids were determined directly on a small portion (about $^1/_{100}$ to $^1/_{200}$) of the whole adrenal lipid extract before chromatography, using the method described by BERENBLUM AND CHAIN[16]. Alternatively, the lipid extract was divided into two portions, giving two "phospholipid + corticosteroid" zones, one of which was extracted with acetone as described above, and the other with warm chloroform–methanol (2:1) in the extractor. The extract containing the

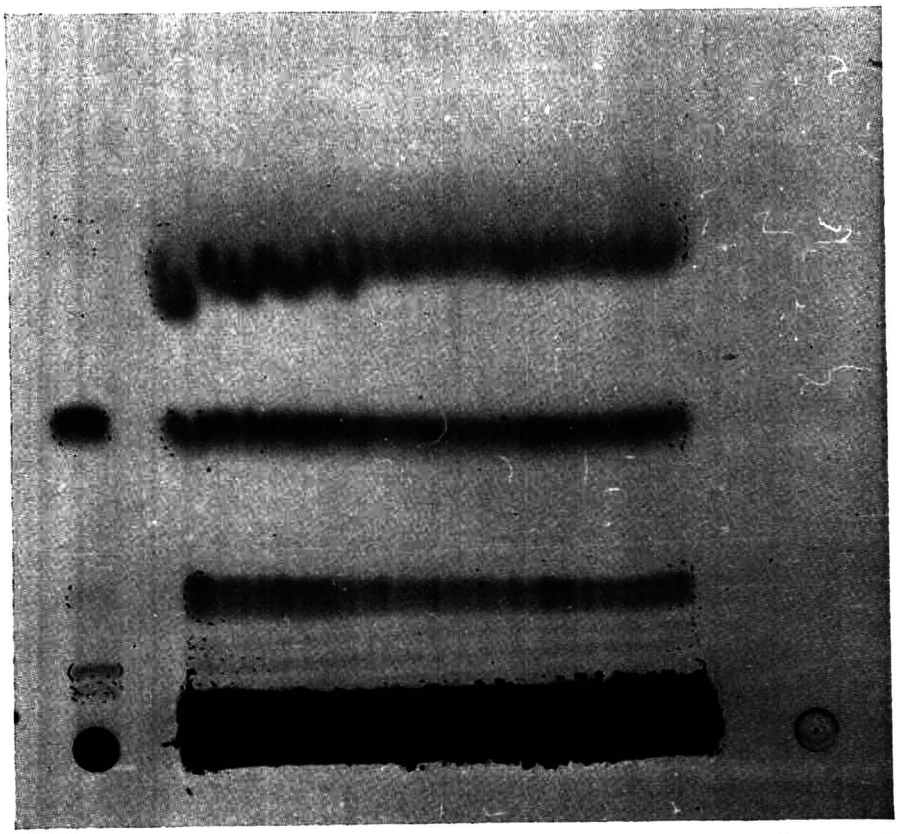

Fig. 1. Chromatographic separation of the lipid constituents of the adrenal extract. Solvent: benzene–ether–acetic acid (70:30:1). Detection with iodine vapour. Plate coated with a 0.5 mm layer of silica gel G. Left: reference lipid mixture. Right: cortisone and deoxycorticosterone used as reference standards. Middle (from the bottom): (1) removed band (phospholipids + corti costeroids); (2) two slight bands of diglycerides; (3) cholesterol; (4) triglycerides; (5) cholesterol esters.

phospholipids was used for the determination of the lipid phosphorus and for the preparation of the methyl esters of the fatty acids for gas chromatography.

Chromatographic purification and fractionation of the steroid constituents of the extracts

The acetone extract containing the corticosteroids (usually 50–200 μg) was evaporated to dryness, and the residue was deposited in a short strip (2 to 3 cm) on a 20 × 20 cm plate coated with a 0.25 mm layer of fluorescent silica gel GF_{254} (Merck), which had previously been washed with chloroform. Cortisone and deoxycorticosterone were used as reference standards. After development with chloroform containing 0.2 % of ethanol the layer between the start and the zone corresponding to deoxycorticosterone was removed by sucking it into a suitable percolator and extracting it with 5–10 ml of ethanol. The various bands situated beyond deoxycorticosterone gave a positive test with tetrazole blue and absorbed in the U.V. region, but they were not corticosteroids. Several alcohol extracts were combined to give 70–200 μg ag-

gregates. The sample was evaporated to dryness, and the residue was deposited in the form of a small strip 1–2 cm long, on a plate prepared as described above, but measuring 20 × 40 cm. The following standards were deposited on the plate alongside with the sample: aldosterone, corticosterone, 11-dehydrocorticosterone, deoxycorticosterone, and a bovine cortical extract. The plate was then developed with a chloroform–methanol–water system (90:10:0.25). Inspection under U.V. light (λ = 254 mμ) permitted the identification of corticosterone and aldosterone. The latter were removed from the plate by suction and eluted with 5 ml of ethanol. The remainder of the plate was sprayed with tetrazole blue (0.5 % in ethanol to which a 2 *N* solution of NaOH had been added in the ratio of 40:60), in order to identify and obtain a semi-quantitative determination of other steroids. If a larger quantity of steroids is available, two or more chromatograms can be developed side by side; one of these is then treated with tetrazole blue, the others are used for the analysis and estimation of purity by paper chromatography, using BUSH's B_1 and C systems, as described in detail in refs. 8 and 9.

Analysis of the steroid constituents of the extracts

The ethanolic eluates of the corticosterone and the aldosterone zones were analysed by U.V. spectrophotometry between 225 and 255 mμ, on the basis of the extinction at the maximum in the region of 240 mμ. A colorimetric determination using tetrazole blue, as described by NOWACZYNSKI *et al.*[17], but modified as indicated in one of our previous papers[9], was carried out on the same solution. This method was also used for the determination of the total content, in the corticosteroid sample, of substances which reduce tetrazole blue. Aldosterone and other steroids present in quantities of 1–5 μg were determined by the micro-technique using tetrazole blue described by DESGREZ *et al.*[18]; the measurements were carried out with the aid of a Beckman DU spectrophotometer using cells of 1 cm thickness. The results were expressed conventionally in terms of hydrocortisone.

Gas-chromatographic analysis of the fatty acids present in the cholesterol ester fraction

The eluates of the cholesterol ester zones were converted into methyl esters using the method of STOFFEL, CHU AND AHRENS[19], with the addition of hydroquinone to prevent the oxidation of the unsaturated fatty acids. The details of this technique were reported in one of our previous papers[20].

The methyl esters were separated on a column 0.125 in. in diameter and about 2 m long and filled with 60–80 mesh silanated Chromosorb W containing 20 % of polyethylene succinate. Nitrogen with a flow rate of 20 ml/min was used as the carrier gas. The initial column temperature of 150° was increased at a rate of 3.3°/min until it reached 200°. A flame-ionisation detector was used in a Perkin-Elmer 800 instrument.

RESULTS AND DISCUSSION

Analysis of the lipid components

Table I gives the analytical data for the individual lipid fractions from the adrenal glands of both normal and pantothenic acid deficient rats.

The composition of the mixture of phospholipids, triglycerides, cholesterol and cholesterol esters in the controls is seen to be very close to that in rat serum,

TABLE I

LIPID CONSTITUENTS OF THE ADRENAL GLANDS OF RATS

	Extract No.	*Number of adrenals*	*Adrenal tissue (mg)*	*Total lipids (mg/100 mg adrenal tissue)*	*Triglycerides*		*Cholesterol*	
					(mg/100 mg adrenal tissue)	*(mg/100 mg total lipids)*	*Free cholesterol*	
							(mg/100 mg adrenal tissue)	*% total cholesterol*
Control rats	25	8	176	12.8	1.9	14.9	0.3	5.4
	26	14	300	13.2	1.7	13.1	0.3	5.7
	29	10	189	16.1	2.0	12.7	0.3	5.0
	39	36	633	12.3	1.6	13.1	0.3	5.7
	41	14	229	13.1	1.7	12.7	0.2	3.3
	43	10	164	12.8	1.8	14.3	0.3	5.0
	45	10	148	13.7	2.0	14.8	0.4	6.2
Mean standard deviation				13.5 ± 1.2	1.8 ± 0.1	13.6 ± 1.0	0.3 ± 0.05	5.2 ± 0.9
Pantothenic acid deficient rats	19	17	257	12.0	2.2	18.4	0.3	7.9
	20	17	259	12.0	2.3	19.4	0.3	7.3
	21	18	298	12.4	2.4	19.2	0.3	7.3
	22	18	296	12.3	2.1	17.5	0.3	7.9
	24	12	211	11.9	1.8	15.0	0.2	7.1
	27	14	291	10.6	1.9	17.8	0.2	6.0
	28	25	370	14.0	1.7	15.2	0.3	6.9
	38	44	669	9.8	1.1	11.6	0.2	7.2
	40	22	346	10.5	1.3	12.3	0.3	8.0
	42	15	178	11.6	1.2	10.5	0.3	9.6
	44	10	155	12.6	2.4	18.8	0.3	7.1
Mean standard deviation				11.8 ± 1.1	1.8 ± 0.5	16.0 ± 3.2	0.3 ± 0.05	7.5 ± 0.9

* $P < 0.005$.
** $P < 0.001$.

taken under indentical conditions (after a 12 h fast) and analysed in the same way[20]. The quantity of free fatty acids is smaller than that in the serum (mean value: 1.5–1.8 as compared with 8.3–7.8 mg/100 mg of lipids).

Adrenal lipid extracts are characterised by consistently high cholesterol ester contents, as had previously been observed by TEPPERMAN *et al.*[21], FIDANZA AND BONOMOLO[22] and POULTON AND REESE[23], using different techniques. Rats which were deficient in pantothenic acid exhibit a lower cholesterol content (4.0 mg/100 mg of adrenal gland, corresponding to 34.5 mg/100 mg of total lipids) than the normal controls (5.7 mg/100 mg of adrenal gland, corresponding to 42.4 mg/100 mg of total lipids). This reduction occurred in the esterified portion, confirming the data reported by FIDANZA AND BONOMOLO[22] and OSBORN, WEAVER AND ANDERSON[24].

On the other hand, an increase in the phospholipid content is observed (4.5 mg/100 mg of adrenal gland, corresponding to 37.9 mg/100 mg of total lipids) as compared with the controls (3.9 mg/100 mg of adrenal gland corresponding to 29.3 mg/100 mg of total lipids).

Table II shows the fatty acid composition as found by gas-chromatographic

Esterified cholesterol		*Total cholesterol*		*Fatty acids*		*Phospholipids*		*Recovery*
(*mg*/100 *mg adrenal tissue*)	% *total cholesterol*	(*mg*/100 *mg adrenal tissue*	(*mg*/100 *mg total lipids*)	(*mg*/100 *mg adrenal tissue*)	(*mg*/100 *mg total lipids*)	(*mg*/100 *mg adrenal tissue*)	(*mg*/100 *mg total lipids*)	(*mg*/100 *mg total lipids*)
4.8	94.6	5.0	39.4	0.10	0.8	—	—	—
5.4	94.3	5.7	42.9	0.23	1.7	4.1	31.0	89.0
6.0	95.0	6.3	39.3	0.09	0.8	4.1	25.7	78.5
4.9	94.3	5.2	42.2	0.15	1.2	3.4	27.4	83.8
6.5	96.7	6.7	49.8	0.10	1.1	3.3	24.8	88.3
4.4	95.0	4.7	36.6	0.40	3.3	3.9	29.2	83.4
6.0	93.8	6.4	46.9	0.50	3.6	4.8	37.7	100.0
5.4**	94.8	5.7**	42.4**	0.22	1.8	3.9	29.3*	84.7
±0.8	±0.9	±0.8	±4.6	±0.16	±1.2	±0.5	±4.5	±8.4
3.8	92.1	4.1	34.8	0.09	0.7	—	—	—
4.2	92.7	4.5	37.6	0.11	0.9	0.9	—	—
3.9	92.7	4.2	33.7	0.10	0.8	—	—	—
3.8	92.1	4.2	33.8	0.09	0.8	—	—	—
3.4	92.9	3.7	31.3	0.09	0.8	—	—	—
3.8	94.0	4.0	39.5	0.13	1.2	—	—	—
3.9	93.1	4.2	37.4	0.13	1.1	3.8	33.8	87.9
2.9	92.8	3.1	31.4	0.20	2.0	3.9	40.0	85.3
3.7	92.0	4.0	38.6	0.20	1.8	4.1	39.6	92.2
3.1	90.4	3.4	29.5	0.30	2.9	5.5	45.8	88.7
3.9	92.9	4.2	32.5	0.40	3.3	5.0	39.2	93.8
3.7**	92.5	4.0**	34.5**	0.17	1.5	4.5	39.7*	89.6
±0.04	±0.9	±0.4	±3.3	±0.03	±0.9	±0.7	±4.2	±3.4

analysis of the cholesterol ester fraction. No appreciable variations exist between the deficient and the normal animals, apart from a moderate increase in poly-unsaturated fatty acids in the deficient animals, at the expense of the other fatty acids in the fraction, as also noted by FIDANZA, CONSTABLE AND WILSON[25,26], working on total lipids.

We believe that a more thorough investigation of these fractions in relation to the lipid composition of the diet would be useful. The fatty acid composition of the other lipid fractions of the adrenal glands will be reported in another paper and discussed in the light of the observations noted in the literature.

Analysis of the steroid components

The analytical data for the corticosteroids are listed in Table III. The quantities of substances which reduce tetrazole blue (expressed as hydrocortisone) are 26.7 μg/100 mg of adrenal gland from the controls, and 32.2 μg/100 mg from the deficient animals, corresponding to 0.25 and 0.2 % of the total lipids, respectively. Thus the difference is not significant.

TABLE II

GAS-CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACIDS PRESENT IN THE CHOLESTEROL ESTER FRACTION

Number of C atoms of the fatty acids	*Pantothenic acid deficient rats*	*Control rats*
12	trace amounts	trace amounts
14	5.7	8.8
16	22.4	24.3
16:1	8.6	10.0
17	0.7	0.7
17:1	0.9	0.9
18	5.8	5.6
18:1	29.1	31.2
18:2	2.0	1.9
18:3	11.5	8.2
20	3.8	2.3
20:4	7.9	4.9
21	trace amounts	0.6
22	1.6	0.6
Saturated fatty acids	40.0	42.9
Mono-unsaturated fatty acids	38.6	42.1
Poly-unsaturated fatty acids	21.4	15.0

The principal steroid component is always corticosterone. This observation clearly corroborates the earlier results[28,29].

Aldosterone has been detected beyond doubt in all the extracts analysed; the quantities present are sufficiently large to permit its determination by the use of larger quantities of extracts than were used in the present work. Fig. 2 shows that traces of 11-dehydrocorticosterone (compound A), and probably also deoxycorticosterone, can be detected in several extracts. Non-steroids, which give a positive test with tetrazole blue, are also observed; some of these remain at the start, whilst others migrate to the front. The presence of these substances explains the fact that the total corticoid contents (cautiously referred to as "substances which reduce tetrazole blue") obtained from the extract to be chromatographed are higher than the values obtained after chromatography. In fact, the combined amounts of corticosterone and aldosterone as obtained after chromatography are about $^1/_5$–$^1/_7$ of the value found for the substances which reduce tetrazole blue (in μg/100 mg of adrenal gland). Unidentified components migrating with the front may be eliminated, as has been mentioned before, by preliminary chromatography of the extract with chloroform containing 0.2 % of ethanol. This operation is not absolutely essential but it is advisable, since otherwise the "tails" of these impurities, if the latter are present in large quantities, could be contaminate the zones of the active steroids and thus lead to positive errors in the determinations.

Check on the recovery and on the chromatographic separation

The substances which react with tetrazole blue and which are situated near the front are accompanied by other, slightly more mobile, components which absorb in the U.V. region, but have not yet been identified; these substances do not interfere in any way with either the chromatographic separation or the recovery of corticosterone and aldosterone.

TABLE III

CORTICOSTEROIDS OF THE ADRENAL GLANDS OF RATS

	Extract No.	Number of adrenals	Adrenal tissue (mg)	Total lipids (mg)	Substances which reduce T.B.* (μg/100 mg adrenal tissue)	Pool No.	Amount analyzed (μg)	Corticosterone (μg/100 mg adrenal tissue)		Aldosterone (μg/100 mg adrenal tissue)	
								U.V.	T.B.*	U.V.	T.B.*
Pantothenic acid deficient rats	19	17	257	30.9	27.1						
	20	17	259	31.9	34.7						
	21	18	298	37.1	37.2	1	200	5.1	4.2	0.89	0.9
	22	18	296	36.6	37.8						
	24	12	211	25.1	31.2						
	27	14	291	31.0	27.5						
	42	15	178	20.7	35.7	2	85	3.6	4.4	—	1.2
	44	10	155	20.0	30.0						
	28	25	370	42.0	27.3		73	2.0	2.9	—	1.6
	38	44	669	66.2	33.8		68	6.6	4.6	2.98	2.7
	40	22	346	36.6	62.1**		129	3.2	4.1	—	1.0
Mean standard deviation					32.2 ± 3.6			4.1 ± 1.7	4.0 ± 0.7		
Control rats	12	36	597	87.2							
	13	14	213	39.0	38.0	3	100	2.8	2.9	0.54	—
	14	20	418	40.3							
	17	10	222	25.5	17.3	4	68	3.0	3.2	0.24	—
	18	10	224	24.0	20.7						
	25	8	176	22.5	22.0						
	39	36	633	78.2	18.6	5	83	4.7	4.8	—	1.8
	43	10	164	21.0	36.6						
	26	14	300	39.7	26.4						
	29	10	189	30.5	36.4	6	115	3.0	3.6	2.40	0.9
	45	10	148	20.3	24.2						
	41	14	229	30.9	65.5**		66	1.6	2.5	—	1.2
Mean standard deviation					26.7 ± 8.2			3.0 ± 1.1	3.4 ± 1.0		

* T.B. = tetrazole blue.

** The mean does not include these two values obtained from chloroform–methanol hot-extracted samples. Nevertheless their contents of corticosteroids is similar to the others after purification.

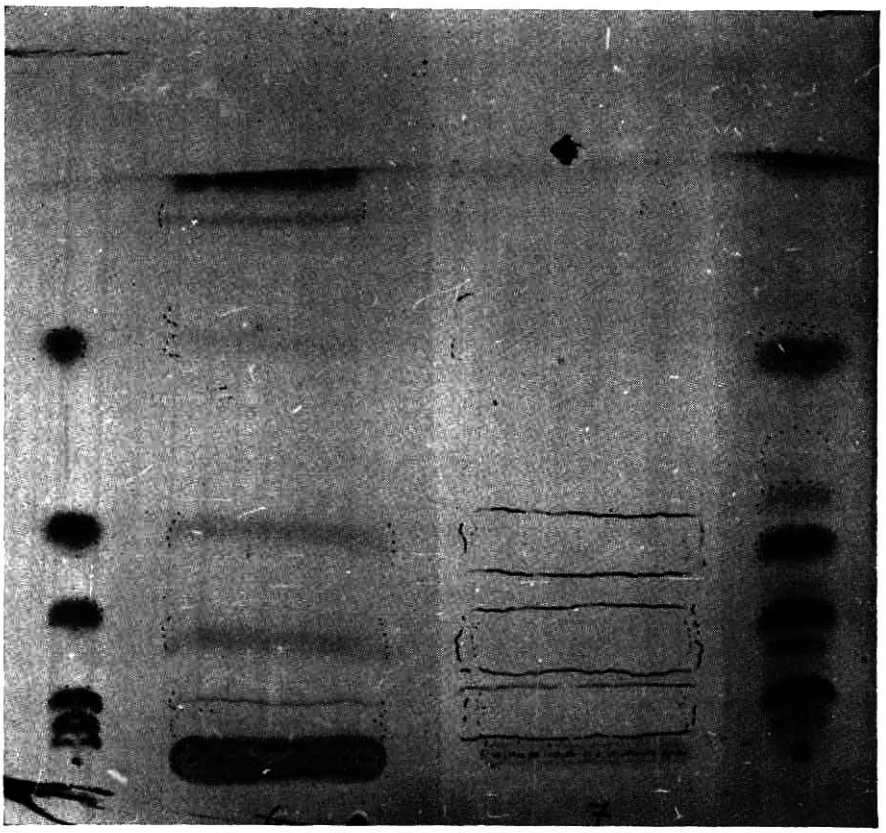

Fig. 2. Chromatographic fractionation of the steroid constituents of the adrenal extracts after their separation from lipids. Solvent: chloroform–methanol–water (90:10:0.25). Detection with tetrazole blue spray. Plate coated with a 0.25 mm layer of fluorescent silica gel G. From left to right: 1st track (from the bottom): THF, THE, cortisol, cortisone, corticosterone, 11-dehydrocorticosterone used as reference standards. 2nd track: crude corticosteroid extract sprayed with tetrazole blue. (It is possible to identify aldosterone, corticosterone, compound A and probably deoxycorticosterone (close to the front).) 3rd track: crude corticosteroid extract protected from the tetrazole blue spray; the bands are later removed. 4th track: bovine adrenocortical extract containing the principal steroids: THF, THE, cortisol, aldosterone, cortisone, corticosterone, compound S, and compound A.

The recovery was checked in the following manner: an extract containing 9.9 mg of lipids per 100 mg of adrenal tissues was divided into 2 equal portions. One portion was used for the determination of the original values, whilst to the other 50 μg of corticosterone were added. The whole analytical procedure was then carried out on both portions, with the following results:

Original extract	corticosterone 12.3 μg/100 mg of clean tissues aldosterone 2.75 μg/100 mg of clean tissues
Original extract + 50 μg corticosterone	corticosterone 64.2 μg/100 mg of clean tissues aldosterone 2.37 μg/100 mg of clean tissues.

The difference between the corticosterone contents of the two portions was 51.9 μg.

As has been stated before, it was found convenient to combine several extracts to obtain larger quantities of steroids. The identity of the corticosterone and aldosterone zones, and the non-steroid character of the other zones which gave a reaction with tetrazole blue could thus be checked by paper chromatography, using BUSH's B_1 and C systems. The analytical results for aggregates Nos. 1, 3 and 4 have clearly established the following points: Zone (a) (start), extending between about —0.5 and +0.5 cm, contains an unidentified non-specific material showing extensive tailing and a weak orange-yellow coloration. Zone (b), between about 0.5 and 3 cm corresponding to the THF and the THE in our thin-layer chromatography system, contains no substances which give a positive test with tetrazole blue with the exception of only a small spot showing blue fluorescence. Zone (c), situated between 4.8 and 6.8 cm, corresponding to aldosterone in our thin-layer chromatography system, contains only one compound, which was identified in BUSH's C system as aldosterone. Zone (d), between 7.8 and 11.3 cm, corresponding to corticosterone in our thin-layer chromatography system, contains only one substance, which was identified in BUSH's C and B_1 systems as corticosterone. Zones (e) and (f), situated between 14.4 and 16.9 cm and 19.2 and 21 cm, resp. contain no components which react with tetrazole blue with the exception of small fluorescent spots in the "tail". These zones correspond in our thin-layer chromatography system to 11-dehydrocorticosterone and 11-deoxycorticosterone, respectively, which are present in trace amounts in some of the extracts. The final zone (g), which lies between 23.5 and 26.2 cm, close to the front, contains one component, which also migrates with the solvent front in paper chromatography, and which gives a weak orange-yellow color with tetrazole blue.

Paper chromatography using BUSH's C system was also carried out directly on the crude extract containing the corticoids obtained after the first chromatographic separation of the lipids. Corticosterone was identified but not aldosterone, since the latter migrates with the impurities which mask the tetrazole blue test.

CONCLUSIONS

The above results show the value of this micro-technique in determining the basic qualitative and quantitative patterns of the lipid and steroid composition of the adrenals, using small quantities of tissue, such as 300 to 400 mg. These determinations are much more difficult to achieve by other methods. However, the data available at present, do not permit any definite conclusions regarding quantitative differences between the steroid production in normal and pantothenic acid deficient rats. They do show, on the other hand, that there are no qualitative differences and that aldosterone is always present in appreciable quantities in the adrenal secretion of rats, together with corticosterone, which should be regarded, as is well known, as the precursor of the former. Cortisol and related metabolic products, such as cortisone and the tetrahydro derivatives of both cortisol and cortisone, are clearly absent, whilst the presence of small quantities of 11-dehydrocorticosterone and 11-deoxycorticosterone is explained by the catabolic and anabolic transformations of the two principal steroids. This micro-technique may be useful in the investigation of the cortical secretions of small animals, as well as in all other research work where only

small quantities of adrenal tissue are available. The chromatographic resolution of the adrenal lipids confirms what was discussed and amply illustrated in one of our earlier investigations.

SUMMARY

A method is described for fractionation and determination of the lipid and steroid components in very small amounts of substrates.

Samples as small as 20 suprarenal glands of either normal or pantothenic acid deficient rats were extracted with chloroform–methanol (2:1). The total lipid extracts were separated by thin-layer chromatograms, the solvent system being benzene–ether–acetic acid (70:30:1). Spots corresponding to cholesterol esters, triglycerides, fatty acids, cholesterol, and phospholipids were obtained; corticosteroids lag behind as the least mobile fraction, together with the phospholipids. The areas were eluted with appropriate solvents and the main components were determined.

Corticosteroids were estimated by a colorimetric method with tetrazole blue and by U.V. spectrophotometry.

A second chromatogram was then performed on the corticosteroid eluate; the solvent system was chloroform–methanol–water (90:10:0.25). A clear-cut separation of the principal corticosteroids present in the suprarenal glands of rats was obtained.

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DÜNNSCHICHTCHROMATOGRAFIE ZUR ANALYSE VON SCHILDDRÜSENHORMONEN

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Nachdem die bereits seit etwa 25 Jahren bekannte[1] Dünnschichtchromatografie (DC) durch die methodische Bearbeitung von STAHL[2] in ständig zunehmendem Masse Eingang in die analytische Technik gefunden hat, sind die Vorteile dieses Verfahrens (kurze Laufzeit, scharfe Trennung etc.) auch der Biochemie zugute gekommen. Durch geeignete Massnahmen war es möglich, die DC als Radiochromatografie (RC) anzuwenden. Dadurch haben sich u.a. neue Möglichkeiten für die medizinische Diagnostik und Forschung ergeben. Hierbei kann, wie nachstehend gezeigt werden soll, eine einfache und billige Ausrüstung zur Erzielung brauchbarer Ergebnisse durchaus hinreichend sein. Ein interessantes Anwendungsgebiet der DC, die Identifizierung von Schilddrüsenhormonen, konnte durch eine systematische Untersuchungsreihe für klinische und wissenschaftliche Zwecke erschlossen werden.

METHODIK[3]

(1) *Dünnschichtchromatografie*

Bei den meisten Versuchen wurde in folgender Weise vorgegangen: 5 g Kieselgel G (Merck) wurden mit 25 ml Wasser im Mörser zu einer gleichmässigen Suspension verrieben und diese auf gründlich gereinigte Glasplatten (20 × 20 cm) durch Hin- und Herschwenken gleichmässig verteilt. Auf ebener Unterlage wurden die Platten über Nacht an der Luft bei Zimmertemperatur getrocknet. Nach entsprechender Markierung und Beschriftung erfolgte das Auftragen der Substanzlösungen mit Mikropipetten. Zur Entwicklung wurden die Platten in Chromatografietröge mit Filterpapierauskleidung gebracht, die bis etwa 0.8 cm Höhe mit dem Elutionsmittel gefüllt waren. Die in $^1/_2$–2 Std. aufsteigend entwickelten Platten wurden durch kurzes Erwärmen im Trockenschrank (bis 60°) getrocknet und anschliessend angefärbt. Zum Jodidnachweis diente eine $PdCl_2$-Lösung[4]. Die Aminosäuren konnten in verschiedener Weise angefärbt werden[5]. Meist kam Ninhydrinreagens in essigsaurer Butanollösung zur Anwendung[6]. Nach abermaligem Trocknen wurden die Platten zwecks Dokumentation mit Neatan bis zur guten Durchfeuchtung besprüht und nach Aufdrücken einer transparenten Klebefolie unter Wasser vorsichtig abgezogen.

(2) *Papierchromatografie*

Die (zur Kontrolle und zum Vergleich durchgeführte) papierchromatografische

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Trennung erfolgte absteigend unter Anwendung des Systems *n*-Butanol–Dioxan–2 *N* NH_4OH (4:1:5) in bekannter Weise[7]. Die Lokalisierung der Tyrosine und Thyronine erfolgte unter der U.V.-Lampe, die des Jodids wiederum durch Ansprühen mit $PdCl_2$.

(3) *Radiochromatografie*

Aus den mit Neatan behandelten Dünnschichtchromatogrammen und den eindimensionalen Papierchromatogrammen wurden die Laufstrecken als 4 cm breite Streifen ausgeschnitten. Die Messung der Aktivitätsverteilung erfolgte mit dem Radiochromatografen FH 417 mit Methandurchflusszähler, schrittweisem Vorschub, Impulsvorwahl und Registrierung mit einem Zeitdrucker. Zur quantitativen Auswertung dienten die Flächenintegrale der aufgenommenen Aktivitätskurven.

Bei den zweidimensionalen Dünnschichtchromatografie wurde zusammen mit der Probe ein Gemisch der Tyrosine (T_1, T_2), Thyronine (T_3, T_4) und Jodid aufgetragen. Nach der Trennung erfolgte die Anfärbung der Referenzsubstanzen. An den entsprechenden Stellen wurde die lose Kieselgelschicht abgekratzt und in Reagensgläser überführt. Die Radioaktivitätsmessung erfolgte im Bohrlochszintillationszähler.

(4) *Serumprobe*

Von einem Patienten, der wegen Schilddrüsenüberfunktion trägerfreies $Na^{131}J$ zur Therapie erhalten hatte[8], wurde 40 Stunden nach Applikation des radioaktiven Präparats eine Blutprobe entnommen. Die Aufarbeitung des abgetrennten Serums geschah in üblicher Weise[9].

ERGEBNISSE UND DISKUSSION

Über die Trennung von Aminosäuren durch DC ist bereits verschiedentlich berichtet worden[5,10–14], jedoch liegen unseres Wissens bisher keine Untersuchungsergebnisse über DC-Analyse von Tyrosinen und Thyroninen vor. Wegen der diagnostischen Bedeutung der Differenzierung von Schilddrüsenhormonen (bzw. ihrer Metaboliten) erschien es daher von Interesse, die Möglichkeiten für ihre Identifizierung durch DC systematisch zu prüfen. Die guten Trenneffekte bei kurzer Laufzeit und die relativ einfache Handhabung der Methode liessen erwarten, dass sie sowohl für klinische, wie auch für medizinisch-wissenschaftliche Zwecke von wesentlichem Nutzen sein könnte.

Die Ergebnisse unserer Untersuchungen sind in Tabelle I zusammengestellt. Es konnten demnach mehrere Systeme ermittelt werden, die eine gute Trennung der Substanzen ermöglichen. Da bekanntlich R_F-Werte nach manueller Absorbensauftragung schlecht reproduzierbar sind, wurden auch die R_S-Werte (S = Jodid) angegeben. Bei Anwendung der Durchlauftechnik oder bei mehrmaligem Entwickeln liessen sich die Trenneffekte noch verstärken. Dabei ergab sich, ebenso wie bei der zweidimensionalen DC, die Möglichkeit, beliebige Systeme miteinander zu kombinieren. Die Entwicklungszeit der eindimensionalen Dünnschichtchromatogramme betrug maximal 2 Stunden, im Vergleich zu *ca.* 24 Stunden bei Anwendung der Papierchromatografie (PC). Die Trennung von Trijodthyronin (T_3) und Thyroxin (T_4) bei Anwendung der PC und DC sind aus Fig. 1 und 2 ersichtlich. Ergänzend ist zu er-

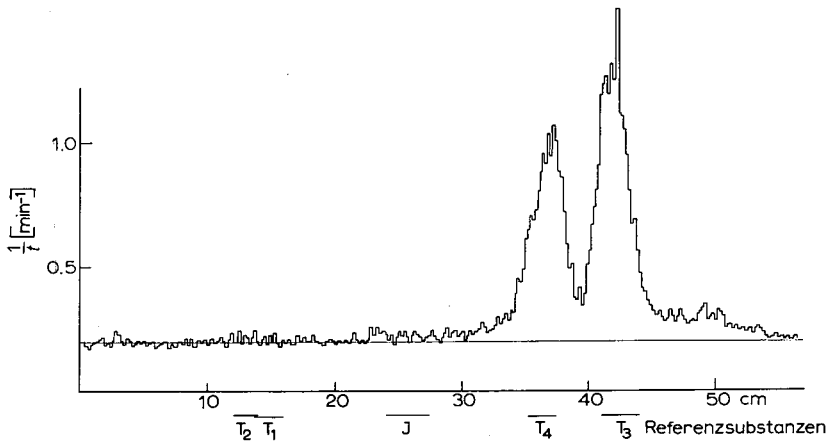

Fig. 1. Papierchromatogramm. System: *n*-Butanol–Dioxan–1 *N* NH_4OH (4:1:5).

wähnen, dass Magnesiumsilikat prinzipiell eine ebenso gute Trennung wie Kieselgel ermöglicht, dass damit jedoch die doppelte Laufzeit benötigt wird. Ausserdem lässt sich die Schicht nach Neatanbehandlung nur schwer quantitativ von der Glasplatte abziehen. Bei Anwendung von Kieselgelmengen unter 5 g stellten wir fest, dass dabei häufig "Tailing-Effekte" auftraten.

In der klinischen Diagnostik bzw. zur Bearbeitung endocrinologischer Probleme haben wir die DC bereits praktisch anwenden können. Im einzelnen soll darüber in gesonderten Mitteilungen berichtet werden[15,16]. Hier sei zur Erläuterung lediglich ein Beispiel angeführt. Als stoffwechselwirksame Inkrete der Schilddrüse finden sich im zirkulierenden Blut T_4 und T_3. Deren Produktions- und Umsatzrate sind verschieden, ebenso wie ihre metabolische Wirksamkeit. T_3 wird eine etwa viermal stärkere Hormonwirkung zugeschrieben als T_4. Demnach kann eine quantitative Verschiebung der Relation zugunsten von T_3 zu Änderungen des Körperstoffwechsels

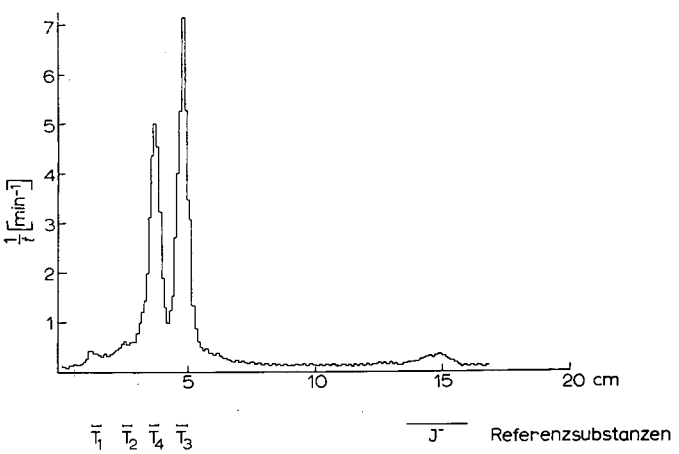

Fig. 2. Dünnschichtchromatogramm. System: Methyläthylketon–Äthanol–1 *N* NH_4OH (8:1:1), 2 × gelaufen.

TABELLE I

DÜNNSCHICHTCHROMATOGRAFIE VON TYROSINEN UND THYRONINEN

System	*Trennung*	R_F-*Werte*[a]					R_S-*Werte*[b]			
		T_1	T_2	T_3	T_4	J^-	T_1	T_2	T_3	T_4
n-Butanol–Dioxan–2 *N* NH_4OH (4:1:5)	+	0.37	0.35	0.49	0.44	0.67	0.55	0.52	0.73	0.66
n-Butanol–Dioxan–0.6 *N* NH_4OH (4:1:5)	—	0.23	0.25	0.35	0.32	0.40	0.57	0.62	0.87	0.80
n-Butanol–Dioxan–1 *N* NH_4OH (4:1:5)	—	0.30	0.29	0.41	0.38	0.41	0.73	0.71	1.00	0.93
n-Butanol–Dioxan–6 *N* NH_4OH (4:1:5)	—	0.41	0.43	0.46	0.45	0.46	0.89	0.93	1.00	0.98
n-Butanol–Dioxan–2 *N* NH_4OH (8:1:1)[c]	+	0.10	0.09	0.22	0.21	0.54	0.19	0.17	0.41	0.39
n-Butanol–Dioxan–2 *N* NH_4OH (8:1:1)[d]	+	0.06	0.06	0.25	0.20	0.49	0.15	0.15	0.64	0.51
n-Butanol–Dioxan–2 *N* NH_4OH (8:1:1)[e]	+	0.05	0.04	0.19	0.12	0.42	0.12	0.10	0.45	0.25
n-Butanol–Dioxan–2 *N* NH_4OH (8:1:1)[f]	++						0.15	0.14	0.48	0.30
n-Butanol–Dioxan–2 *N* NH_4OH (8:1:1)[g]	++						0.13	0.12	0.47	0.30
n-Butanol–Dioxan–2 *N* NH_4OH (8:1:1)[h]	++						0.13	0.10	0.35	0.19
n-Butanol–Dioxan–2 *N* NH_4OH (8:1:2)	+	0.13	0.12	0.23	0.19	0.28	0.44	0.43	0.82	0.68
n-Butanol–Dioxan–2 *N* NH_4OH (4:1:1)	++	0.18	0.14	0.33	0.27	0.42	0.43	0.33	0.79	0.64
n-Butanol–Dioxan–2 *N* NH_4OH (2:2:1)	+	0.22	0.18	0.37	0.31	0.41	0.53	0.44	0.90	0.76
n-Butanol–Methyläthylketon–1 *N* NH_4OH (4:1:1)	+	0.25	0.21	0.35	0.32	0.45	0.56	0.47	0.78	0.71
n-Butanol–Methyläthylketon–2 *N* NH_4OH (2:2:1)	++	0.14	0.17	0.23	0.27	0.49	0.29	0.35	0.47	0.55
n-Amylalkohol–Aceton–1 *N* NH_4OH (1:4:1)	+	0.28	0.18	0.64	0.60	0.72	0.39	0.25	0.89	0.83
n-Butanol–Aceton–1 *N* NH_4OH (1:4:1)	++	0.35	0.28	0.55	0.50	0.62	0.56	0.45	0.89	0.81
Benzylalkohol–Aceton–1 *N* NH_4OH (1:8:1)	++	0.09	0.07	0.26	0.17	0.81	0.11	0.09	0.32	0.21
Benzylalkohol–Aceton–2 *N* NH_4OH (1:8:1)	+	0.04	0.03	0.14	0.08	0.60	0.07	0.05	0.23	0.13
Äthylenglykol–Aceton–1 *N* NH_4OH (1:8:1)	+	0.33	0.32	0.55	0.45	0.83	0.40	0.39	0.66	0.54
Äthylenglykol–Aceton–1 *N* NH_4OH (1:4:1)	+	0.09	0.04	0.25	0.15	0.28	0.32	0.14	0.89	0.53
Äthylenglykol–Methyläthylketon–2 *N* NH_4OH (1:8:1)	+	0.03	0.02	0.10	0.08	0.17	0.18	0.12	0.59	0.47
Äthylenglykol–Methyläthylketon–2 *N* NH_4OH (1:8:1)[i]	+	0.01	0.01	0.13	0.06	0.21			0.62	0.29

Äthanol–Methyläthylketon–2 *N* NH_4OH (1:4:2)	+	0.46	0.43	0.56	0.49	0.67	0.69	0.64	0.84	0.74
Äthanol–Methyläthylketon–2 *N* NH_4OH (2:7:1)	+	0.09	0.07	0.25	0.17	0.64	0.14	0.11	0.40	0.27
Äthanol–Methyläthylketon–2 *N* NH_4OH (1:8:1)	++	0.07	0.03	0.24	0.09	0.40	0.17	0.08	0.36	0.23
Äthanol–Methyläthylkcton–2 *N* NH_4OH (1:4:1)	++	0.39	0.31	0.56	0.48	0.73	0.51	0.42	0.76	0.67
Äthanol–Methyläthylketon–2 *N* NH_4OH (1:4:1)	++	0.31	0.24	0.45	0.39	0.59	0.52	0.41	0.76	0.67
Isopropanol–Methyläthylketon–1 *N* NH_4OH (1:4:1)	+	0.08	0.05	0.17	0.12	0.56	0.14	0.09	0.30	0.21
Benzylalkohol–Methyläthylketon–1 *N* NH_4OH (1:4:1)	+	0.08	0.04	0.14	0.10	0.37	0.22	0.11	0.38	0.27
tert.-Amylalkohol–Dioxan–1 *N* NH_4OH (2:2:1)	+	0.23	0.14	0.36	0.27	0.39	0.59	0.36	0.92	0.69
tert.-Amylalkohol–Dioxan–1 *N* NH_4OH (2:2:1)[i]	+	0.33	0.18	0.46	0.36	0.57	0.58	0.32	0.81	0.63
Dichloräthan–Aceton–1 *N* NH_4OH (1:8:1)	+	0.05	0.02	0.14	0.09	0.24	0.21	0.08	0.58	0.38
n-Butanol–Dichlormethan–1 *N* NH_4OH (8:1:1)	+	0.16	0.14	0.33	0.24	0.45	0.36	0.31	0.73	0.53
n-Butanol–Dichloräthan–1 *N* NH_4OH (8:1:1)	+	0.13	0.09	0.26	0.20	0.43	0.30	0.21	0.60	0.46
n-Amylalkohol–Äthanol–2 *N* NH_4OH (8:1:1)	+	0.05	0.03	0.14	0.08	0.28	0.18	0.11	0.50	0.29
sec.-Butanol–Benzylalkohol–1 *N* NH_4OH (8:1:1)	++	0.04	0.03	0.19	0.12	0.38	0.11	0.08	0.50	0.32
n-Butanol–Äthanol–2 *N* NH_4OH (4:1:5)	+	0.26	0.25	0.37	0.34	0.49	0.53	0.51	0.75	0.69
n-Butanol–Äthanol–1 *N* NH_4OH (4:1:5)	+	0.26	0.19	0.37	0.31	0.50	0.52	0.38	0.75	0.62
n-Butanol–Äthanol–1 *N* NH_4OH (8:1:1)	++	0.08	0.07	0.21	0.15	0.45	0.18	0.16	0.47	0.30
n-Butanol–Äthanol–1 *N* NH_4OH (4:1:1)	+	0.19	0.17	0.31	0.26	0.47	0.40	0.36	0.66	0.55
Äthanol–Aceton–Acetatpuffer (pH 4.8) (1:4:1)	—	0.35	0.37	0.46	0.45	0.85	0.41	0.43	0.54	0.53
Phenol–Wasser (3:1)	+	0.43	0.55	0.61	0.65	0.28	1.53	1.96	2.18	2.32
n-Butanol–Aceton–Diäthylamin–Wasser (10:10:2:5)	—	0.47	0.49	0.60	0.68	0.52	0.50	0.94	1.15	1.30
Methyläthylketon–Pyridin–Wasser–Eisessig (70:15:15:2)	—	0.45	0.49	0.54	0.53	0.64	0.70	0.76	0.84	0.83
Aceton–*p*-Hydroxybenzoesäuremethylester–1 *N* NH_4OH (16:1:3)	++	0.15	0.12	0.33	0.23	0.65	0.23	0.18	0.48	0.35
n-Butanol–Methylcellosolve–1 *N* NH_4OH (8:1:1)	+	0.18	0.19	0.30	0.24	0.51	0.36	0.38	0.60	0.48
Methyläthylketon–Cellosolve–1 *N* NH_4OH (8:1:1)	++	0.09	0.06	0.24	0.15	0.44	0.21	0.14	0.55	0.34
Methyläthylketon–Methylcellosolve–Formamid (4:1:1)	+	0.18	0.23	0.26	0.30	0.77	0.23	0.30	0.34	0.39
Aceton–Methylcellosolve–Formamid (4:1:1)	+	0.17	0.14	0.32	0.28	0.46	0.37	0.30	0.70	0.61

[a] T_1 = Monojodtyrosin; T_2 = Dijodtyrosin; T_3 = 3,5,3′-Trijodthyronin; T_4 = Thyroxin.
[b] R_F-Wert in Bezug auf Jodid.
[c] 3 g Kieselgel.
[d] 10 g Kieselgel.
[e] 5 g Kieselgel.
[f] Zweimalige Laufzeit (erneutes Einsetzen).
[g] Doppelte Laufzeit (Durchlauftechnik).
[h] Dreimalige Laufzeit (3 Einsetzen). Magnesiumsilikat als Adsorbens.

im Sinne einer Schilddrüsenüberfunktion führen. Bei pauschaler Bestimmung des Hormonjods im Serum (PB^{127}I) und (BE^{127}I) mit üblichen Methoden[17] wird ein pathologisch verändertes Mengenverhältnis T_4:T_3 nicht erfasst, und es ist eventuell möglich, dass bei "im Normbereich" liegenden Werten unter Umständen trotzdem eine hyperthyreote Stoffwechsellage bestehen kann. In Fig. 1 und 2 sind die Chromatogramme eines unserer Hyperthyreosefälle dargestellt. Es zeigt sich eine gute Übereinstimmung im Hinblick auf die durch Papierchromatografie und Dünnschichtchromatografie nachgewiesenen Verhältnisse von T_3 zu T_4. Zum quantitativen Vergleich sind die ermittelten Werte bei ein- und zweidimensionaler Dünnschichtchromatografie, sowie bei Papierchromatografie in Tabelle II zusammengestellt.

TABELLE II

VERGLEICH DER AUS DREI METHODEN GEWONNENEN PROZENTUALEN AKTIVITÄTSWERTE

Substanz	*Zwei-dimensionale DC*	*Ein-dimensionale DC*	*Papier-chromato-grafie*	*Mittelwert*
3,5,3'-Trijodthyronin	50.0	50.7	50.7	50.47
Thyroxin	38.9	39.5	37.9	38.77
Jodid	2.9	3.8	3.6	3.43
Rest	7.0	5.9	7.6	6.83

Die klinische Problematik des erwähnten Falles kann hier ebensowenig besprochen werden, wie etwa Probleme der thyreoidalen Jodfehlverwertung[18] etc. Nur soviel sei gesagt, dass bei der Untersuchung der ^{131}J-markierten Hormone und ihrer Metaboliten die Jodkinetik von entscheidendem Interesse ist. Serienuntersuchungen von Blutproben können daher zur Bearbeitung spezieller Fragestellungen erforderlich sein. Die schnell und einfach durchführbare Technik der Dünnschichtchromatografie bietet hierfür günstige Voraussetzungen.

ZUSAMMENFASSUNG

Anhand der Ergebnisse systematischer Untersuchungen wird die Anwendungsmöglichkeit der Dünnschichtchromatografie zur Analyse von Jodhormonverbindungen aufgezeigt. Zur Erläuterung ist ein Fall der klinischen Praxis angeführt. Als entscheidende Vorteile der Dünnschichtchromatografie-Technik wird auf deren relativ einfache Handhabung, die kurze Laufzeit und die ausgezeichneten Trenneffekte hingewiesen.

SUMMARY

The results of systematic investigations have shown that thin-layer chromatography can be applied to the analysis of hormonal iodo-compounds. As an illustration a clinical case is discussed. It is emphasized that the primary advantages of thin-layer chromatography are its relative simplicity, rapidity and the excellent separations obtainable.

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DETECTION OF TRITIUM ON PAPER AND THIN-LAYER CHROMATOGRAMS

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INTRODUCTION

Tritium can be incorporated into a very large number of organic compounds using simple exchange techniques which have recently been developed[1]. Paper chromatography and thin-layer chromatography are powerful techniques for the separation of small quantities of very complex mixtures of compounds. It is therefore important to have methods for the detection of tritium on paper and thin-layer chromatograms. Many common isotopes important in biochemistry, for example ^{14}C, ^{35}S and ^{32}P, can be easily detected on thin-layer and paper chromatograms by laying a film on top of the chromatogram[2]. The β-particles pass into the emulsion of the film and on development their tracks leave darkened areas on the film which indicate the position of the radioactive compounds on the chromatogram. Tritium, however, emits only a very weak β-particle (maximum energy 0.018 MeV, mean energy 0.006 MeV) even the most energetic of which will only penetrate 2 μ in the photographic emulsion[3]. This presents a problem in that all commercial sheet film has a protective layer of gelatin covering the photographic emulsion. In the case of Kodak Royal Blue, for example, this overcoat is $0.8 \pm 25\ \%$ μ thick[4]. For this reason special techniques must be adopted for the detection of tritium on thin-layer and paper chromatograms.

DETECTION OF TRITIUM ON CHROMATOGRAMS

There are three general techniques which have been used for the detection of tritium on paper and thin-layer chromatograms.

1. Radioautography

The chromatogram is impregnated with liquid nuclear emulsion, allowing time for exposure and the whole is then developed in photographic developer. For the purpose of this article this procedure will be called radioautography. It has the advantage of being in principle very sensitive for water-insoluble materials which will not diffuse in the emulsion, but has the disadvantage of destroying the compounds involved so that they cannot be later used for co-chromatography. It has the further disadvantage that some compounds, for example reducing materials, will interact chemically with the emulsion and give "spots" even if they do not contain radioactivity. This

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method has been applied to paper chromatograms by ROGERS[5] and MARKMAN[6] and there seems to be no reason why it should not be applied to thin-layer chromatograms. SHEPPARD AND TSIEN[7] made their own photographic plates from liquid emulsion (*i.e.*, with no overcoat) and used these for radioautography on thin-layer chromatograms.

2. Sublimation autography

This technique involves the sublimation of some of the radioactive material from the chromatogram into the emulsion of the film where it produces an image. It can be used to detect not only volatile radioactive materials but also volatile materials which, because of their chemical properties, will develop the film. This technique has not been suggested as such by any workers in the field, but is clearly the mechanism involved in many of the suggested detection procedures. For example, MOSES AND CALVIN[8] claimed that tritiated water was incorporated predominantly into glycolic acid in a photosynthesising system. Our work has shown this not to be the case and the heavy spot due to glycolic acid was due to its sublimation into the emulsion where it was being detected with much greater efficiency than other much more heavily labelled compounds. Again, PARUPS, HOFFMAN AND JACKSON[9] suggested a technique for impregnating a paper chromatogram with anthracene crystals and exposing it to a light sensitive film. This is a valuable suggestion, as is discussed below, but PARUPS *et al*[9]. tested the procedure using nicotine, which is quite volatile and, from their published results, clearly distilled into the film.

More recently, RICHARDSON *et al.*[10] have detected tritium-labelled steroids by laying a sheet of X-ray film on top of thin-layer chromatograms. They claim that they obtain spots, but that with the interposition of a thin cellophane sheet (3.35 mg/cm^2) only a faint spot was obtained. It is clear that the phenomena they were using was sublimation autography. They observed that estradiol gave a spot even although it contained no radioactivity. This is probably because it sublimed into the film and interacts chemically with the emulsion to produce an image. This phenomenon is well-known in conventional ^{14}C radioautography in paper chromatography. Here the "ghost" spots can be recognized by their "graininess."

Scintillation autography could be used to detect sublimable reducing material on paper and thin-layer chromatograms. The technique of sublimation autography could be a valuable additional tool used in conjunction with scintillation autography (described below) for the detection and identification of labelled compounds, for example, use with and without transparent screens.

3. Scintillation autography

In 1958 one of the present authors[11,12] suggested the technique which involves soaking a paper chromatogram in a liquid scintillator so that the energy of the disintegrating tritium atoms is converted into light which is detected by a fast photographic film. Thus the weak β-particle of tritium is converted into light quanta which can travel into the emulsion of the film to produce an image. It is proposed to call this technique "scintillation autography". This is a photographic technique and is, in principle, quite distinct from any radioautographic procedure discussed above; it is, however, only about one-tenth as sensitive as impregnating a paper chromatogram with nuclear emulsion, but has the advantage that the radioactive compounds are not

destroyed and can be used for co-chromatography. It has the disadvantage that it is of no use with compounds that are soluble in toluene. However, many can be rendered insoluble by turning them into their salts. In practice, it can be used directly with most biological materials, the principal exceptions being lipids and steroids. PARUPS *et al.*[9] suggested the modification of impregnating the paper chromatogram with anthracene crystals; this can be done by dipping or spraying, using anthracene or some other scintillator dissolved in a suitable solvent such as benzene. For the reasons discussed above, the figures as to sensitivity given by PARUPS *et al.*[9], are not valid, but the procedure has been tested by us and shown to be one-tenth as sensitive as the liquid scintillator technique, but having the advantage of being usable with toluene soluble materials.

APPLICATION OF SCINTILLATION AUTOGRAPHY TO THIN-LAYER CHROMATOGRAMS

In our laboratories we use the technique of scintillation autography for the detection of tritium-labelled compounds on thin-layer chromatograms. We have found that commercial silica gel is itself a scintillator and that it gives off light when bombarded with ionising radiation including the weak β-particles from tritium. Thus to detect tritium on thin-layer chromatograms where the stationary phase contains silica gel (*e.g.*, Merck silica gel G) all that is necessary is to lay a sheet of fast photographic film on top of the thin-layer chromatogram. In our work we use Kodak "Royal Blue", which has superceded Kodak "Blue Band" as one of the fastest films presently commercially available. For a thin-film chromatogram made from Merck silica gel G of thickness 0.2 mm, a spot of area 3 mm^2 having an activity of 0.01 μC of tritium can be detected in seven days. The sensitivity can be doubled by spraying the thin-layer chromatogram with a saturated solution of anthracene (in benzene) until crystals of anthracene can be seen to be distributed evenly over the surface of the chromatogram.

In order to line up the image and the thin-layer chromatogram, the following procedure is used. Some tritium compound is placed on a piece of filter paper. The filter paper is then sprayed with anthracene or terphenyl and cut into segments with the radioactive material at the apex of each segment. A small amount of the stationary phase is scraped from two corners of the thin-layer chromatogram, exposing the glass plate, and two marker segments are taped onto the glass plate. The image of the apex of the marker segments can then be lined up with the apex of the marker segments after the film has been developed. If sublimation is suspected, then a thin sheet of a suitable transparent plastic is interposed between the film and the thin-layer chromatogram. This will only slightly reduce the light transmitted, but will reduce the rate of sublimation considerably. Scintillation autography and sublimation autography taken together can yield additional information of the physical and chemical properties of the compounds being investigated. Rates of sublimation depend on the heat of vaporization rather than on boiling point and some materials, for example glycolic acid, sublime very rapidly.

It should be emphasized that scintillation autography, unlike autoradiography and sublimation autography, is a photographic process and is therefore subject to reciprocity failure[12]. For a radioautographic process the darkening of the film is due to β-particle tracks and this darkening is approximately proportional to exposure time.

This is not the case for a photographic process. From these considerations it should be clear that it is not valid to select an exposure period, say 24 h, as has been done by CHAMBERLAIN *et al.*[13], and compare the various techniques. The shorter the period taken, the better will a scintillation autographic technique appear with respect to any technique based on radioautography or sublimation autography.

ULTIMATE SENSITIVITY OF SUBLIMATION OR SCINTILLATION AUTOGRAPHY

Clearly, if one wishes ultimate sensitivity, techniques should be used to keep the spots as compact as possible. In this respect thin-film chromatograms have an advantage over paper chromatograms in that because they run quickly there is less diffusion and the spots are more compact. In principle, the sensitivity could be improved by placing a reflector behind the chromatogram. The authors have used sheets of tinfoil as reflectors behind paper chromatograms during scintillation autography and it does improve the sensitivity slightly.

In practice, if the spots produced on the film are not sufficiently dense or if the exposure time is inconveniently long, the simplest procedure is to use more activity on the chromatogram but, if this is not possible or leads to overloading, then resort must be made to other methods. We have found that improvements in film processing offer the easiest solution; for example, having selected the fastest film available, greater contrast and hence greater sensitivity can be obtained by developing the film for longer times at lower temperatures than recommended by the manufacturers. Very long development times, however, will bring up the background. Since there is emulsion on both sides of fast commercial sheet film and since sublimation or scintillation autography will only expose the emulsion on one side of the film, half this background can be avoided if the film to be developed is fixed to a supporting sheet by means of waterproof adhesive tape, with the unwanted emulsion layer in contact with the sheet. The supporting sheet may be a discarded piece of film and should be the same size as, or larger than, the film to be processed. Alternatively, when several sheets of film are to be processed, they may be taped together in pairs with their unwanted sides together. The development of the film is carried out as usual, washed, and placed in the fixer. During fixing the tape is stripped off the film, separated from its supporting sheet and the fixing continued until both sides of the film have cleared. Image intensifying procedures are also available if even greater sensitivity is desired[14].

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SUMMARY

The techniques which have been used to detect tritium on paper and thin-layer chromatograms are reviewed and their theory of operation and experimental limitations discussed. A simple technique is described whereby small amounts of tritium-labelled compounds may be detected on thin-film chromatograms.

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PAPER CHROMATOGRAPHIC SEPARATION OF NUCLEOTIDES, NUCLEOSIDES, PURINES, AND PYRIMIDINES*

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During the course of studies of nucleotide catabolism in kidney slices and in ischemic organs[1] numerous nucleotides and their dephosphorylated degradation products (nucleosides, purines, pyrimidines) had to be separated completely by means of paper chromatography. Among the different separation procedures described so far (see refs. 10–12), none was found to be quite so satisfactory for this particular purpose. Some of the methods known involved too many chromatographic steps or gave inadequate separations of the compounds under study, other ones caused, owing to the solvents used, serious disturbances during the final spectrophotometric quantitation. A new method of paper chromatography was therefore developed for the separation, identification and quantitation of mononucleotides, nucleosides, purines, and pyrimidines; the solvents, originally developed for chromatography of phosphorylated compounds[2], could be used with some modifications in the mode of application.

METHODS AND RESULTS

Chromatography was carried out in glass jars; Whatman No. 1 papers (20 × 50 cm) were washed by immersion in 1 *N* HCl for 60 min and then in distilled water until neutral. This method of washing gave reproducible and low blanks for U.V. absorption.

Reference compounds were obtained from commercial sources. Compounds were dissolved in water or dilute acid except for uric acid which was dissolved in 0.03 *M* $NaHCO_3$. In this $NaHCO_3$ solution, uric acid was stable for at least one week. Concentrations used were approximately 2 mg/ml and test spots were 1–10 μg.

Preparation of tissues and media

Tissues were ground at the temperature of liquid nitrogen and 0.3–0.5 g were extracted in 1–2 ml of 0.3–0.6 *N* perchloric acid at 0° by high-speed stirring for 3 min. Media from *in vitro* experiments were chilled to 0° and, after the removal of tissues, were centrifuged. The media supernatants were made up to 0.6 *N* acidity by the addition of 10 *N* perchloric acid, and then centrifuged. The acid-soluble fractions, after centrifugation, were neutralized by the addition of 6 *N* KOH and, after half an hour, potassium perchlorate was removed by centrifugation.

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Solvents for chromatography

I. Isopropyl ether–*n*-butanol–98% formic acid (30:30:20 by vol.). Run in downward direction at 20–24° for 5 h.

II. *n*-Butanol–*n*-propanol–ethanol–25% ammonium hydroxide–water (40:40:10:45:15 by vol.). Run in downward direction at 26° for 12 h in solvent-saturated atmosphere.

III. 80% (v/v) formic acid–*n*-butanol–*n*-propanol–acetone–30% (w/v) trichloroacetic acid (25:40:20:25:15 by vol.). Run in ascending direction at 20–24° for 18 h in solvent-saturated atmosphere.

IV. Methanol–isopropanol–25% ammonium hydroxide–water (45:30:15:10 by vol.). Run in downward direction at 20–24° for 12 h in partially water-saturated atmosphere.

V. Water–*n*-propanol–25% ammonium hydroxide (90:10:1 by vol.). Run in upward direction at 20–24° for 3–4 h.

R_F values for test compounds are summarized in Table I.

TABLE I

R_F VALUES OF NUCLEOTIDES, PURINES, PYRIMIDINES, AND RELATED COMPOUNDS*

Compounds	*Solvents*				
	I	*II*	*III*	*IV*	*V*
Adenine	0.34	0.46	0.74	0.55	0.48
Adenosine	0.17	0.54	0.59	0.54	0.55
AMP	0.17	0.16	0.38	0.17	0.81
Deoxyadenosine	0.31	0.63	0.66	0.64	0.58
Deoxy-AMP	0.26	0.18	0.50	0.22	
Hypoxanthine	0.23	0.43	0.52	0.62	
Inosine	0.12	0.42	0.36	0.66	0.79
IMP	0.14	0.10	0.24	0.10	0.89
Deoxyinosine	0.20	0.49	0.47	0.69	0.82
Guanine	0.12	0.29	0.45	0.43	
Guanosine	0.10	0.30	0.41	0.53	0.76
GMP	0.12	0.08	0.26	0.10	
Deoxyguanosine	0.18	0.41	0.48	0.55	0.79
Deoxy-GMP	0.17	0.09	0.36	0.13	
Xanthine	0.19	0.33	0.38	0.51	0.64
Xanthosine	0.35	0.31	0.33	0.62	0.75
Uracil	0.38	0.48	0.60	0.70	0.80
Uridine	0.18	0.40	0.46	0.72	0.86
UMP	0.22	0.15	0.36	0.26	0.92
Deoxyuridine	0.29	0.45	0.51	0.73	0.88
Cytosine	0.31	0.51	0.73	0.64	
Cytidine	0.14	0.46	0.58	0.65	0.75
CMP	0.15	0.13	0.36	0.22	0.88
Deoxycytidine	0.22	0.56	0.66	0.73	0.77
Deoxy-CMP	0.22	0.15	0.48	0.28	
Thymine	0.52	0.56	0.71	0.72	
Thymidine	0.42	0.54	0.67	0.77	0.85
TMP	0.36	0.17	0.50	0.32	
Orotic acid	0.22	0.31	0.54	0.49	0.88
Uric acid	0.19	dec.	0.19	dec.	
Allantoin	0.27	0.32	0.28	0.62	

* All values determined on Whatman No. 1 paper using solvents and running times as given in the text. Partial decomposition in a solvent indicated by dec.

Chromatography of extracts

Up to 1 ml of extract was usually applied to the paper as a band (5–10 cm) using an airstream to speed drying. Initial chromatography was done in solvent III resulting in separation into several mixed fractions (Table II). These mixed fractions were then eluted, rechromatographed as shown in Table II, and quantitated.

Nucleoside di- and tri-phosphates were determined in a second sample of extract using methods described elsewhere[2].

TABLE II

PAPER CHROMATOGRAPHIC SEPARATION OF NUCLEOTIDES, NUCLEOSIDES, PURINES, AND PYRIMIDINES FROM TISSUE EXTRACTS

Initial separation solvent III		*Further separation*
R_F values	*Fractions*	*Solvent and running time*
0.68–0.77	Adenine*	V (3 h)
0.57–0.67	Adenosine, adenine*	II (12 h)
0.47–0.55	Hypoxanthine, uracil, adenosine*	II (12 h)
0.38–0.45	Uridine, xanthine	II (12 h)
0.27–0.37	Inosine, AMP, xanthine	II (12 h)
0.22–0.26	CMP, UMP, uric acid**	II (2 × 24 h)
0.17–0.21	IMP, GMP, uric acid**	II (2 × 24 h)
0.00–0.11	Tri- and diphosphonucleotides of adenine, guanine, uridine and cytidine with NAD, NADP, nucleotide coenzymes, etc.	

* Since adenosine and adenine each appear in 2 fractions when extracts are chromatographed, their final quantitation is done after a third chromatographic separation of the appropriately combined eluates. For this purpose, solvent V is used for 3 h.

** Uric acid is unstable in alkaline solvents and rechromatography of a second sample is done with solvent I (2 × 8 h) instead of solvent II prior to quantitation.

Detection of compounds

All purine and pyrimidine compounds could be detected on the chromatograms by viewing with transmitted U.V. light at 253.7 nm using a Mineralight. Photoprints, according to the method of MARKHAM AND SMITH[3], were used to locate trace quantities. Allantoin was detected on chromatograms by spraying with 0.25 % mercuric acetate in 95 % ethanol, drying, and then spraying with 0.05 % diphenylcarbazone in 95 % ethanol[4].

Elution

Elution was done according to the method of SANGER AND TUPPY[5] using water or *N* HCl. The eluate was collected from the tip of the filter paper in a glass vial or test tube. Elution was ordinarily complete after 0.2–0.4 ml eluate had been collected. Recoveries of known amounts after chromatography ranged from 90 % for uric acid to 100 % for more soluble compounds.

Identification and quantitation

Nucleotides, nucleosides, purines and pyrimidines were identified by use of the following methods: U.V.-spectra (220–300 nm) at different pH, comparison of ratios

of optical densities at different wave lengths (250/260, 280/260, 290/260), estimation of the content of phosphorus for nucleotides, comparison of R_F values against known compounds in different solvents, and the specific color reaction of GERLACH AND DÖRING[6] for adenine-containing compounds.

After separation, all substances except allantoin were quantitated by U.V. absorption using known molecular absorption coefficients[7,8]. The application of solvents increased the blank U.V. absorption value of the paper depending on the distance from the starting line (Fig. 1). Thus, the U.V. absorption values of eluates had to be corrected by subtracting the U.V. absorption given at the same wave length by the eluate from an equivalent amount of paper taken from a blank region at the same R_F as the isolated compound.

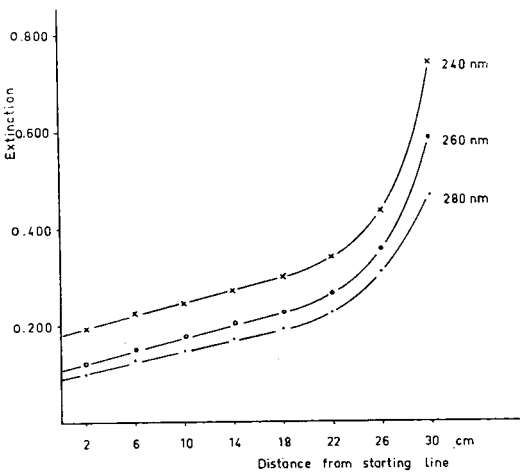

Fig. 1. Blank values *vs.* R_F for acid-washed Whatman No. 1 paper run in solvent II (see text). Paper cut into 4 cm wide strips centering at the indicated distance from the starting line, eluted in 0.3–0.4 ml distilled water, diluted to 0.65 ml and read at the indicated wavelength. The individual extinction values were corrected to correspond to 150 mg of paper strip.

Allantoin was determined by the method of YOUNG AND CONWAY[9], scaled down to a final volume of 2.5 ml. The method, as modified, was sensitive to 0.005 μM allantoin when read at 520 nm in a spectrophotometer. Only uric acid, besides allantoin, gave appreciable color in this reaction, amounting to one third on a molar basis. Since the determination of allantoin could not be carried out after application of the spray reagent, the position of the allantoin fraction was located by running a separate spot of pure allantoin on the same paper and spraying this as a separate strip after chromatographic development.

SUMMARY

A method of paper chromatography is described for separation, identification, and quantitation of nucleotides, nucleosides, purines and pyrimidines. The method has been used for studies of nucleotide catabolism in tissues *in vivo* and *in vitro*.

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PRÄPARATIVE ZONENELEKTROPHORESE IM GIPSBLOCK

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Die Methodik der Papierelektrophorese — des Prototyps der Zonenelektrophorese — lässt sich nicht ohne weiteres in den grösseren präparativen Masstab übertragen. Die Verwendung von Cellulose als stabilisierendes Medium führt zu Schwierigkeiten, die insbesonders in der Inhomogenität der Stabilisierungsfüllung und deren adsorptiven Eigenschaften begründet sind. Andere Stabilisierungsmittel, wie gequollene Stärke, Polyvinylchlorid u.a. vermeiden zwar diese Nachteile, aber sie geben breiförmige Füllungen, deren Aufarbeitung nach der elektrophoretischen Zerlegung der Substanzgemische Nachteile bietet, welche bei der Verwendung eines formbeständigen Füllmaterials nicht auftreten.

Bei der Suche nach einem formbeständigen Füllmaterial, welches nach Beendigung der Elektrophorese freigelegt werden kann, sodass die Zonen der Einzelkomponenten visuell — im sichtbaren oder U.V.-Licht, oder nach Anfärbung eines Abklatsches — festgestellt und durch Zerteilen des Füllkörpers isoliert werden können, zeigte sich, dass Gips eine für viele Zwecke sehr gut verwendbare Stabilisierungsfüllung ergibt.

GIPS ALS STABILISIERENDES MEDIUM

Wird gebrannter Gips ($CaSO_4 \cdot 0.5H_2O$) mit vier Gewichtsteilen Wasser angeteigt, so entsteht eine dünnflüssige Suspension, welche nach einiger Zeit zu einer kompakten Masse erstarrt. Wie die Rechnung ergibt, besteht diese zu etwa 12 Vol. % aus dem Dihydrat $CaSO_4 \cdot 2H_2O$. Dieses günstige Verhältnis von Festkörper zu Flüssigkeit hat bei der Elektrophorese zur Folge, dass die Poren der Füllung, welche aus verfilzten Nädelchen des Dihydrats besteht, relativ weit sind und nur eine geringe Elektroosmose verursachen. Ein lufttrockenes Formstück der Gipsfüllung schwimmt zunächst auf Wasser, bis es durchfeuchtet ist.

Für elektrophoretische Zwecke kann käuflicher gebrannter Gips nicht verwendet werden, er ist zu unrein. Ein genügend reines Präparat gewinnt man leicht aus käuflichem "Calciumsulfat, gefällt, gepulvert" $CaSO_4 \cdot 2H_2O$, Fa. Merck, Darmstadt, welches dicht gepresst in einer Schale im elektrischen Ofen etwa 5 Std. auf 150° erhitzt, anschliessend gepulvert und durch ein Sieb (Din 20, 400 Maschen/cm²) gegeben wird. Das Präparat wird in verschlossener Flasche aufbewahrt.

Der so gebrannte Gips erstarrt, mit vier Gew.Teilen Wasser verrührt, sehr langsam; nach 30 Min. hat sich ein klarer Überstand über der noch flüssigen Suspension gebildet. Der Gips ist jedoch nicht totgebrannt, sondern es fehlen ihm nur die Impfkristalle des Dihydrats. Die Kristallisationsgeschwindigkeit der Gips-Suspen-

sion, und damit die Grösse der Dihydrat-Nädelchen kann durch Zugabe von "Impfgips" zum gebrannten Gips geregelt werden: je rascher die Suspension des gebrannten Gipses erstarrt, desto feiner werden die Nädelchen des Dihydrats und die Poren der Füllung.

Zur Herstellung von Impfgips werden 40 g gebrannter Gips in 1 l Wasser etwa 2 Std. auf der Machine geschüttelt. Das sehr feinkörnige Dihydrat wird abgesaugt, kurz mit Gips-gesättigtem Wasser, dann mit Alkohol gewaschen und auf der Nutsche trocken gesaugt. Das Produkt wird anschliessend nicht im Mörser zerrieben, sondern zwischen Papierblättern vorsichtig zu einem lockeren Pulver zerdrückt.

Als optimal für elektrophoretische Zwecke hat sich eine Erstarrungszeit der Gips-Suspension von 6–8 Min. erwiesen. Dies wird durch Zusatz von 1–3 % Impfgips zum gebrannten Gips erreicht; meistens werden 2.5 % Imfgips benötigt, wovon man sich durch einen Versuch überzeugt: 4 g gebrannter Gips werden mit 0.1 g Impfgips durch Schütteln (*nicht* durch Reiben in der Reibschale!) in einem Kölbchen vermischt, worauf 16 ml Wasser zugesetzt werden. Die Suspension wird 2 Min. durch Umschwenken gerührt, worauf man sie ruhig stehen lässt und von Zeit zu Zeit durch leichtes Neigen des Kolbens prüft, ob der Gips "steht".

Die Füllung der Elektrophorese-Kammer erfolgt stets mit rein wässriger Gips-Suspension, also nicht mit Pufferlösung. Hierbei ist es wichtig, die Anwesenheit von Luftblasen in der Suspension zu vermeiden, da sie beim Aufsteigen in der erstarrenden Füllung feine senkrechte Flüssigkeitskanäle hinterlassen würden. Aus diesem Grund geschieht die Herstellung der Gips-Suspension im Vakuum.

In einem 2 l Rundkolben befinden sich 300 g gebrannter Gips und 4.5 g Impfgips, welche durch Schütteln gemischt werden. Der Kolben, welchem mittels Gummistopfen ein 1.5 l Tropftrichter aufgesetzt ist, wird durch den Tropftrichter an einer Ölpumpe auf etwa 0.1 mm Luftdruck evakuiert, worauf der Hahn des Trichters geschlossen wird. Nachdem in den Tropftrichter 1200 ml destilliertes Wasser gegeben wurden, wird der Hahn des Trichters so lange geöffnet, bis das Wasser bis auf einen kleinen Rest in den Kolben eingeströmt ist, worauf der Hahn verschlossen und der Kolbeninhalt stark geschüttelt wird. Gleichzeitig tritt eine Stoppuhr in Gang. Nach einer Minute Schütteln bleibt der Kolben zur Auflösung des Schaums bis zum Ablauf der zweiten Minute ruhig stehen, worauf der Hahn geöffnet und der Tropftrichter entfernt wird. Das Giessen der Gips-Suspension erfolgt durch einen Trichter gegen die Wand des Gefässes, um die Schaumbildung möglichst gering zu halten. Dann wird der Gips 1 Min. lang mit einem Glasstab zügig gerührt, um etwa an der Gefässwand haftende Luftblasen zu zwingen aufzusteigen und um die ungewollte, aber unvermeidbare Ausrichtung der Impfgips-Nädelchen, welche beim Einfüllen der Suspension vorzugsweise die Richtung des Flüssigkeitsstroms eingenommen hatten, zu zerstören. Aus diesem Grund wird der Rührstab zum Schluss nicht einfach hochgezogen, sondern unter ständigem Rühren langsam immer höher gehoben. Ein kleiner, im Ansatzkolben verbliebener Rest der Gips-Suspension lässt erkennen, wann die Füllung abgebunden ist.

Sobald dies der Fall ist, verschwindet auch ein sehr kleiner wässriger Überstand über der Gips-Suspension, der vorübergehend aufgetreten war. Die abgebundene Gipsmasse wird nun mit Gips-gesättigtem Wasser überschichtet und mindestens eine Stunde sich selbst überlassen. Sie kann in diesem Zustand beliebig lange stehen. Da an der Oberfläche der Gipsmasse fast stets kleine Unebenheiten zu finden

sind, wird die Oberfläche unter dem Gipswasser auf etwa 5–10 mm Tiefe mit Hilfe eines schräg gehaltenen Bleches abgeschabt. Der Abrieb wird mit Gipswasser herausgespült, die nun völlig glatte Oberfläche wieder mit Gipswasser abgedeckt, der Guss ist fertig.

Die Löslichkeit des Dihydrats $CaSO_4 \cdot 2H_2O$ in Wasser, etwa 0.2 %, muss beim Arbeiten mit diesem Medium berücksichtigt werden. Dies bedeutet zunächst, dass Elektrolytlösungen, welche sich mit Ca^{2+} oder SO_4^{2-} unter Bildung von schwer löslichen Verbindungen umsetzen, ausgeschlossen sind. So ist Phosphat als Puffersubstanz nicht verwendbar, dagegen sind (für die Elektrophorese von Proteinen) Veronalpufferlösungen gut geeignet, ebenso Boratlösungen (pH 9,6 für die Elektrophorese von Kohlehydraten).

Grundsätzlich muss jede Lösung, Pufferlösung wie Substanzlösung, welche mit der Gipsfüllung in Kontakt kommt, mit Gips gesättigt sein. Würde die Gipsfüllung mit reinem Wasser durchgewaschen, so würden sich in der Masse feine senkrechte Kanäle bilden, welche sich infolge der Löslichkeit des Calciumsulfats rasch vergrössern. Sofern nicht besondere Verhältnisse vorliegen, stört die Löslichkeit des Calciumsulfats und die Anwesenheit der Ca-Ionen den Verlauf der Elektrophorese nicht, so z.B. bei der Elektrophorese von Human- und Tierseren, den Komponenten des Tuberkulins, d.h. seinen Proteinen, Kohlehydraten und Nucleinsäuren. Stoffe, welche schwer lösliche Ca-Salze bilden, können im Gipsblock natürlich nicht wandern; ein Bleispiel hierfür ist der Farbstoff Congorot. Als besonderer Vorteil für die Anwendung von Gips als Stabilisierungsmedium erweist sich die Tatsache, dass der Gips keine Proteine adsorbiert, sodass sowohl der Trenneffekt wie Ausbeute sehr zufriedenstellend sind; ausserdem erlaubt dieses Verfahren die Anwendung von relativ grossen Elektrophorese-Kammern.

DIE ELEKTROPHORESE-APPARATUR

Die Elektrophorese-Apparatur ist eine Weiterentwicklung der früher in unserem Laboratorium gebauten Apparatur für präparative Elektrophorese in Agar-Gel[1]. Das Prinzip der flachen, kastenförmigen, senkrecht stehenden Elektrophorese-Kammer, aus der das Trägermedium nach Beendigung der Elektrophorese in seiner ursprünglichen Form herausgebracht wird und des Elektrolyt-Kreislaufs, welcher gleichzeitig die Kühlung der Elektrophorese-Kammer bewirkt, wurde beibehalten, da die Dimension der Apparatur die Zerlegung von etwa 1 g Substanz in einem Arbeitsgang erlauben soll.

Die Bauelemente der Apparatur bestehen aus:
(a) Elektrophorese-Kammer;
(b) Kathode;
(c) Trog mit Anode, Elektrolyt-Zulauf und -Ablauf;
(d) Pufferreservoir mit Umlaufpumpe und Kühleinrichtung.

(*a*) *Die Elektrophorese-Kammer*

Die aus Plexiglas gebaute Elektrophorese-Kammer (Fig. 1) besitzt oben einen lichten Querschnitt von 200 × 32 mm, unten von 200 × 22 mm. Ihre Höhe beträgt 320 mm. Diese Form bietet eine genügende Kühlfläche für die Ableitung der Stromwärme, das keilförmige Profil gibt der erstarrten Gipsfüllung den mechanischen Halt.

Die 5 mm starke Vorder- und Rückwand der Kammer ist durch je vier massive Plexiglasrippen versteift. Die Vorderwand ist abnehmbar, sie wird beiderseits durch sieben Plexiglasschrauben gehalten, die übrigen Plexiglasteile sind untereinander durch Verschraubung und Verkittung verbunden. Die Kammer besitzt oben zwei Querträger zum Einhängen in den Trog. Die Querträger besitzen zwei senkrechte

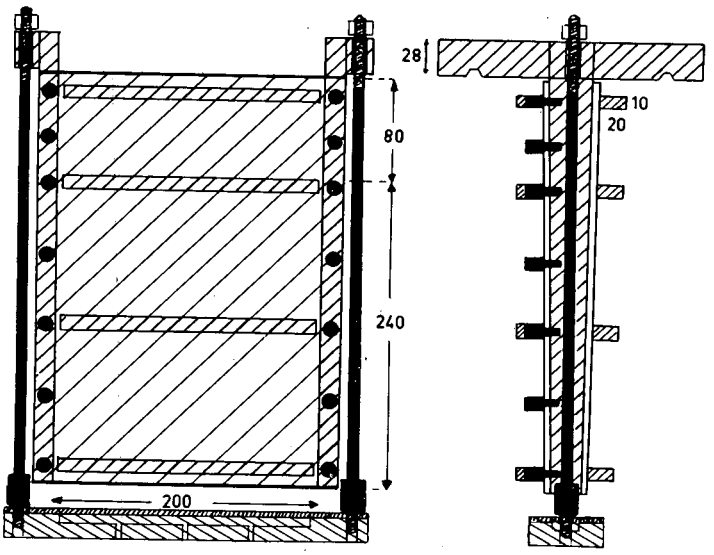

Fig. 1. Die Elektrophorese-Kammer.

Bohrungen, durch welche die Haltestangen für den Kammerboden laufen. Der Kammerboden ist abnehmbar, besitzt oben eine 6 mm tiefe Aussparung im Format 190 × 17 mm, in welche drei senkrechte Luftlöcher gebohrt sind. Der Kammerboden bildet so einen Rahmen für den unteren Abschluss der Kammer; der Rahmen ist mit einem 5 mm starken Schaumgummibelag versehen. Er wird zum Füllen der Kammer mit einer Polyäthylenfolie abgedeckt, welche zwei Bohrungen besitzt. Mit den beiden Haltestangen (Plexiglas) wird die Folie an der Bodenplatte angeschraubt. Mit Hilfe der beiden oberen Muttern wird schliesslich der Kammerboden dicht mit der Kammer verbunden. Durch die Vertiefung im Kammerboden ragt die fertige Füllung unten etwas aus der Kammer kissenförmig abgerundet heraus, sodass sich keine Luftblasen an der Bodenfläche der Gipsfüllung sammeln können.

(b) Die Kathode

Oberhalb der Gipsfüllung wird in die Elektrophorese-Kammer die aus Plexiglas gefertigte Kathode eingesetzt, welche in den Elektrolyt-Überstand der Kammer eintaucht. Die Kathode besteht aus dem Sockel-Teil und dem Kopf-Teil, welche in Fig. 2 durch die unterschiedliche Schraffur gekennzeichnet sind; beide Teile sind durch zwei Plexiglasschrauben verbunden. Der unten offene, 19 cm lange Sockel-Teil, welcher einen horizontalen Platindraht trägt, ist so dimensioniert, dass er gerade in einen Cellophanschlauch (40 mm ø) eingeschoben werden kann. Der Schlauch wird an beiden Enden verknotet, die Enden werden später am Kopf-Teil befestigt. Im

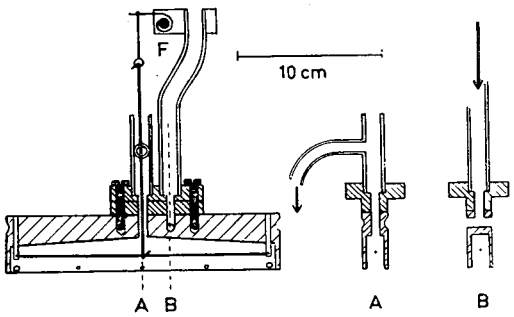

Fig. 2. Die Kathode.

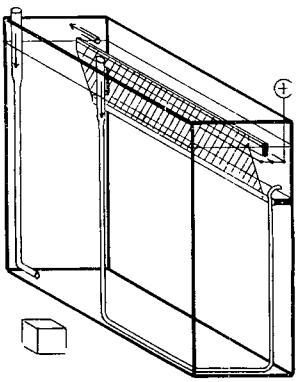

Fig. 3. Der Trog mit Anode.

Sockel-Teil befinden sich oben zwei Gewinde und zwei Durchlässe für die Elektrolytlösung, denen die Bohrungen im Kopf-Teil entsprechen. Mit einem Korkbohrer werden die den Bohrungen entsprechenden vier Fenster in den Cellophanschlauch geschnitten, worauf Sockel- und Kopf-Teil mit zwei Plexiglasschrauben verbunden werden; der dazwischen verbliebene Teil des Cellophanschlauchs bildet die Dichtung. Nachdem der horizontale Platindraht mit einem Pt-Zwischenstück mit dem federnden Stromanschluss F verbunden ist, ist die Kathode gebrauchsfertig.

Der Elektrolyt fliesst in lebhaftem Strahl ständig in den Zufluss bei F, tritt in den Sockel-Teil und teilt sich an der Querbohrung (Schnitt B). Eine Längsrille in der Höhe der Querbohrung, welche um den ganzen Sockel-Teil verläuft, verteilt den eintretenden Elektrolyt innerhalb der Cellophanhülle auf die beiden Längsseiten des Sockel-Teils. Durch je fünf Bohrungen in diesen Längsseiten strömt der Elektrolyt von unten her zur Pt-Kathode, steigt in den Ablauf (Schnitt A), den er durch einen seitlichen Überlauf verlässt. Der Kopf-Teil der Kathode ist oben so breit (45 mm, Schnitt A), dass er beim Einsenken in die Elektrophorese-Kammer auf deren Vorder- und Rückwand aufsitzt, wobei der untere Teil der Kathode 50 mm tief in die Elektrophorese-Kammer hineinragt und in den Elektrolyt-Überstand der Kammer eintaucht. Auf diese Weise wird das Diaphragma ständig von innen mit frischer Pufferlösung bespült und der gebrauchte Elektrolyt entfernt; da die Kathodenflüssigkeit anschliessend mit dem anodenseits sauer gewordenen Elektrolyt ständig im Kreislauf gemischt wird, bleibt das ursprünglich eingestellte pH der Elektrolytlösung auch bei höherer Stromstärke (bis 1.5 A) tagelang konstant, obwohl in 26.8 A·Std. ein Mol-Äquivalent Säure bzw. Lauge an den Elektroden gebildet wird.

(c) Der Trog mit Anode und Elektrolyt-Umlauf

Fig. 3 zeigt den aus Plexiglas gefertigten Trog. Die Innenmasse des Troges sind: Länge 32 cm, Breite 16 cm, Höhe 36 cm; die Vorder- und Rückwand ist 0.5 cm stark, die Seitenwände 1.5 cm und der Boden 2 cm. Alle Teile sind mit Metallschrauben verschraubt, ihre Berührungsflächen mit Araldit gekittet. Der Trog fasst bis zum Niveau des in der linken Seitenwand angebrachten Trog-Abflusses ca. 16 l Elektrolyt.

Die Anode, drei horizontale Platindrähte 2 cm unter dem Flüssigkeitsniveau, ist parallel zur Rückwand fest im Trog eingebaut. Der Anodenraum ist durch Cellophan gegen den übrigen Flüssigkeitsraum fast ganz abgeschlossen; das Cellophan

ist an zwei (herausnehmbaren) horizontalen Leisten befestigt, welche auf seitlich angebrachten Trägern ruhen. Auf der linken Seite des Troges reicht das Cellophan bis zur Seitenwand, auf der rechten Seite ist im Cellophan ein Fenster ausgeschnitten, durch welches der Flüssigkeitsstrom eintritt; die Flüssigkeit wird so, entlang den Anoden, in den Trog-Ablauf geführt.

Der Elektrolyt-Zulauf ist geteilt: die Hauptmenge tritt in das in Fig. 3 links gezeichnete Glasrohr ein, welches bis auf den Boden des Troges führt; die Flüssigkeit umspült und kühlt die Kammer und tritt schliesslich durch das rechts oben liegende Cellophan-Fenster in den Anodenraum ein, den sie durch den Trog-Abfluss verlässt. Der andere Teil (etwa ein Viertel) des Elektrolytzulaufs wird in den Kathodenzulauf geführt; er verlässt die Kathode durch ihren Überlauf, welcher knapp über dem in Fig. 3 gezeigten zweiten Glasrohr endet. Dieses Kathodenrohr führt zum Cellophanfenster des Anodenraums. Die Flüssigkeit im Kathodenrohr bildet einen geringen elektrischen Nebenschluss für die Elektrolytwanderung; aus diesem Grund wurde das Rohr nicht auf dem kürzesten Wege zum Anodenraum geführt. Der elektrische Nebenschluss kommt praktisch nicht zur Geltung, da das Verhältnis des Widerstandes dieser relativ engen und langen Leitung zum Widerstand des Stromweges durch die geräumige Elektrophorese-Kammer und den umgebenden Troginhalt nur einige Promille Nebenschluss-Leistung erwarten lässt.

(d) Das Puffer-Reservoir mit Umlaufpumpe und Kühleinrichtung

Der aus dem Trog-Überlauf abfliessende Elektrolyt fliesst frei in einen Trichter, der zum Puffer-Reservoir führt, einem runden Glastrog von 12 l Fassungsvermögen.

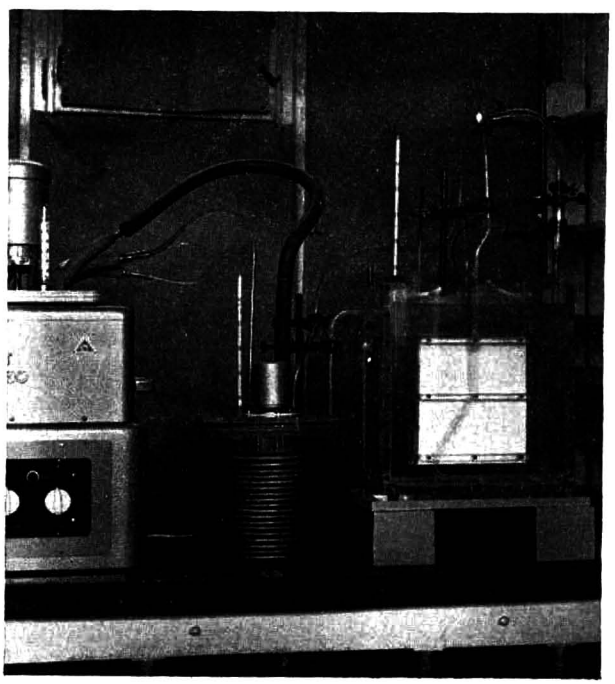

Fig. 4. Elektrophorese-Apparatur.

Der Plexiglasdeckel des Troges trägt eine elektrische Förderpumpe aus V2A-Stahl (Fa. Heidolph, Schwabach) mit einer Förderleistung von 3 l/Min., im Trog befindet sich eine 14 m lange Kühlschlange aus V4A-Stahl (8 × 1 mm), welche von einem Kühlaggregat (Ultra-Kryomat Typ TK 30, Fa. Messgerätewerk Lauda/Tauber) gespeist wird. Fig. 4 zeigt die komplette Apparatur.

DIE ARBEITSMETHODE

(*a*) *Die Pufferlösungen*

Für den in ständigem Umlauf kreisenden Elektrolyt sind nur solche Pufferlösungen verwendbar, welche mit Calciumsulfat nicht reagieren. Zu dieser Einschränkung treten noch folgende Erfordernisse: Die Puffersubstanz darf elektrochemisch nicht verändert werden — also ist z.B. Acetat nicht brauchbar, da das Acetat-Ion bei seiner Entladung zu CO_2 und Äthan umgesetzt wird, und letzten Endes soll die Puffersubstanz in Wasser nicht schwer löslich sein, da sie sonst an der Elektrode kristallin abgeschieden werden könnte. Unter dieser letzten Schwierigkeit leidet z.B. der übliche Veronal–Veronal-Na-Puffer, denn bei längerem Stromdurchgang fällt Veronal aus, welches sich zunächst auf der Anode abscheidet, später auch ungelöst fein verteilt in der gekühlten Pufferlösung (5–15°) verbleibt. Diese Schwierigkeit lässt sich, z.B. bei der elektrophoretischen Zerlegung von Seren, leicht dadurch umgehen, dass als Grundlage des Elektrolyten Natriumsulfat (0.1 *M*) verwendet wird, dessen Lösung durch 0.01 *M* Veronal-Na und 0.36 g /l Veronal auf pH 8.5 eingestellt ist. Nun ist unter Gewährleistung des gewünschten pH die Konzentration an Veronal so gering, dass dieses während der ganzen Elektrophoresedauer in Lösung verbleibt. Voraussetzung für die Anwendbarkeit dieses gepufferten Elektrolyten ist natürlich, dass er mit Calciumsulfat gesättigt ist; eine Abscheidung von Calciumhydroxyd findet an der Kathode nicht statt.

Für die elektrophoretische Trennung von Kohlehydratgemischen hat sich ein Gips-gesättigter *M*/20 Boratpuffer vom pH 9.6 bewährt; Kohlehydrate, welche benachbarte *cis*-ständige Hydroxylgruppen besitzen, wandern unter diesen Umständen zur Anode, während die übrigen durch die geringe osmotische Strömung sehr langsam in die Richtung zur Kathode verlagert werden.

(*b*) *Die Vorbereitung der Elektrophorese-Kammer*

Wie schon geschildert wurde, erfolgt das Giessen des Gipsblockes mit reinem Wasser. Die Kammer wird nach dem Anbringen des Kammerbodens in einem Gestell senkrecht gelagert und etwa 25 cm hoch mit Gips-Suspension gefüllt (etwa 1.2 l). Nachdem der Gips abgebunden ist und die oberste Schicht der Füllung mit dem Schaber abgenommen wurde, entfernt man den Kammerboden und gibt auf die Oberfläche der Gipsfüllung etwa 20 ml einer mit Methylenblau stark gefärbten Gipslösung. Diese dringt im Laufe einiger Minuten in die Gipsfüllung ein, der Ablauf tropft in eine Schale. Das Nachwaschen der Methylenblau-Zone erfolgt mit gipsgesättigter Pufferlösung. Dieses Verfahren zeigt nicht nur, wann der ganze Gipsblock genügend mit Pufferlösung durchgewaschen wurde, sondern es lässt Fehler im Guss, insbesonders (sehr selten auftretende) Kanäle, die durch verspätet aufgestiegene Luftblasen verursacht wurden, mit Sicherheit erkennen. Die horizontale Farbstoffzone bewegt sich ohne Verzerrung (mit einer Geschwindigkeit von rund 5 cm in 40 Min.) durch

die ganze Füllung abwärts. Kleine, manchmal sichtbar werdende Unebenheiten machen sich im späteren elektrophoretischen Lauf nicht geltend. Sobald der Ablauf farblos geworden ist, nach etwa 4–5 Std., kann die Eintragung der Substanzlösung beginnen.

(c) Die Eintragung der Substanzlösung

Die Substanzlösung muss Gips-gesättigt sein. Hierfür ist der feinkörnige "Impfgips" besonders geeignet: Nach Zugabe einer kleinen Gipsmenge zu dem bereits dialysierten Serum oder der in Puffer gelösten Substanz (Konzentration bis etwa 5 %) überlässt man die Suspension etwa 1 St. sich selbst, worauf zentrifugiert wird und die klare Substanzlösung leicht von dem Gipssediment getrennt werden kann.

Sobald in der horizontal aufgehängten Kammer der letzte Rest des Pufferüberstandes in das Gipsbett eingedrungen ist, gibt man 20–25 ml der Substanzlösung auf die Kammerfüllung; bei einem Kammerquerschnitt von rund 50 cm^2 ergibt dies eine Flüssigkeitsschicht von etwa 5 mm Höhe, welche im Lauf von einigen Minuten einzieht. Vorher hat man sich etwa 80 ml Pufferlösung bereitgestellt, mit welchem Volumen man die Substanzschicht durch "Einwaschen" schrittweise, d.h. durch Aufgiessen von jeweils 10 ml Flüssigkeit, tiefer setzt. Nach Zusatz des gesamten Einwaschvolumens befindet sich die Oberkante der Substanzfüllung etwa 1.5 cm unter der Gips-Oberfläche, eine Tiefe, welche für den Start einer Serum-Elektrophorese ausreicht, da die Fraktion mit der geringsten Wanderungsgeschwindigkeit, das γ-Globulin, praktisch an der Startposition verbleibt und sich dort nur durch Diffusion ausbreitet. Da die Gipsfüllung relativ dicht ist, besteht keine Gefahr, dass die Gipsfüllung, über der sich vorübergehend kein flüssiger Überstand mehr befindet, "trocken läuft"; durch Kapillarwirkung bleibt die gesamte Füllung gleichmässig feucht und es wird keine Luft in das Gipsbett eingezogen.

Nun setzt man die (innen mit Pufferlösung gefüllte) Kathode in die Elektrophorese-Kammer und das Ganze in den mit Puffer gefüllten Trog. Überschüssige Pufferlösung läuft aus dem Trog — in welchem vorher schon die Cellophanabsperrung des Anodenraums und das Kathodenrohr angebracht war — rasch durch den Trogablauf ab. Gleichzeitig füllt man in die Elektrophorese-Kammer den Elektrolyt-Überstand bis zum endgültigen Niveau auf; dies soll möglichst rasch geschehen, damit die Füllzone der Substanz im Gipsblock nicht durch unbeabsichtigte Flüssigkeitsströmung verlagert wird.

Sobald dies geschehen ist, wird möglichst bald der "Niveau-Heber" in eine der Ecken der Elektrophorese-Kammer eingesetzt. Er hat etwa 4 mm lichte Weite, taucht in die Elektrophorese-Kammer bis etwa 2 cm oberhalb der Gipsfüllung ein und reicht auf der Aussenseite der Kammer bis fast an deren unteres Ende hinab (der durch den Niveau-Heber bedingte elektrolytische Nebenschluss ist bei dessen Querschnitt und Länge unerheblich). Dieses U-Rohr besitzt oben einen Glasansatz mit kurzem Gummischlauch-Ende, sodass es nach dem Einsetzen durch Ansaugen gefüllt werden kann, worauf das Ende mit einem Quetschhahn verschlossen wird. Der Niveau-Heber hat sich beim Lauf der Elektrophorese als sehr wertvoll erwiesen, da er Niveauunterschiede zwischen Kammer und Aussenflüssigkeit, welche durch Elektroosmose entstehen, unterdrückt. Es hat sich gezeigt, dass zwar in der Gipsfüllung (in Na-Sulfat–Veronal-Puffer) keine ins Gewicht fallende elektroosmotische Strömung auftritt, wohl aber im Cellophandiaphragma der Kathode. Dort wandert

Wasser aus dem Kammerüberstand in die Kathoden-Zelle, wodurch das Niveau des Kammerüberstandes absinkt; ohne Niveau-Heber würde sich deshalb von unten her Elektrolyt durch die Gipsfüllung nachschieben, wodurch sehr langsam wandernde Substanzen in den Elektrolytüberstand verlagert würden; nicht dialysierbare Stoffe verbleiben zwar dort und lassen sich aus dem Überstand isolieren, aber sie entziehen sich dem später durchzuführenden Positionsnachweis der Zonen im Abklatsch des Gipsblockes.

Hier sei noch auf eine Eigentümlichkeit von Proteinlösungen hingewiesen. Wie schon geschildert wurde, bleiben Farbstoffzonen im Gipsblock geschlossen und horizontal, wenn in der Füllung eine Flüssigkeitsströmung herbeigeführt wird. Unternimmt man den gleichen Versuch mit einer angefärbten Proteinlösung grösserer Konzentration, etwa mit Serum, das mit Amidoschwarz versetzt ist, so zeigt sich ein abweichendes Verhalten, welches auf die grössere Viskosität solcher Lösungen zurückzuführen ist. Die Vorderfront der Proteinzone bleibt bei ihrer Wanderung zunächst völlig eben und horizontal; bei weiterem Durchwaschen der Zone bekommt diese auf ihrer Rückseite unweigerlich zackenförmige Ausbuchtungen. An solchen Stellen, an denen (auch in einwandfrei gefertigten Gipsfüllungen) zufällig eine stärkere Vermischung mit der nachdringenden Flüssigkeit erfolgt ist, ist die Viskosität geringer, die Strömung schneller, ein Prozess, der sich ständig vergrössert. Aus diesem Grund wird das Einwaschen der Proteinzone in den Gipsblock beim Füllen der Kammer unter möglichst geringem Flüssigkeitsüberdruck, d.h. durch schrittweise Zugabe der Einwasch-Flüssigkeit durchgeführt, und die Zone wird nicht tiefer gesetzt, als es erforderlich erscheint. Die gleichen Gründe sind dafür massgeblich, dass die Zonen nach Beendigung der Elektrophorese aus dem Gipsblock durch Herausschneiden isoliert werden, denn das Durchwaschen der intakten Füllung (nach Art der Chromatographie) kann auch bei ursprünglich guter Schichtung der Zonen keine einwandfreien Eluate liefern.

Die Viskositätsunterschiede zwischen Protein- und Pufferlösung wirken sich im allgemeinen nur bei der Strömung der Flüssigkeiten, nicht aber bei der elektrophoretischen Wanderung der Zonen in der stehenden Flüssigkeit aus, solange die Proteinkonzentration nicht zu hoch ist. Bei höheren Proteinkonzentrationen, wie z.B. bei der Albuminzone der (unverdünnt gelaufenen) Seren führt der Temperaturunterschied zwischen der Aussenschicht und dem Inneren des Gipsbettes dazu, dass diese Zone nicht die sonst übliche horizontale Schichtung besitzt, sondern in der Mitte schneller läuft als aussen, wie ein Abklatsch des Querschnitts durch den Gipsblock zeigt. Die Wölbung dieser Zone gestattet aber immer noch die einwandfreie Abtrennung des nachfolgenden α_1-Globulins; dieses und die übrigen Globulinzonen sind auch im Querschnitt der Füllung horizontal geschichtet.

(d) Die Durchführung der Elektrophorese

Sobald der Niveau-Heber funktioniert, wird der Elektrolytumlauf in Gang gesetzt; das Kühlaggregat war schon vorher in Betrieb und hatte den im Pufferreservoir befindlichen Puffer auf die Arbeitstemperatur (5–15°) gekühlt, sodass nun die Elektrophorese-Kammer im Laufe etwa einer Stunde ohne schroffen Temperaturwechsel auf die Arbeitstemperatur gebracht wird. Mit Beginn der Kühlung kann der Elektrophorese-Stromkreis eingeschaltet werden. Die Badtemperatur wird im Kühlaggregat, im Pufferreservoir und im Trog (in der Nähe des Anodenfensters) gemessen.

Eine Regulierung der Stromstärke ist nur solange nötig als sich die Temperatur der Elektrophorese-Kammer durch die Kühlung erniedrigt; bei konstanter Arbeitstemperatur bleibt die Stromstärke der Elektrophorese unverändert. Zweckmässig ist ein Gleichstromanschluss von 140 und 220 V mit 2 A Maximalbelastung. Anders als bei der Papierelektrophorese wird bei dieser Apparatur nicht die Spannung, sondern die Stromstärke gemessen und konstant gehalten; bei den konstanten Dimensionen der Apparatur und Anwendung des gleichen Puffers bei gleicher Temperatur ist die Wanderungsgeschwindigkeit einer bestimmten (Protein-)Fraktion proportional der Stromstärke (d.h. A/cm² Kammerquerschnitt). Einer Halbierung der Elektrolytkonzentration entspricht unter sonst unveränderten Bedingungen dann etwa eine doppelte Laufgeschwindigkeit dieser Fraktion.

(e) Die Isolierung der Elektrophorese-Fraktionen

Nach Beendigung der Elektrophorese wird der in der Kammer befindliche Überstand — in dem sich eine Fraktion befinden kann — entnommen, die Kammer flach gelegt und die Vorderwand der Kammer entfernt. Schon im Tages- oder U.V.-Licht lassen sich oft Zonen im Gipsblock erkennen (so das stets durch Bilirubin gefärbte Albumin des Humanserums). Im allgemeinen werden die Zonen durch das Abklatschverfahren lokalisiert. Zu diesem Zweck wird auf die nun freiliegende Fläche des Gipsblockes eine grössere Plexiglasscheibe aufgelegt, das Ganze gewendet und in den Abstreifer gelegt. Der Abstreifer ist eine am Tisch befestigte Holzplatte, auf welcher ein massiver senkrecht stehender Plexiglasstreifen befestigt ist, der mit seiner Länge von 20 cm in den lichten Querschnitt der Kammer passt. Beim Abziehen der Kammer in Richtung zur Kathode hält er den Gipsblock fest, der Block liegt dann frei und unbeschädigt auf seiner Unterlage.

Die Oberfläche des Blockes ist zunächst noch zu feucht für die Abnahme eines guten Abklatsches; deshalb werden die beiden ersten Abklatsche, bei denen die Flüssigkeit sehr schnell in das Filterpapier einzieht, verworfen. Zur Abnahme des Abklatsches wird Papier Whatman Nr. 1 oder 4 verwendet: ein passendes Format ist mit Tesafilm an der Aussenwand eines Glaszylinders von etwa 15 cm ø befestigt. Auf dem Papier ist mit einem Querstrich die Stelle markiert, welche beim Abrollen des Zylinders auf die Kathodenkante des Blockes zu liegen kommt. Das Abrollen des Zylinders erfolgt nach Massgabe der gut sichtbaren Durchfeuchtung des Papiers an der Auflagestelle. Nach dem Abnehmen der Abklatsche wird der Gipsblock bis zu seiner Aufarbeitung mit einer Schale abgedeckt. Für die Anfärbung der Zonen der Serumproteine und anderer Proteine, welche durch Pikrinsäure fällbar sind, hat sich das folgende, rasch ausführbare Verfahren bewährt, welches auf der Anfärbung der Protein–Pikrinsäure-Fällung beruht: der im Ofen (110°) getrocknete Abklatsch kommt für 10 Min. in ein Bad, hergestellt durch Zusatz von 200 ml 3 %iger Essigsäure zu der Lösung von 0.5 g Bromphenolblau + 2.5 g Pikrinsäure in 50 ml Methanol. Anschliessend wird das Blatt in wiederholt gewechselter 3 %iger Essigsäure gewaschen, bis die proteinfreien Flächen rein weiss erscheinen. Der Bogen wird dann zwischen Filtrierpapier stark abgepresst; beim Einbringen in eine NH_3-Atmosphäre färben sich die Proteinzonen tiefblau. Die Färbemethode ist wesentlich rascher durchführbar (in ca. 25 Min.) als die übliche mit Amidoschwarz und gibt stärker angefärbte Zonenbilder.

Der Abklatsch lässt erkennen, dass die (Protein-) Zonen an den Schmalwänden

der Kammer, wo die Kühlung sich am stärksten auswirkt, etwas zurückgeblieben sind. Deshalb wird vor der Zerlegung des Blockes auf beiden Längsseiten ein etwa 5–10 mm breiter Streifen mit einem langen Messer abgeschnitten und verworfen. Vorher hat man sich die beabsichtigte Zerteilung des Gipsblocks auf dem Abklatsch markiert, welcher nun nochmals auf den Block gelegt wird. Die Zonenmarkierung wird mit der Kante eines Blechstreifens in den Gipsblock leicht eingedrückt, worauf der Block im gewünschten Sinn mit Hilfe eines Messers oder durch Eindrücken eines entsprechend gebogenen Blechstreifens zerteilt wird.

Die in Wasser oder Pufferlösung gebrachten Gipsstücke lassen sich leicht zu einer homogenen Suspension zerteilen; der Gips wird abfiltriert, nach kurzem Nachwaschen ist er frei von Protein. Eine Adsorption von Protein an den Gips wurde nie beobachtet.

DIE ELEKTROPHORETISCHE TRENNUNG EINES FARBSTOFF-GEMISCHES

Ein erstes Beispiel zeigt die elektrophoretische Zerlegung eines Farbstoffgemisches (*o*-Nitranilin, Methylorange und Amido-schwarz 10B) in dem eingangs erwähnten Na-Sulfat–Veronal-Puffer (*M*/10, pH 8.5). Das *o*-Nitranilin wandert unter diesen Umständen nicht elektrophoretisch, es könnte nur durch eine elektro-osmotische Strömung verlagert werden. Die Farbstofflösung war vor Beginn der Elektro-

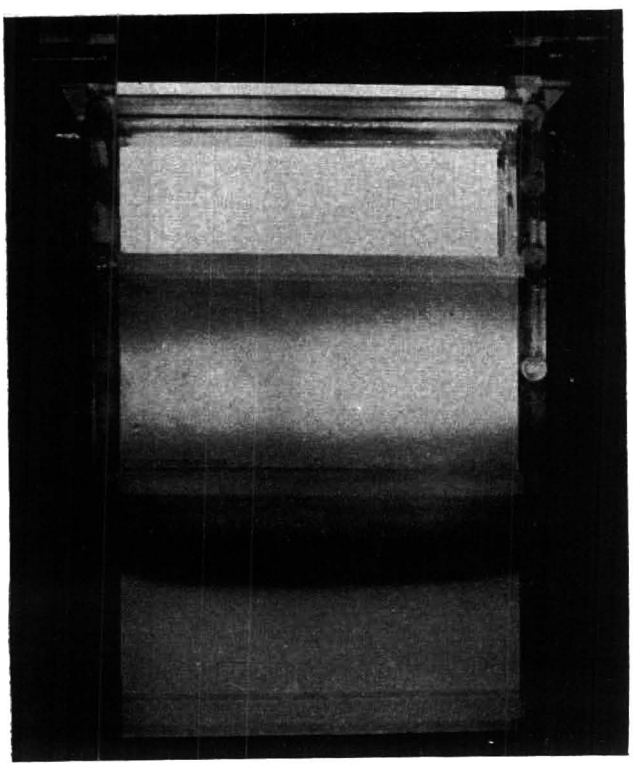

Fig. 5. Elektrophorese-Kammer mit Farbstoff-Zonen.

phorese auf 4 cm Tiefe eingespült worden, was ohne Schwierigkeit gelingt, da die Farbstofflösung nicht viskos ist. Die technischen Daten der Elektrophorese sind in Tabelle I zusammengefasst.

TABELLE I

ELEKTROPHORETISCHE ZERLEGUNG EINES FARBSTOFFGEMISCHES

Pufferlösung:	0.1 *M* Na_2SO_4–Veronal, pH 8.5
Substanzmenge:	10 ml Farbstofflösung
Startposition:	4 cm Tiefe
Spannung:	110 V =
Stromstärke:	0.5 A
Temperatur:	12°
Dauer:	18 St.

Die Fig. 5 zeigt die nach dem Abschluss der Elektrophorese herausgenommene Kammer. Die oberste Farbstoffzone, das *o*-Nitranilin, hat sich durch Diffusion verbreitert und wurde durch die sehr geringe Elektroosmose nur wenig nach oben verlagert. Das Amidoschwarz erweist sich im Gipsblock als die am schnellsten wandernde Substanz; in Cellulose, die es stark adsorbiert, wäre es nicht vom Nitranilin getrennt worden. Das Methylorange ist vom Amidoschwarz gut abgesetzt, die Farbstoff-Fronten liegen bis auf die schmale Randzone horizontal.

DIE ELEKTROPHORETISCHE TRENNUNG VON HUMANSERUM

Dialysiertes Humanserum wurde mit Gips gesättigt. Wir überzeugten uns, dass das Serum hierbei nicht verändert wird, insbesondere wird an den überschüssigen Gips kein Serumbestandteil adsorbiert.

Nach Beendigung der Elektrophorese (siehe Fig. 6 und Tabelle II) wurde der

TABELLE II

ELEKTROPHORETISCHE TRENNUNG VON HUMANSERUM

Pufferlösung:	0.1 *M* Na_2SO_4–Veronal, pH 8.5
Substanzmenge:	26 ml Serum
Startposition:	1.5 cm Tiefe
Spannung:	110 V =
Stromstärke:	1.5 A
Temperatur:	15°
Dauer:	30 St.

Gipsblock, dem Abklatsch entsprechend, mit Hilfe eines gebogenen Blechstreifens unterteilt, wobei die zwischen den Proteinzonen liegenden Gipsstücke verworfen wurden. Zur Isolierung des β-Globulins ist zu bemerken, dass dieses aus einem Protein-(β_0)- und einem Lipoprotein-(β_L)-Anteil besteht. Das durch Ultrafiltration (Filter: Lsg 60, Membranfilter-Ges., Göttingen) von anorganischen Bestandteilen

befreite Protein wird bei der anschliessenden Gefriertrocknung teilweise denaturiert, denn das Lipoprotein geht dann mit Puffer nicht mehr in Lösung (siehe unten). Die Ausbeute der Proteinfraktionen betrug:

Albumin	744.2 mg
α_1-Globulin	57.9 mg
α_2-Globulin	160.5 mg
β-Globulin	85.3 mg
γ-Globulin	220.2 mg

Unter der Voraussetzung, dass die in die Elektrophorese-Kammer eingebrachte Serum-Menge 7 % Protein enthält (1820 mg), entsprechen die isolierten Proteinfraktionen (1268.1 mg) einer Ausbeute von rund 70 % des Ausgangsmaterials.

Die Prüfung auf die elektrophoretische Einheitlichkeit der isolierten Fraktionen wurde mit der von uns entwickelten interferometrischen Agar-Zonenelektrophorese[2] vorgenommen. Fig. 7 zeigt das Ergebnis dieser Kontrolle*. Das oberste

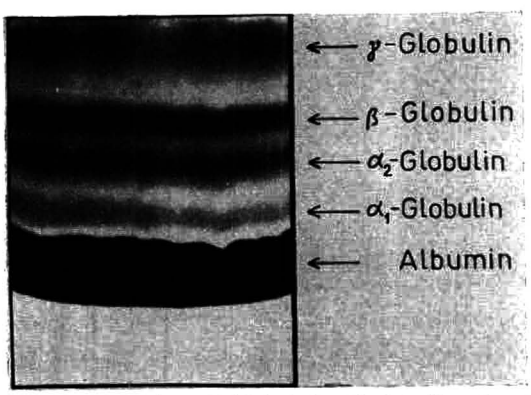

Fig. 6. Angefärbter Abklatsch der Protein-Zonen von Humanserum.

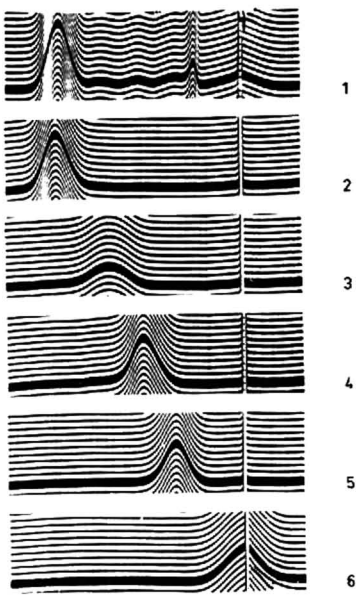

Fig. 7. Analytische Zonenelektrophorese von Humanserum und seinen Protein-Komponenten. (1) Humanserum; (2) Albumin; (3) α_1-Globulin; (4) α_2-Globulin; (5) β-Globulin; (6) γ-Globulin.

Diagramm zeigt das Ausgangsserum, von links nach rechts die Gipfel des Albumins, α_1-, α_2-, β_0-, β_L-Globulins und den γ-Globulingipfel, welcher infolge der Elektroosmose im Agar-Gel nicht wandert, sondern an der Startstelle, dem Füllkanal stehen bleibt. Wie man sieht, fehlt dem löslichen β-Globulin (Diagramm 5) die im Gesamtserum als steiler Gipfel auffallende Fraktion des β_L-Lipoproteins, welches infolge Denaturie-

* Zur besseren Kenntlichmachung der Elektrophorese-Ergebnisse wurde auf den Interferenzdiagrammen ein Zwischenraum zwischen zwei Interferenzlinien schwarz ausgezogen; die senkrechte Doppellinie ist der Füllkanal (Startzone).

rung unlöslich geworden ist. Die isolierten Serumfraktionen erweisen sich als elektrophoretisch einheitlich.

Die Literatur über präparative Elektrophorese-Verfahren ist zwar schon umfangreich — trotzdem entschlossen wir uns, sie um diesen Beitrag zu vermehren, da damit nicht nur ein neuartiges Füllmaterial, sondern auch eine Methodik, die sich unverändert seit vier Jahren bewährt hat, bekannt gemacht werden soll.

DANK

An dieser Stelle sei Herrn Dr. W. REUTER für seine Mitwirkung bei den Vorarbeiten, Herrn W. BRUDER für seine Mitarbeit in der Institutswerkstätte, und Herrn D. KÖHLER für die Durchführung der Versuche bestens gedankt.

ZUSAMMENFASSUNG

Für die präparative Elektrophorese hat sich reiner Gips als stabilisierendes Füll-Medium bewährt. Die Vorteile dieses Mediums sind darin zu erblicken, dass die Gips-Füllung keine Proteine oder Kohlehydrate adsorbiert, dass mit Gips auch relativ grosse Elektrophorese-Kammern leicht homogen gefüllt werden können, dass die Elektroosmose infolge der weiten Poren des Gipsbetts sehr gering ist, und dass die einzelnen Zonen nach der Beendigung der Elektrophorese durch Zerschneiden der freigelegten Gips-Füllung genau getrennt werden können. Für die Elektrophorese von Stoffen, welche schwer lösliche Sulfate oder Ca-Salze bilden, ist die Gips-Füllung nicht geeignet. Die beschriebene Apparatur gestattet die elektrophoretische Zerlegung von etwa 1 g Substanz in einem Arbeitsgang in guter Ausbeute.

SUMMARY

Pure gypsum has proved to be a good stabilizing medium for preparative electrophoresis. The advantages of this medium are that it does not adsorb proteins or carbohydrates, that it can be used to fill relatively large electrophoresis chambers homogeneously, that electro-osmosis is very slight owing to the wide pores of the material and that after completion of electrophoresis the individual zones can be clearly separated by cutting up the gypsum layer. Gypsum cannot be used in the case of substances that form sparingly soluble sulphates or calcium salts. Using the apparatus described it is possible to resolve about 1 g of substance in one operation in good yield.

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IDENTIFICATION OF AROMATIC SUBSTANCES BY "ELECTROPHORETIC SPECTRA" USING PAPER ELECTROPHORESIS

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The majority of papers dealing with electrophoresis in papers are concerned with the behaviour of different higher-molecular-weight organic substances, predominantly of animal or vegetable origin. On the other hand, the number of papers on the separation of low-molecular-weight substances is comparatively small, and, further, a considerable number of them deal with organic acids.

The number of publications on electrophoresis of simpler aromatic substances[1–11] is still smaller, although, as already previously pointed out[12], this method is very suitable as a supplement to chromatographic procedures, since it was found that some factors which play an important role in chromatography on paper exert no influence upon the mobility in the electric field. This mainly relates to some functional groups and to the mobility of isomeric compounds (however, in as far no interaction in the form of hydrogen bridges takes place). On the other hand, electrophoresis on paper permits the estimation of the number and type of functional groups, whose contribution to the total mobility is roughly additive[12].

In the present work we attempted to use paper electrophoresis for identification purposes, but from another viewpoint. Determination of the mobilities for pH values of 1–10 for one and the same compound enables the construction of a graph (electrophoretic spectrum), from whose curve certain conclusions can be drawn as to the arrangement of the molecule. This result is analogous to that obtained in earlier papers where either paper or gas chromatography[13,14] is used for a similar purpose. In paper chromatography, the R_F values are determined for twenty different solvent systems, and in gas chromatography on four columns with various stationary phases. The chromatographic behaviour and the structure of the substances subjected to chromatography are closely interconnected, and this manifests itself in the course of establishing the curves (chromatographic spectra).

In order to attain easy reproducibility of the mobilities measured at various pH values, buffers of a certain pH value and a certain ionic strength were selected as conductive media. In addition to this, a special piece of simple equipment was designed which permits simultaneous electrophoretic separation at various pH values in a single experiment. For control of the function of the instrument and, if necessary, correction of mobilities, a standard substance was added to each compound subjected to electrophoresis.

For determination of the electrophoretic spectra, a number of compounds of the benzene, naphthalene and diphenyl series were selected.

EXPERIMENTAL

Apparatus

The apparatus for the determination of the electrophoretic spectra is illustrated in Fig. 1. The essential part of the equipment is the arrangement of the cathode (A_1) and the anode (A_2) section, which consists of ten chambers, each of which is filled with an electrolyte (100 ml) of different pH value. All ten chambers have a platinum electrode in common. The entire apparatus is made of Plexiglas. In principle it is an instrument for electrophoresis of current type with a so-called humid chamber.

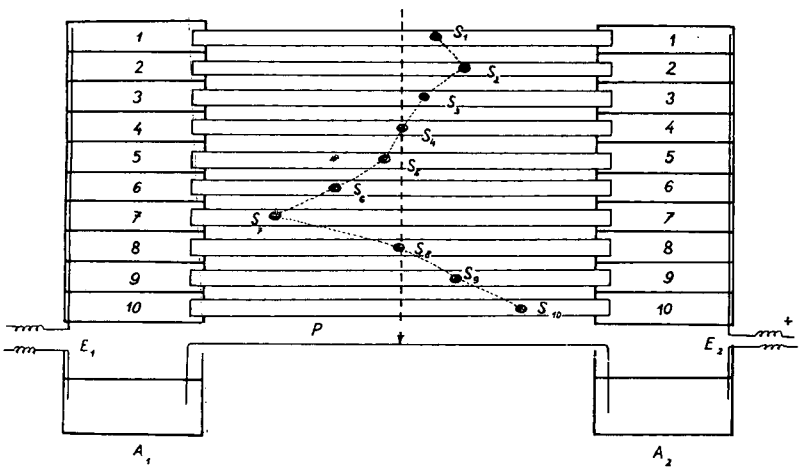


Fig. 1. Schematic representation of equipment for determination of electrophoretic spectra.

Each of the ten anodic chambers is connected with the corresponding cathodic chamber by a strip of Whatman No. 1 paper (size 2.5 × 55 cm), onto which, at the starting place in the middle, the substance under investigation is applied together with the standard (3-nitrophthalic acid), which makes it possible to apply corrections to the mobilities and, thus also, to compare individual experiments.

The voltage used is 250 V, the duration of electrophoresis is 3 h, the current intensity amounts to about 6 mA.

Electrolytes

A number of electrolytes of different pH values were prepared in sufficient amounts; the pH value of the solutions thus obtained was measured by a hydrogen electrode at 18°.

Composition and measured pH values of the electrolytes are given in Table I.

Evaluation

Detection of unknown constituents is first undertaken by a number of detection procedures such as were described in the paper dealing with chromatographic spectra obtained by paper chromatography[13]. This detection is also a part of the identification.

This is followed by a check of the mobility of the standard (3-nitrophthalic acid) to make sure that all ten chambres are intact and that no exchange of buffers has

TABLE I

COMPOSITION AND MEASURED pH VALUES OF THE ELECTROLYTE

Electrolytes Nos. 2–10 were further diluted with distilled water in the ratio 1:1.

No.	*Electrolyte composition*	*pH*
1	100 ml 0.1 *N* HCl	1.75
2	0.40 ml 0.2 *M* K_2HPO_4 + 19.60 ml 0.1 *M* citric acid	2.35
3	4.11 ml 0.2 *M* K_2HPO_4 + 15.99 ml 0.1 *M* citric acid	3.1
4	7.71 ml 0.2 *M* K_2HPO_4 + 12.29 ml 0.1 *M* citric acid	4.2
5	10.30 ml 0.2 *M* K_2HPO_4 + 9.70 ml 0.1 *M* citric acid	5.2
6	12.63 ml 0.2 *M* K_2HPO_4 + 7.37 ml 0.1 *M* citric acid	5.8
7	16.47 ml 0.2 *M* K_2HPO_4 + 3.53 ml 0.1 *M* citric acid	7.2
8	19.45 ml 0.2 *M* K_2HPO_4 + 0.55 ml 0.1 *M* citric acid	8.7
9	0.05 *M* borax	8.9
10	6.0 ml 0.1 *N* borax + 4.0 ml 0.1 *N* NaOH	9.7

occurred. 2-Nitrophthalic acid was chosen as standard because it is very easy to detect in U.V. light as a dark spot, and also because it has favourable mobility values in nearly all the buffers employed. Then the mobilities of the identified substance are calculated on the basis of the mobility of the standard, most advantageously in buffer No. 4 ($u = 11.4 \cdot 10^{-5}$ cm$^2 \cdot$V$^{-1} \cdot$s^{-1}). The electrophoretic spectra are established at least twice. The mobilities of various aromatic compounds in the individual buffers are presented in Table II.

Aromatic substances with zero mobility

Since in paper electrophoresis only those substances that are capable of carrying a positive or a negative charge migrate, there is a long list of compounds containing miscellaneous functional groups which exhibit zero mobility in all the buffers employed.

Among the measured substances are the following aromatic compounds: 1-amino-2,3-dichlorobenzene, 4-chlorobenzaldehyde, 4-nitrotoluene, 1,4-dinitrobenzene, 3-nitrophenol, 2-nitrophenol, 1-nitronaphthalene, 1,5-nitrochloronaphthalene, 4,4′-N-tetramethyl-2,2′-dinitrobenzidine, 4-nitrodiphenyl, 2,2′-dinitrobenzidine, 4-hydroxydiphenyl, 2-hydroxydiphenyl, 4,4′-dichloro-2-nitrodiphenyl, 4,4′-dinitrodiphenyl, 2-nitrodiphenyl, 3-nitrodiphenyl, 4,2′-dinitrodiphenyl, 3,3′-dinitrophenyl, 2-nitrophenanthrene, 9-nitrophenanthrene, 2-nitro-4,4′-dichlorodiphenyl, 3,3′-dimethoxy-4,4′-dichlorodiphenyl, 3,3′-dimethyldiphenyl, and 3,3′-dimethoxydiphenyl.

DISCUSSION

From Table II it is obvious that the electrophoretic spectra (Fig. 2) of compounds of the diphenyl series were those predominantly determined. Our attention was devoted to these substances, since they are for the greater part strongly carcinogenic and their determination in biological material is of great importance. For this reason, any identification method is desirable.

If a reference standard is available, identification of an unknown substance is relatively easy, and comparison of the R_F values by paper chromatography and of the curves of electrophoretic spectra permits correlation of the data obtained. The

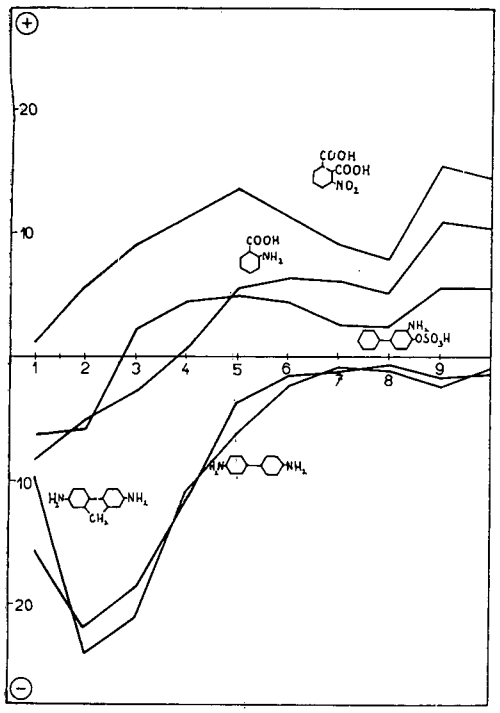


Fig. 2. Electrophoretic spectra of various aromatic compounds; on X-axis: pH values of buffers employed; on Y-axis: mobility $u \cdot 10^5$.

identity can be confirmed in this case also by means of various detection reagents[13].

Use of the mobility values u (Table II), either for comparison with the standard or for further treatment in cases where no reference standard is available, necessitated the establishment of the experimental error for the individual buffers.

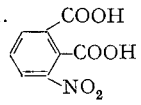
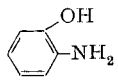
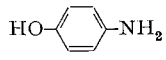
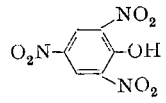
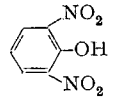
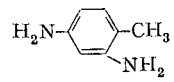
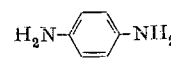
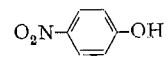
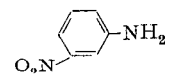
Although simultaneous electrophoresis in various buffers leads to considerable reproducibility, a certain scattering of the values of u is encountered, mainly caused by personal errors (in particular in determination of the centres of diffuse spots etc.). For this reason, statistical evaluation of errors was carried out, and it was found that the variation coefficient for all buffers amounts to 8.4%. Consequently, comparison of results necessitates taking into account a scatter corresponding to this error.

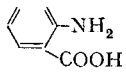
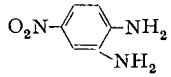
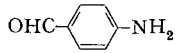
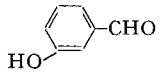
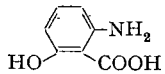
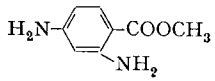
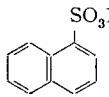
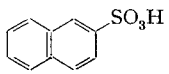
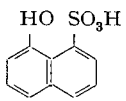
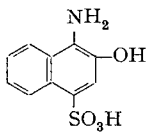
Some compounds form diffuse spots in certain buffers. Although this fact leads to less accurate mobility data, it is, on the other hand, a reproducible and thus a characteristic property of these substances. In some cases, the spots are even diffusely distributed on both sides of the start. These substances are not given in Table II (aniline, N-dimethylaniline, 2-methyl-6-chloroaniline, 2,5-dimethylaniline, 4-chloroaniline, 3-chloroaniline, 4-methylaniline).

Comparison of the curves of the electrophoretic spectra of compounds with various functional groups, and also with different numbers of them, requires the mobilities obtained to be related on a uniform basis. If all mobilities are divided by one of them, the curve of the electrophoretic spectrum is simplified to such an extent that the influence of the number of functional groups in the molecule disappears and

TABLE II

MOBILITY u IN INDIVIDUAL BUFFERS AND COLORATION OF SPOTS

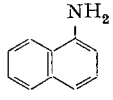
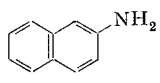
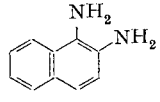
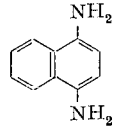
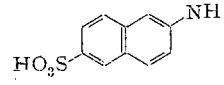
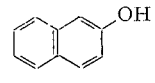
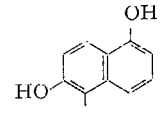
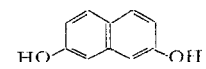
		Buffer No.										*After spray with Ehrlich reagent*		*Without spray*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Vis.*	*UV*	*UV*
Stand.		1.3 +	5.8 +	9.1 +	11.4 +	13.8 +	11.7 +	9.4 +	8.1 +	15.6 +	14.9 +	och	yg	d
1		10.4 —	18.6[a] —	14.0[a] —	12.6 —	3.3 —	0.4 —	0.4 —	0.3 —	0.5 —	0.9 —	ry	1–4 y 5–10	d
2		11.0 —	17.8 —	14.4 —	10.8 —	6.8 —	1.9 —	1.5 —	0.4 —	0.4 —	0.2 —	y	yg	d
3		1.1 +	1.8 +	1.6 +	1.7 +	1.4 +	1.3 +	1.2 +	1.2 +	3.0 +	3.0 +	ochy[a]	ory	d
4		0.0	0.0	1.5 +	5.9 +	8.1 +	7.2 +	5.3 +	4.9 +	11.8 +	10.2 +	r	ror	d
5		19.8[a] —	18.0[a] —	17.7[a] —	14.1[a] —	5.6 —	1.9 —	1.7 —	1.7 —	3.3 —	3.0	g (dull)	gy	—
6		18.2 —	25.8 —	18.7 —	13.9 —	8.3 —	3.0 —	1.7 —	0.8 —	0.6 —	2.7	c	orr	bl
7		0.0	0.0	0.0	0.00	0.0	2.3 +	3.9 +	3.8 +	9.2 +	8.5 +	y	y	d
8		11.4 —	8.4 —	4.0 —	1.3 —	1.4 —	1.1 —	1.3 —	1.7 —	3.0 —	4.3 —	y	•—	d

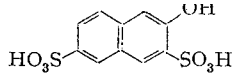
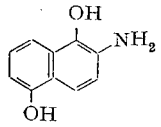
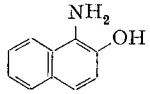
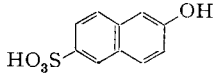
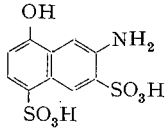
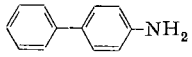
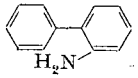
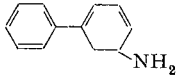
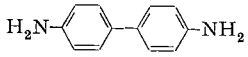
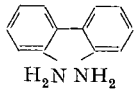
9		8.1 —	5.0 —	2.6 —	0.8 —	5.6 +	6.7 +	6.3 +	5.4 +	11.0 +	10.4 +	bry	y	bl
10		6.2 —	7.1 —	3.1 —	0.8 —	0.2 —	0.7 —	0.3 —	0.1 —	0.8 —	0.2 —	y	—	d
11		5.5 —	2.5 —	1.3 —	1.3 —	0.4 —	0.7 —	0.7 —	0.7 —	1.7 —	1.3 —	y	y	y
12		0.0	0.6 —	1.4 —	1.7 —	1.5 —	1.2 —	1.2 —	1.3 +	2.3 +	3.4 +	—	—	d
13		5.8 —	3.9 —	1.6 —	1.5 —	3.1 +	5.1 +	5.3 +	4.8 +	8.9 +	9.5 +	y	y	bl
14		9.4 —	14.5 —	5.8 —	4.4 —	2.6 —	2.0 —	1.0 —	1.0 —	3.4 —	2.6 —	ochy	d	blv
15		9.4 +	10.6 +	9.0 +	8.4 +	8.8 +	8.2 +	6.3 +	6.6 +	9.3 +	9.4 +	—	—	d
16		7.4 +	11.3 +	7.3 +	8.0 +	7.0 +	6.5 +	5.6 +	4.2 +	10.5 +	8.7 +	—	—	d
17		7.1 +	12.6 +	13.1 +	8.9 +	7.6 +	6.8 +	5.2 +	4.7 +	7.8 +	8.8 +	—	—	bl
18		0.0	0.0	0.0	2.9[a] +	3.7[a] +	5.5 +	3.2 +	3.2 +	7.0 +	7.9 +	c	y	bl

For footnote see p. 111.

(*continued on p. 106*)

TABLE II (*continued*)

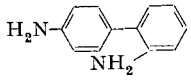
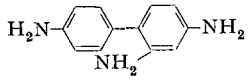
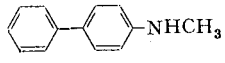
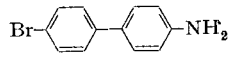
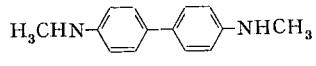
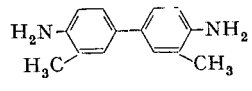
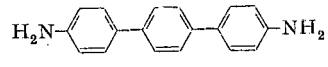
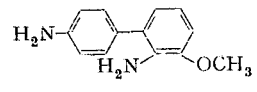
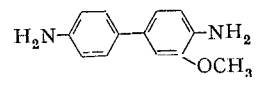
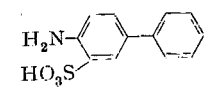
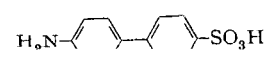
		Buffer No.										*After spray with Ehrlich reagent*		*Without spray*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Vis.*	*UV*	*UV*
19		9.5[a] —	15.3[a] —	7.7[a] —	4.1[a] —	1.3 —	0.9 —	0.7 —	0.7 —	2.4 —	2.6 —	y	gy	bl
20		7.7 —	13.5 —	10.5 —	4.1 —	0.9 —	0.8 —	0.0 —	0.0 —	2.5 —	2.0 —	y	y	bl
21		4.0[a] —	7.0[a] —	5.0[a] —	2.5[a] —	1.3 —	0.7 —	1.2 —	1.3 —	1.5 —	1.2 —	y	y	bl
22		4.0[a] —	8.6[a] —	6.2[a] —	3.0[a] —	1.8 —	1.1 —	1.4 —	0.8 —	1.6 —	2.3 —	y	y	bl
23		0.0	0.0	0.0	0.2 +	3.8 +	3.7 +	3.2 +	2.7 +	8.2 +	8.7 +	yor	yg	blv
24		0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.0	0.0	1.2 +	—	—	bl
25		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2 +	2.2 +	—	—	d
26		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1 +	—	—	bl

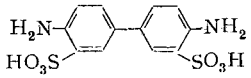
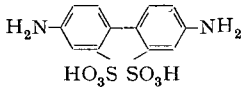
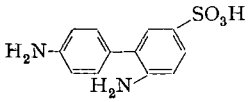
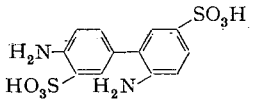
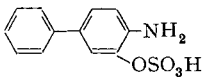
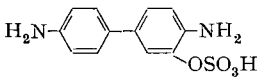
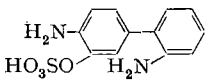
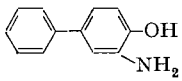
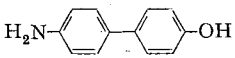
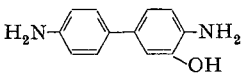
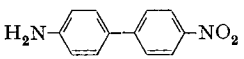
27		9.2 +	18.8 +	19.0 +	14.6 +	13.0 +	12.9 +	10.1 +	10.1 +	16.7 +	18.1 +	—	—	d
28		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6 +	1.8 +	br	br	bl
29		0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1 +	5.5 +	5.5 +	y	y (dull)	d
30		1.3 +	5.8 +	1.2 +	11.4 +	6.6 +	5.8 +	4.2 +	3.4 +	8.5 +	10.7 +	—	—	d
31		8.3 +	8.3 +	6.2 +	9.0 +	8.8 +	7.5 +	6.5 +	6.2 +	11.2 +	12.7 +	y	y	
32		9.8 —	13.5 —	9.8 —	7.8 —	3.1 —	2.0 —	0.9 —	1.3 —	1.5 —	2.2 —	y	y	bl
33		8.2 —	12.3 —	10.9 —	8.9 —	1.9 —	0.4 —	0.0 —	0.0 —	1.5 —	1.4 —	y	yg	bl
34		12.5 —	13.9 —	12.6 —	11.3 —	3.6 —	0.9 —	1.2 —	1.1 —	4.2 —	3.4 —	ochy	y	bl
35		15.4 —	21.8 —	18.5 —	11.4 —	3.4 —	1.3 —	1.0 —	0.4 —	1.3 —	1.1 —	or	or	bl
36		11.5 —	16.1 —	10.8 —	7.9 —	3.1 —	1.0 —	1.1 —	1.9 —	3.3 —	3.0 —	y	dg	bl

For footnote see p. 111.

(continued on p. 108)

TABLE II (*continued*)

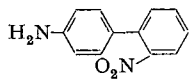
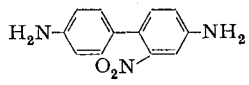
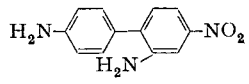
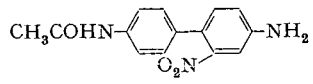
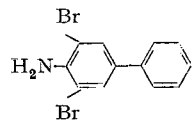
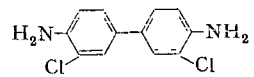
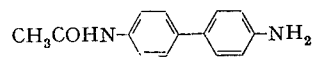
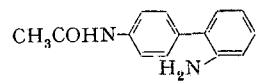
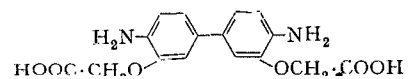
		Buffer No.										*After spray with Ehrlich reagent*		*Without spray*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Vis.*	*UV*	*UV*
37		15.2 —	20.8 —	15.0 —	8.7 —	4.0 —	0.0 —	0.2 —	0.6 —	1.4 —	1.9 —	yoch	yg	vbl
38		18.5 —	25.2[a] —	16.9[a] —	9.5[a] —	3.1 —	2.9 —	1.4 —	1.4 —	1.8 —	2.7 —	brr	bror	blv
39		7.7[a] —	15.1[a] —	10.3[a] —	4.5[a] —	1.3 —	1.1 —	0.8 —	0.0 —	2.6 —	1.5 —	y	ochy	bl
40		1.4[a] —	0.8 —	0.0 —	0.0 —	0.0 —	0.0 —	0.0 —	0.0 —	0.0 —	0.0 —	—	—	bl
41		13.4 —	25.0 —	17.0 —	7.3 —	0.7 —	0.5 —	0.6 —	0.1 —	1.8 —	1.5 —	y	y	bl
42		12.1[a] —	20.2[a] —	12.8[a] —	5.7[a] —	1.5 —	1.1 —	0.9 —	0.7 —	1.3 —	1.0 —	ror	or	bl
43		6.6 —	8.0 —	3.3 —	1.6 —	0.9 —	1.0 —	0.8 —	0.8 —	1.3 —	1.7 —	y	y	bl
44		10.5 —	21.2 —	14.8 —	8.8 —	1.7 —	1.9 —	0.8 —	1.7 —	1.5 —	2.0 —	y	yg	v
45		10.1 —	16.8 —	11.2 —	3.9 —	0.7 —	0.7 —	0.0 —	0.0 —	0.7 —	0.7 —	r	orr	v
46		0.4 +	0.2 +	0.1 +	0.3 +	3.7 +	3.9 +	3.0 +	2.9 +	5.6 +	6.4 +	y	gy	bl
47		0.1 —	0.1 -	0.0	0.5 +	3.1 +	4.0 +	2.9 +	2.7 +	7.1 +	6.8 +	ochy	yg	bl

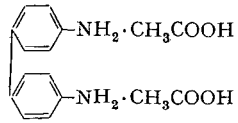
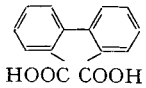
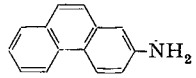
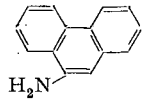
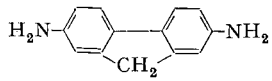
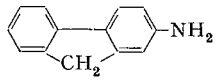
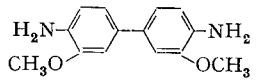
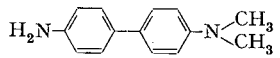
48		1.3 —	0.9 —	0.7 +	10.7 +	9.2 +	7.6 +	7.0 +	4.4 +	13.8 +	11.9 +	ochy	—	—
49		0.4 —	0.7 —	0.7 +	4.4 +	5.5 +	7.8 +	6.2 +	5.2 +	12.6 +	11.8 +	ochy	—	—
50		2.8 —	4.3 —	2.1 —	0.0	3.9 +	4.0 +	4.2 +	3.0 +	6.1 +	5.2 +	y	yg	bl
51		1.3 +	12.6 +	14.0 +	12.8 +	12.3 +	10.7 +	7.9 +	7.2 +	14.5 +	13.5 +	ochy	y	vbl
52		6.1 —	5.8 —	2.3 +	4.8 +	5.0 +	4.6 +	2.8 +	2.9 +	5.8 +	5.8 +	y	y	—
53		5.3 —	7.5 —	3.1 —	1.0 +	3.4 +	3.1 +	2.6 +	2.1 +	5.8 +	6.1 +	or	or	—
54		3.6 —	6.9 —	1.3 —	3.3 +	3.5 +	3.2 +	3.2 +	3.1 +	5.2 +	5.2 +	y	gy	—
55		15.8 —	21.3 —	16.5 —	8.8 —	2.8 —	0.8 —	0.9 —	0.8 —	0.8 —	0.8 —	ochy	y	—
56		4.4 —	8.4 —	6.5 —	3.3 —	1.5 —	0.0	0.0	0.0	0.0	0.0	yor	y	—
57		12.3 —	16.3 —	12.7 —	5.9 —	1.8 —	0.7 —	0.1 —	0.3 —	0.8 —	0.2 —	r	ory	—
58		5.0[a] —	13.5[a] —	5.1[a] —	2.8[a] —	0.9 —	0.4 —	0.0 —	0.0 —	0.0 —	0.0 —	or	dor	—

For footnote see p. 111.

(*continued on p. 110*)

TABLE II (*continued*)

		Buffer No.										*After spray with Ehrlich reagent*		*Without spray*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Vis.*	*UV*	*UV*
59		11.3 —	19.1 —	12.1 —	5.9 —	2.7 —	0.2 —	1.9 —	2.5 —	3.7 —	2.5 —	y	d	—
60		8.5 —	10.0 —	6.0 —	1.4 —	0.7 —	0.7 —	0.9 —	0.0 —	1.1 —	1.2 —	orr	dr	—
61		8.5 —	12.6 —	6.9 —	4.0 —	1.2 —	1.0 —	1.8 —	1.6 —	2.8 —	2.8 —	ory	—	d
62		2.8 —	3.4 —	0.0 —	0.0 —	0.7 —	0.5 —	0.7 —	0.7 —	2.3 —	2.3 —	y	d	—
63		7.5[a] —	14.5[a] —	12.5[a] —	7.8[a] —							y	yg	—
64		5.9 —	4.4 —	1.6 —	0.8 —	0.7 —	0.7 —	0.7 —	0.3 —	0.6 —	0.9 —	or	or	—
65		3.4 —	5.2 —	4.3 —	2.6 —	1.6 —	1.0 —	0.4 —	0.7 —	1.7 —	1.2 —	ory	ochy	—
66		4.1 —	7.3 —	4.4 —	2.0 —	0.7 —	0.4 —	0.2 —	0.3 —	1.3 —	1.8 —	y	gy	—
67		3.6 —	2.9 —	2.4 —	1.6 —	2.3 +	3.4 +	2.1 +	1.8 +	6.3 +	7.2 +	or	cr	bl

68		16.3 —	23.6 —	19.9 —	8.1 —	0.9 —	0.3 —	0.1 —	0.1 —	0.3 —	0.2 —	brr	or	bl
69		0.8 +	1.6 +	2.1 +	3.0 +	6.9 +	8.4 +	4.3 +	6.0 +	11.7 +	11.7 +	—	—	bl
70		0.5 +	0.5 +	0.0	0.0	0.00	0.0	0.0	0.0	0.0	0.0	y	dy	bl
71		5.2[a] —	8.5[a] —	2.5[a] —	0.5 —	0.5 —	0.0	0.0	0.3 —	0.9 —	0.5 —	ochy	y	bl
72		9.9 —	23.8 —	21.0 —	10.9 —	0.8 —	2.1 —	0.6 —	1.0 —	2.1 —	0.7 —	orr	or	bl
73		3.3 —	8.0 —	5.5 —	3.3 —	1.3 —	0.5 —	0.6 —	0.6 —	1.9 —	1.7 —	y	y	bl
74		10.4 —	19.7 —	13.7 —	3.6 —	1.3 —	0.8 —	0.8 —	1.0 —	2.0 —	1.6 —	brr	r	bl
75		4.4 —	7.1 —	5.4 —	1.1 —	0.0	0.0	0.0	0.0	0.0	0.0	ochy	yor	bl

[a] Considerably elongated spot.

Coloration of spots: r = red, och = ochre, y = yellow, g = green, or = orange, br-r = brick-red, d = dark, c = carmine, br = brown, bl = blue, v = violet.

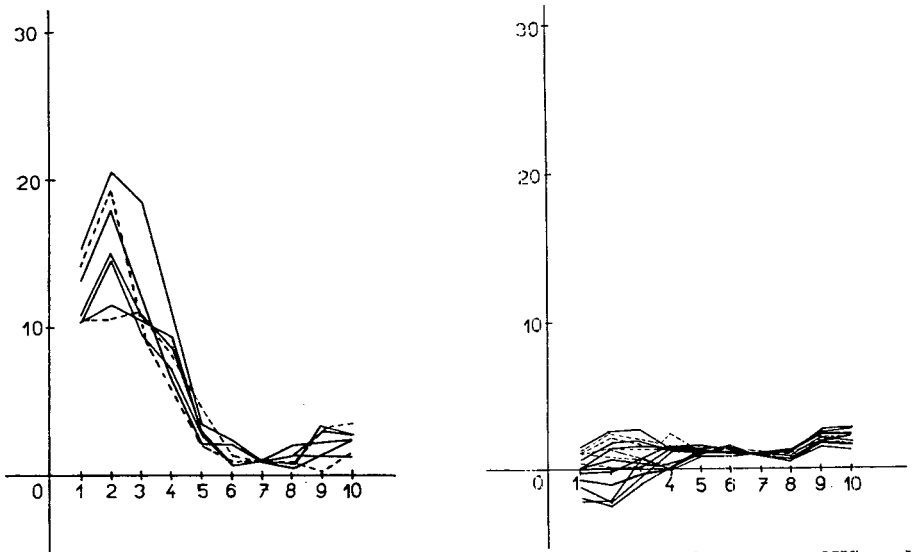


Fig. 3. Graphically represented mobility ratio u_x/u_7; for functional groups: —NH_2, —NH_2; migration region: — .

Fig. 4. Graphically represented mobility ratio u_x/u_7; for functional groups: —NH_2, —SO_3H, —OSO_3H; migration region: — + .

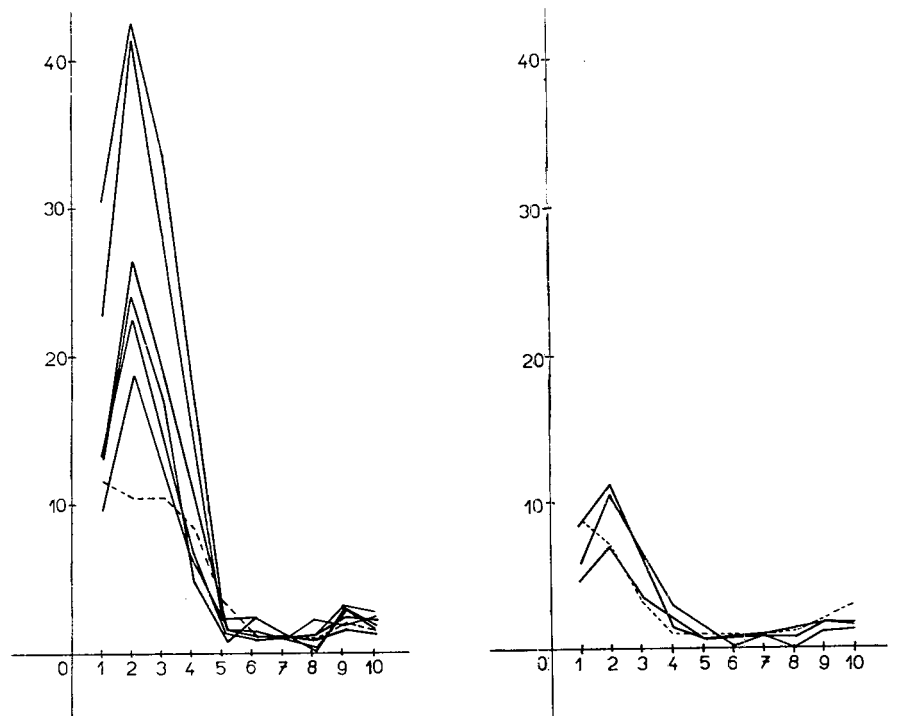


Fig. 5. Graphically represented mobility ratio u_x/u_7; for functional groups: —NH_2, —$NHCH_3$, —CH_3, —OCH_3; migration region: — .

Fig. 6. Graphically represented mobility ratio u_x/u_7; for functional groups: —NH_2, —NO_2; migration region: — .

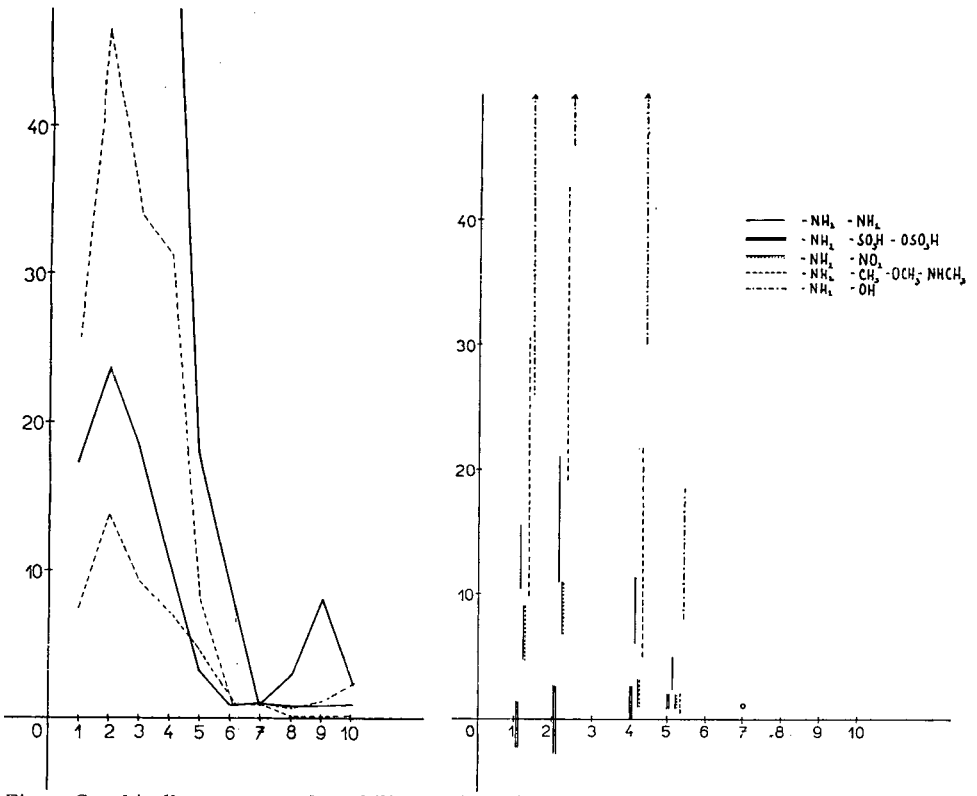


Fig. 7. Graphically represented mobility ratio u_x/u_7; for functional groups: —NH_2, —OH; migration region: — . Dashed line: benzene series, two curves with the lowest values belong to compounds having a hydrogen bridge.

Fig. 8. Summarized expression of the graphs from Figs. 3–7 for individual functional groups (mobility ratio u_x/u_7).

only their character becomes manifest. In our case we selected as a base the mobility at pH 7.2 (u_7), which we divided into the other mobility values (u_x/u_7). In this way we obtained values whose connecting lines are illustrated in Figs. 3–7. The curves in each of these figures belong to substances with the same type of functional groups. At the first glance we see that the course of this curve is characteristic for different functional groups, and thus it was possible to construct a graph (Fig. 8) in which the regions of the values of u_x/u_7 are delimited for various functional groups for which sufficient experimental material was available. By means of this graph it is thus possible to determine to a certain degree the type of the functional group on the aromatic nucleus (benzene, naphthalene, diphenyl). It is obvious that various positional interactions (such as internal hydrogen bridges etc.) may cause deviation from the given data. For example, a valuable finding is that, in this way, it is possible to ascertain the presence of CH_3– and –$NHCH_3$ groups. Even the position of such a group can be determined in many cases (u_x/u_7 is higher for substitution in the *p*-position). Other mobility ratios may, of course, also be utilized; however, this only leads, in some cases, to a clearer conception, and not to any new conclusions (Fig. 9).

Another characteristic feature of each compound is the direction of its migration in all the buffers employed, whether it proceeds to the anode or to the catode, or, in the case of ampholytes, to both. For the last type of substance mentioned, the shape of the electrophoretic spectra allows the very easy determination of the position of the isoelectric point, which is likewise characteristic for each compound.

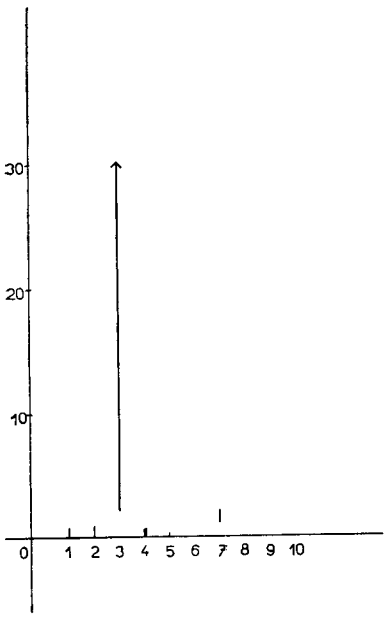


Fig. 9. Expression of mobility ratio u_{10}/u_1 for the functional groups: (1) —NH_2, —NH_2; (2) —NH_2, —CH_3, —OCH_3, —$NHCH_3$; (3) —NH_2, —SO_3H, —OSO_3H; (4) —NH_2, —NO_2; (5) —NH_2, —OH; (6) —COOH, —COOH; (7) —COOH, —NH_2, —OH; (8) —NO_2, —COOH; (9) —NO_2, 2-COOH; (10) —NH_2, —Cl.

The method described can also serve as an aid for determining in which buffer given compounds may be separated in the best way[15].

In general it may be stated that this method is a suitable supplement to other identification procedures.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. E. BOYLAND, D.Sc., Royal Cancer Hospital, London, Great Britain, Dr. CLAYSON, University of Leeds, Great Britain, and Dr. G. B. PLISS, Institute of Oncology, Leningrad, USSR, for some compounds and valuable information.

SUMMARY

The relationship between electrophoretic migration on paper and the type, number and position of the functional groups of a series of aromatic compounds was

investigated. The compounds were mainly diphenyl derivatives, which for the greater part are strongly carcinogenic substances and whose identification in biological material causes certain difficulties.

Application of the so-called "electrophoretic spectra" offers the possibility of more detailed identification of these substances.

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STUDIES ON THE HETEROGENEITY OF HEMOGLOBIN

VIII. CHROMATOGRAPHIC AND ELECTROPHORETIC INVESTIGATIONS OF VARIOUS MINOR HEMOGLOBIN FRACTIONS PRESENT IN NORMAL AND *IN VITRO* MODIFIED RED BLOOD CELL HEMOLYSATES

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(Received August 17th, 1964)

SCHROEDER *et al.*[1-3] have demonstrated the presence of three to five fast moving minor human hemoglobin components in red blood cell hemolysates of normal healthy adults using a specific Amberlite IRC-50 column chromatographic procedure. These hemoglobins were designated as Hb-A_{Ia}, Hb-A_{Ib}, Hb-A_{Ic}, Hb-A_{Id} and Hb-A_{Ie} respectively according to the order of elution. These investigators obtained a reasonably complete and rather consistent separation of the first three hemoglobin fractions, while the Hb-A_{Id} and/or Hb-A_{Ie} were less frequently observed in normal erythrocytic hemolysates. In the preceding paper of this series[4] the authors reported the results of the quantitative analyses of these minor hemoglobin components in red blood cells of patients with various hematological diseases using the same Amberlite IRC-50 chromatographic procedure. Surprisingly, only the fractions A_{Ia}, A_{Ib} and A_{Ic} were demonstrated. The quantities of these fractions often varied from the normal, the pattern being dependent upon the disorder studied. These results led to the studies to be reported in this paper dealing with the possibly increased *in vitro* formation of any of these hemoglobin fractions under various experimental conditions, while the electrophoretic behavior of the isolated components was also investigated.

METHODS

Amberlite IRC-50 chromatography was performed using the method described by SCHROEDER *et al.*[1-3] and described in more detail in the preceding communication[4]. Developer No. 5 was used as eluting buffer in all column chromatographic experiments. The isolated components were concentrated in the cold after a five-fold dilution with distilled water using short columns of carboxymethylcellulose (CMC) following the procedure described previously[5]. The hemoglobin components from the CMC columns were eluted with a small volume of a Tris–EDTA–borate buffer, pH 8.1, used in starch gel electrophoresis[6]. The components were subjected to starch gel electrophoresis following the technique described by SMITHIES and modified for hemoglobin electrophoresis[6]. Because of the rather low concentration of some of the isolated and concentrated hemoglobin fractions it was found advanta-

geous to stain the gels for a prolonged time with the *o*-dianisidine stain[6]. It is noteworthy that the components A_{Ia} and A_{Ib} were separated in many instances to such an extent that they could be analyzed separately; when quantitative data were desired the two minor fractions were considered together as was done in the preceding paper[4].

The effect of incubation upon the quantities of the components A_{Ia}, A_{Ib} and A_{Ic} and of hemoglobin fractions with different chromatographic mobilities was studied by incubating either red blood cell hemolysates or sterile blood samples. The hemolysates were incubated without further additions or after adding oxidized glutathione (GSSG) or reduced glutathione (GSH). In most experiments the hemoglobin was present as oxy-hemoglobin, while monocarboxy(CO)-hemoglobin was also occasionally used. The incubation was carried out in a tonometer with 100 % O_2, air or nitrogen at 37° for a specific period ranging from 16 to 48 h. The samples were finally dialyzed separately against a phosphate buffer (developer No. 5) prior to Amberlite IRC-50 chromatography. Blood samples from the two authors were collected in Vacutainer tubes*, containing EDTA as anticoagulant. The sterile blood samples were incubated at 37° for 24 h, at room temperature for 7 days and at 4° for 18, 28 and 49 days. At the end of these incubation periods the blood samples were centrifuged, the red blood cells washed three times with 0.9 g % NaCl solutions and lysed with distilled water and 0.4 volume of toluene. The types and quantities of the minor hemoglobin components, separated by Amberlite IRC-50 chromatography, were compared with those found for freshly prepared red blood cell hemolysates of the same donors.

Possible differences in the types and quantities of the minor hemoglobin components in old and young red blood cells were studied by analyzing hemolysates of "top" red cells and "bottom" red cells, separated by fractional centrifugation. Whole blood samples were placed in plastic tubing and centrifuged for 60 min at 2,000 r.p.m. The packed red cell segments were separated by clamping with surgical hemostats dividing the sample into a top segment and a bottom segment, each composing about 25 % of the total red blood cell column. The middle segment was discarded. Blood samples obtained from the two authors and from three patients with hemolytic anemia, described in the preceding paper[4], were studied. When appropriate, reticulocyte counts were made on the top and bottom fractions using one of the conventional laboratory procedures.

RESULTS

Fig. 1. presents tracings of the starch gel electrophoretic patterns of the three minor hemoglobin components which were isolated by Amberlite IRC-50 chromatography from normal red blood cell hemolysates. The heterogeneity of these fractions was surprising. The Hb-A_{Ic} was composed of one major fraction with an electrophoretic mobility slightly faster than that of the major adult hemoglobin fraction, Hb-A_{II}, while on several occasions a minor fraction with an electrophoretic mobility similar to that of Hb-F_{II} of cord blood red cell hemolysates was observed. Two fast moving fractions were observed in Hb-A_{Ia} which were well separated. The slowest

* Registered Trademark of Becton, Dickinson and Company, Rutherford, N.J.

of the two appeared to be the major fraction although occasionally the fast component was present in relatively large amounts. Heterogeneity was also observed for Hb-A_{Ib}; a minute amount of a hemoglobin fraction, which was slightly faster than

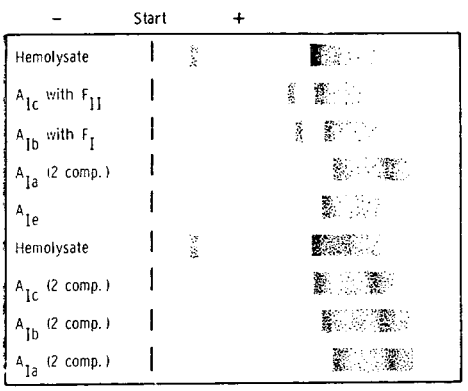

Fig. 1. Tracing of the starch gel electrophoretic patterns of several minor hemoglobin components isolated by Amberlite IRC-50 chromatography.

Hb-F_{II} and definitely slower than Hb-A_{II} in electrophoresis, was demonstrable in a few instances. All isolated minor fractions showed consistently fast moving "tails" suggesting further heterogeneity, which may be due to the handling of the isolated components.

As was mentioned in the preceding paper[4], the percentages of the minor fractions Hb-A_{Ia+b} and Hb-A_{Ic}, present in freshly prepared hemolysates of normal adult erythrocytes, were found to be rather constant, namely Hb-A_{Ia+b} 1.3–2.7 % and Hb-A_{Ic} 4.3–6.6 % (range of 14 cases). The amounts demonstrable in old and young red blood cells obtained from two normal healthy adults and three patients with hemolytic anemia, are presented in Table I. In three of the five blood samples (cases A, B and III) the percentages of the minor fractions in the top and bottom layers were essentially identical. In Case I the percentage of Hb-A_{Ic} was slightly

TABLE I

THE PERCENTAGES OF THE MINOR HEMOGLOBIN FRACTIONS IN HEMOLYSATES OF OLD AND OF YOUNG RED BLOOD CELLS, AS DETERMINED BY AMBERLITE IRC-50 CHROMATOGRAPHY

		Hb-A_{Ia+b}	*Hb-A_{Ic}*	*Reticulocytes* (%)
Normal A	Top	2.2	5.1	—
	Bottom	2.4	5.2	—
Normal B	Top	2.8	5.2	—
	Bottom	2.4	4.9	—
Patient I	Top	1.1	2.1	48.5
	Bottom	1.0	1.3	20.9
Patient II	Top	3.0	4.3	3.3
	Bottom	3.8	7.6	2.0
Patient III	Top	1.9	3.7	1.9
	Bottom	1.7	3.5	0.7

higher in young cells than in the old cell layer, while in Case II Hb-A_{Ia+b} and Hb-A_{Ic} were present in smaller percentages in the top layer than in the bottom layer. It is noteworthy that no other minor hemoglobin fractions were observed in any of these hemolysates.

The effects of incubation at 37° of normal red blood cell hemolysates on the percentages of the various minor hemoglobin components are presented in the Figs. 2 and 3. An additional component, presumed to be Hb-A_{Ie}, was formed during incubation. This component was eluted as a broad band behind the Hb-A_{Ic} component (Fig. 2). The percentages produced were largely dependent on the duration of the incubation; as much as 11.5 % was present after a 48 h incubation period (Fig. 3).

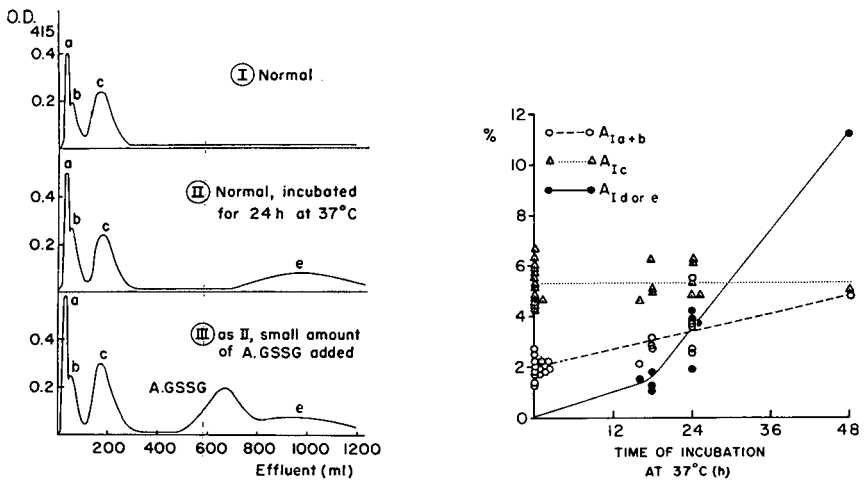

Fig. 2. Amberlite IRC-50 chromatograms of the fast moving minor hemoglobin components of adult red cell hemolysates under different experimental conditions, using developer No. 5.

Fig. 3. The effect of incubation of hemolysates of normal erythrocytes on the quantities of the fast moving minor hemoglobin components.

No significant change in the percentages of Hb-A_{Ic} was observed, while a definite increase in the amounts of Hb-A_{Ia+b} could be demonstrated (Fig. 3). The results of incubation experiments of whole blood samples at different temperatures and for different time periods are summarized in Table II. It should be noted that the chromatographic separation of the minor fractions under these conditions was for unknown reason inferior to that of freshly prepared hemolysates. Nevertheless, the presence of notable quantities of Hb-A_{Ie} was demonstrable after prolonged incubation at 25° and at 37°, while the quantities of Hb-A_{Ia+b} were also significantly elevated. Storage of a sterile blood sample for 4 to 7 weeks at 4° seemed to form less of Hb-A_{Ie} than shorter incubation at a higher temperature.

As has been described before[7], incubation of red blood cell hemolysates with oxidized glutathione (GSSG) results in the formation of a hemoglobin-glutathione complex (Hb-A·GSSG) in which each of the two active sulfhydryl groups, one in each β-chain, binds a glutathione residue. This mixed disulfide shows distinct electrophoretic and chromatographic properties and can therefore easily be separated

TABLE II

THE EFFECT OF PROLONGED INCUBATION OF WHOLE BLOOD SAMPLES ON THE PERCENTAGES OF THE MINOR HEMOGLOBIN COMPONENTS, DEMONSTRABLE BY AMBERLITE IRC-50 CHROMATOGRAPHY

Subject	*Days of incubation*	*Temp. (°C)*	*Hb-A_{Ia+b}*	*Hb-A_{Ic}*	*Hb-A_{Ie}*
A	0		2.3	5.1	—
A	1	37	3.0	6.8	4.1
A	7	25	4.8	5.1	4.0
A	18	4	2.3	6.0	<0.5
A	49	4	4.9	6.1	<0.5
B	0		1.8	4.5	—
B	1	37	2.6	5.0	0.6
B	7	25	4.9	5.7	8.2
B	28	4	2.7	5.3	<0.5

from the major normal hemoglobin fraction. The possibility that an increased formation of such a complex in aged red blood cells and in old red cell hemolysates does occur has been suggested[7]. The behavior of the Hb-A·GSSG complex in Amberlite IRC-50 chromatography was studied by separating the various minor hemoglobin fractions from a mixture composed of 95 % of a freshly prepared hemolysate, being incubated at 37° for 24 h, and 5 % of the same hemolysate after incubation with GSSG at 37° for 24 h in an oxygen atmosphere. An example of the results obtained is presented in chromatogram III of Fig. 2. The chromatographic mobility of the Hb-A·GSSG complex was found to be distinctly different from that of any of the other minor hemoglobin fractions in that it was eluted between Hb-A_{Ic} and Hb-A_{Ie}. A chromatographic separation of the Hb-A·GSSG complex and the minor hemoglobin fractions A_{Ic} and A_{Ie} was not possible when the total quantity of the hemolysate was incubated with GSSG as the large quantity of Hb-A·GSSG, present in such hemolysates, overlapped these two minor fractions almost entirely.

Freshly prepared hemolysates, either as oxy-hemoglobin or as monocarboxy (CO)-hemoglobin, have also been chromatographed after incubation with GSH and with GSSG. Some of the results obtained are tabulated in Table III. Incubation with

TABLE III

THE EFFECT OF INCUBATION OF RED BLOOD CELL HEMOLYSATES WITH GSSG OR GSH ON THE PERCENTAGES OF THE MINOR HEMOGLOBIN FRACTIONS

Hb-type	*Incubation period (h)*	*Component added**	*Atmosphere*	*A_{Ia+b}*	*A_{Ic}*	*A_{Ie}*	*A·GSSG*
CO-Hb	18	None	100% N_2	2.7	6.2	1.3	0
CO-Hb	18	GSH	100% N_2	7.2	6.2	1.6	0
CO-Hb	18	None	100% N_2	2.6	7.4	1.5	0
CO-Hb	18	GSSG	100% N_2	6.2	6.9	—	25.6**
Oxy-Hb	24	None	Air	2.1	4.6	1.5	0
Oxy-Hb	24	GSH	Air	10.7	5.7	—	51.9**

* Molar ratio Hb: GSH (or GSSG) = 1:20. These incubation experiments were carried out in a tonometer of 100 ml capacity at 37°.

** Hb-A_{Ie} and Hb-A·GSSG were not separable due to the excessive amounts of the A·GSSG component.

GSH in a nitrogen atmosphere resulted in a 2 to 3 fold increase of the Hb-A_{Ia+b} components while the formation of Hb-A_{Ic} was not prevented. No Hb-A GSSG was detectable. A similar increase in the Hb-A_{Ia+b} fractions was observed when the hemolysates (either as oxy-hemoglobin or as CO-hemoglobin) were incubated with GSSG and when the hemolysate (as oxy-hemoglobin) was incubated with GSH in an air atmosphere. Excessive amounts of the Hb-A·GSSG component were demonstrable in each of these experiments; the formation of the mixed disulfide in the hemolysate when exposed to GSH in an atmosphere of air can likely be explained by the formation of a notable quantity of oxidized glutathione during the incubation period.

DISCUSSION

Heterogeneity of the hemoglobin of normal adult human red blood cell hemolysates has been demonstrated by electrophoretic procedures[8,9] and by different chromatographic techniques[1,5,10]. Because of somewhat conflicting results obtained with these different procedures it is at present not possible to state the exact number of hemoglobin fractions with reasonable certainty. Moreover the number seems to increase with the age of either blood sample or red cell hemolysate. From the evidence obtained so far, it can be concluded that at least five hemoglobin fractions have been recognized in a freshly prepared normal adult red blood cell hemolysate in addition to the major Hb-A fraction and the Hb-A_2 component. These components are: Hb-A_{Ia}, Hb-A_{Ib}, Hb-A_{Ic}, Hb-F_I and Hb-F_{II} and can be distinguished from each other by their behavior in Amberlite IRC-50 chromatography and/or by their electrophoretic mobility after chromatographic isolation. Indication has also been obtained that some of these fractions, notably the hemoglobins A_{Ia} and A_{Ib}, are heterogeneous in electrophoresis (Fig. 1), but these results are certainly not unequivocal. It seems important at this point to emphasize that positive identification of all these minor fractions has not been made. Although the complete structures of F_I and F_{II}, as found in cord blood samples, are known[11,12], no universal agreement exists that the same components are also present in small quantities in adult red blood cell hemolysates[13]. Many studies are, however, strongly indicative in this respect[6,14–16]. The studies reported in this paper also demonstrate the presence of small quantities of hemoglobin components with electrophoretic and chromatographic mobilities identical to those of F_I and F_{II} in the isolated Amberlite IRC-50 fractions A_{Ib} and A_{Ic}. The amino acid composition of Hb-A_{Ic} was found to be identical to that of Hb-A[17] while recent studies by HOLMQUIST AND SCHROEDER[18] have offered evidence that the N-terminal amino acid of one of the two β-chains is conjugated with an unknown component.

In addition to these five minor components there seems to exist an additional component that behaves as a chromatographically fast moving fraction in CMC chromatography, while it is not separable from the major hemoglobin fraction in Amberlite IRC-50 chromatography. The evidence of this is derived from two sources. It has been observed that the fast hemoglobin component obtained by CMC chromatography comprises about 12 % of the total, while the fast moving hemoglobin components in Amberlite IRC-50 chromatography comprise only about 7.5 % of the total. Moreover, when the fast moving hemoglobin components isolated by CMC chromatography (eliminating the overlap between the fast components and the major component) were rechromatographed on Amberlite IRC-50 approximately 40 %

showed a mobility similar to that of the major normal hemoglobin fraction, Hb-A_{II} and Hb-A_{III} (unpublished data).

The question also arises as to how many of these components may be artificially produced during the process of lysing the red blood cells. A definite answer cannot be given; the relatively small deviations in the amounts of the different fractions, as they are present in red cell hemolysates obtained from different normal individuals, certainly points away from this possibility. Artifacts certainly are produced during the *in vitro* aging process of the red blood cells and of hemolysates. Two additional components (Hb-A_{Id} and Hb-A_{Ie}) can be recognized. The Hb-A_{Id} fraction is probably identical to the Hb-A·glutathione complex, which can easily be produced by *in vitro* incubation of a hemolysate with oxidized glutathione. The incubation experiments with oxidized and reduced glutathione reported in this paper would point away from the possibility that normally occurring minor hemoglobin components are chiefly due to the attachment of glutathione residues to the major component because the chromatographic mobility of Hb-A·GSSG is different from any of these components of or the components arising on incubation of 37° (except Hb-A_{Id}). The increase in components Hb-A_{Ia+b} observed during incubation might very well be due to attachment of either GSH or GSSG to some hemoglobin component as an increase in Hb-A_{Ia+b} was noted during the incubation of hemolysates with either component.

The effect of *in vitro* aging of red blood cells and of hemolysates can mainly be measured by the formation of the Hb-A_{Ie} fraction and the increase of the Hb-A_{Ia+b} components. When the influence of incubation of blood at 4° for 49 days is compared with that observed after incubation for 1 day at 37° it seems likely that the Hb-A_{Ie} formation is primarily influenced by an increase in temperature while the increase in the Hb-A_{Ia+b} components is more dependent of the time of incubation. Hb-A_{Ie} has never been observed by us in freshly prepared red blood cell hemolysates. *In vivo* aging of red blood cells does not result in the formation of additional minor hemoglobin fractions nor in changes in the quantities of the normally occurring minor hemoglobin components, as demonstrable by Amberlite IRC-50 chromatography.

ACKNOWLEDGEMENT

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SUMMARY

The results of studies into the nature of the minor hemoglobin components of normal red blood cell hemolysates as obtained by Amberlite IRC-50 chromatography have been reported.

All three minor hemoglobin fractions (A_{Ia}, A_{Ib} and A_{Ic}) were found to be inhomogeneous in starch gel electrophoresis. A_{Ia} was composed of two fast moving components. The fractions A_{Ib} and A_{Ic} consisted of fast moving hemoglobin components with specific electrophoretic mobilities, while at times components with the electrophoretic mobilities of Hb-F_I and Hb-F_{II}, respectively, were also present. No additional minor hemoglobin fractions were found in freshly prepared hemoly-

sates. No significant differences between the quantities of the A_{Ia}, A_{Ib} and A_{Ic} fractions in old and young red cells could be demonstrated.

Incubation of either hemolysates or sterile blood samples at 37° produced notable amounts of an additional fraction (A_{Ie}), while the quantities of the fractions A_{Ia+b} were also increased. The formation of the A_{Ie} fraction was primarily temperature dependent while the increase in the amounts of A_{Ia+b} was mainly dependent upon the time of incubation. Incubation of hemolysates with oxidized glutathione produced a component with a unique chromatographic mobility; this component is probably identical with the A_{Id} component, occasionally seen in aged red cell hemolysates. Incubation of hemolysates with either oxidized or reduced glutathione also increased the amount of the A_{Ia+b} fractions, while no change in the amounts of A_{Ie} was observed.

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REVERSED-PHASE CHROMATOGRAPHY OF Al, Ga, In, Tl AND THE TRANSITION METALS OF THE IRON GROUP ON PAPER TREATED WITH DI-(2-ETHYLHEXYL) ORTHOPHOSPHORIC ACID IN CHLORIDE MEDIUM

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INTRODUCTION

In a previous paper[1] the possibility of a good separation of Al, Ga, In and Tl was demonstrated by means of reversed-phase paper chromatography. Such a separation was based on the use of paper treated with solutions of di-(2-ethylhexyl) orthophosphoric acid (HDEHP) and elution with hydrochloric acid.

Similar chromatographic techniques have already been applied to the separation of rare earths[2], alkali metals and alkaline earths[3,4].

All the chromatographic investigations performed so far have shown that valuable information can be obtained on the extraction behaviour of the elements with respect to the extractant used: in this case HDEHP. Therefore it appeared that some hypotheses could be made on the chemical interaction between the various components of the system, namely the metallic ion, the extractant molecule and the aqueous solution of the anion.

To continue this investigation and to get a more detailed picture of the various phenomena, a systematic study carried out with the elements aluminium, gallium, indium, thallium, iron, cobalt, and nickel is described below. Criteria for evaluation of results were almost the same as in previous publications[3,4]; some special consideration was given to aluminium, and occasionally to iron, because of their peculiar behaviour toward HDEHP.

As a consequence of the systematic study some theoretical conclusions were obtained and, at the same time, interesting separations of such elements from one another and from additional ones, such as barium, calcium, titanium, strontium, manganese, yttrium, zirconium and uranium, were carried out.

EXPERIMENTAL

Reagents and equipment

Di-(2-ethylhexyl) orthophosphoric acid (HDEHP) was a Virginia-Carolina Chemical Co. (Richmond, U.S.A.) product supplied by Soc. Eigenmann and Veronelli (Milan).

Reagents were all analytical grade: 0.1 *N* stock solutions of each element were prepared and then diluted to 0.005 *N* to obtain solutions for spotting. The Al, Ga, In

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and Tl stock solutions were obtained as described in a previous paper[1]. The stock solutions of Fe, Co and Ni were obtained by dissolving the respective chlorides (RP grade, Carlo Erba, Milan) and kept slightly acidic to avoid hydrolysis.

Chromatographic paper Whatman No. 1 (CRL/1 type) was treated with cyclohexane solutions of HDEHP which had been previously equilibrated with 2.5 *M* HCl, in the usual manner[2].

The equipment used in this work has already been described[3]. Spots of about 0.05 ml each were applied to the paper according to the standard procedure. After development with HCl, iron and cobalt were detected on the paper by spraying with a saturated aqueous solutions of KCNS, and nickel with a 5 % (w/v) dimethylglyoxime solution in ethanol. Aluminium and indium were detected with a 0.1 % alcoholic morin solution, and gallium and thallium with a similar quercetin solution[1].

Experiments were generally performed with HDEHP as supplied, but some of them were also carried out with HDEHP purified by a method derived from that described by STEWART AND CRANDALL[5]. Elution of various cations on paper treated with this purified HDEHP gave the same R_F values as those on paper treated with unpurified HDEHP.

RESULTS AND DISCUSSION

General

In Table I, experimental R_F values for the metal ions are collected as functions of the molarity of hydrochloric acid used as eluent, the papers being treated with five concentrations of HDEHP solution in cyclohexane, *viz.* 0.010 *M*, 0.025 *M*, 0.050 *M*, 0.075 *M*, and 0.0100 *M*. In this table values in parentheses refer to tailing spots.

In Fig. 1 R_F values are plotted against the logarithm of the hydrochloric acid concentration. The experiments were carried out over a range of acidity for HCl from 10 *M* to $1 \cdot 10^{-4}$ *M*, but the lowest concentrations were neglected in drawing Fig. 1 since the relative results showed no interesting features. A decrease of R_F value for some of the elements can be seen from Fig. 1, *e.g.* gallium, when very concentrated hydrochloric acid is used as the eluent; this behaviour, reported also in the literature on liquid–liquid extraction and ion-exchange experiments, is very similar to that of the alkaline earths with both hydrochloric and acetic acid[3,4]. It deserves special consideration and will be discussed in a future paper. Our present discussion will deal essentially with the behaviour of cations in the ranges of concentration of eluent in which their R_F values increase with increasing acidity.

When the HCl concentration was between 5.5 and 6.5 *M*, gallium showed two spots, one of which was with the front of the eluent, and the other corresponding to the value reported in Table II and in the figure; this phenomenon will also be discussed in the future.

As already mentioned[2], the R_F values for a given metallic ion on paper treated with a liquid extractant and eluted with an aqueous solution can be related to the extraction coefficient E_a° of the same ion in the analogous liquid–liquid system through the relationship:

$$\log \left(\frac{1}{R_F} - 1 \right) = \log E_a^\circ + \log k \qquad (1)$$

TABLE I

R_F VALUES FOR ALUMINIUM, GALLIUM, INDIUM, THALLIUM AND THE TRANSITION METALS OF THE IRON GROUP, AS FUNCTIONS OF THE MOLARITY OF THE HCl ELUENT

Paper treated with HDEHP. Operating temperature 23 ± 1°.

HDEHP molarity	*Cations*	R_F									
		HCl molarity									
		10	*9*	*7*	*6*	*3*	*1*	*0.5*	*0.3*	*0.2*	*0.1*
0.010	Al^{3+}	0.88	0.91	(0.83)	(0.66)	(0.47)	0.00	0.00	0.00	0.00	—
	Ga^{3+}	0.46	0.47	0.56	0.63	0.90	0.94	0.83	0.75	0.54	0.22
	In^{3+}	0.86	0.86	0.84	—	—	0.65	0.25	0.03	0.00	—
	Tl^{3+}	0.71	0.73	—	0.79	0.78	0.82	—	0.80	—	0.82
	Fe^{3+}	0.28	0.43	0.69	0.79	0.63	0.00	0.00	0.00	0.00	—
	Co^{2+}	0.86	—	0.88	0.88	0.89	0.91	—	0.92	0.92	0.91
	Ni^{2+}	0.85	—	0.88	0.89	0.90	0.92	—	—	0.93	0.92
0.025	Al^{3+}	0.90	(0.86)	0.87	(0.80)	(0.40)	0.00	—	—	—	0.00
	Ga^{3+}	0.16	0.20	0.29	0.51	0.90	0.89	0.81	0.62	0.38	0.15
	In^{3+}	0.88	0.89	0.86	0.84	0.82	0.61	0.18	0.00	0.00	0.00
	Tl^{3+}	0.38	0.44	0.57	0.66	0.75	0.80	0.80	—	0.82	0.82
	Fe^{3+}	0.02	0.08	0.40	0.75	0.40	0.00	—	0.00	0.00	—
	Co^{2+}	0.78	0.79	0.86	0.86	0.93	0.93	—	0.92	0.93	0.93
	Ni^{2+}	0.82	0.84	0.86	0.86	0.93	0.93	—	0.92	—	0.94
0.050	Al^{3+}	0.90	(0.85)	(0.73)	(0.74)	(0.32)	0.00	0.00	0.00	—	0.00
	Ga^{3+}	0.10	0.12	0.20	0.26	0.90	0.91	0.73	0.52	—	0.08
	In^{3+}	0.88	0.85	0.84	0.85	0.82	0.54	0.10	0.00	—	0.00
	Tl^{3+}	0.22	0.30	0.50	0.57	0.77	0.81	—	0.80	—	—
	Fe^{3+}	0.00	0.05	0.40	0.72	0.32	0.00	0.00	—	—	0.00
	Co^{2+}	0.86	0.81	0.87	0.85	0.90	0.88	0.90	0.89	—	0.90
	Ni^{2+}	0.85	0.81	0.87	0.86	0.87	0.90	0.92	0.91	—	0.91
0.075	Al^{3+}	0.90	0.84	(0.78)	0.64	(0.20)	0.00	0.00	0.00	—	0.00
	Ga^{3+}	0.00	0.06	0.12	0.26	0.86	0.86	0.65	0.33	0.19	0.05
	In^{3+}	0.83	0.86	0.84	0.83	0.82	0.46	0.07	0.00	0.00	0.00
	Tl^{3+}	0.09	0.16	0.39	0.47	0.70	0.74	0.76	0.76	0.75	0.76
	Fe^{3+}	0.00	0.04	0.41	0.70	0.19	0.00	0.00	0.00	0.00	0.00
	Co^{2+}	0.77	0.78	0.87	0.85	0.88	0.90	0.89	0.89	0.90	0.89
	Ni^{2+}	0.81	0.82	0.87	0.86	0.88	0.90	—	—	0.90	—
0.100	Al^{3+}	0.86	0.84	(0.62)	(0.54)	0.09	0.00	0.00	0.00	0.00	0.00
	Ga^{3+}	0.00	0.00	0.02	0.22	—	0.90	0.58	0.32	0.18	0.00
	In^{3+}	0.87	0.90	0.85	0.86	0.80	0.35	0.04	0.00	0.00	0.00
	Tl^{3+}	0.10	0.12	0.35	0.53	—	0.80	0.77	—	0.77	0.81
	Fe^{3+}	0.00	0.04	0.40	0.75	0.16	0.00	0.00	0.00	0.00	0.00
	Co^{2+}	0.84	0.79	0.86	0.90	0.88	0.94	0.94	0.94	0.95	0.94
	Ni^{2+}	0.85	0.82	0.88	0.90	0.88	0.94	0.95	0.95	0.94	0.95

where k is a constant which depends on the experimental conditions. Further, the extraction coefficient E_a° may be related to the activities of the chemical species involved in the reaction postulated as the extraction mechanism through an equilibrium constant. As already pointed out[4], the equilibrium generally accepted as representing the extraction mechanism with HDEHP may be written as:

$$M^{b+} + c(HDEHP)_2 \rightleftharpoons M(DEHP)_b \cdot b(HDEHP) \cdot (c - b)\,(HDEHP)_2 + bH^+ \cdot$$

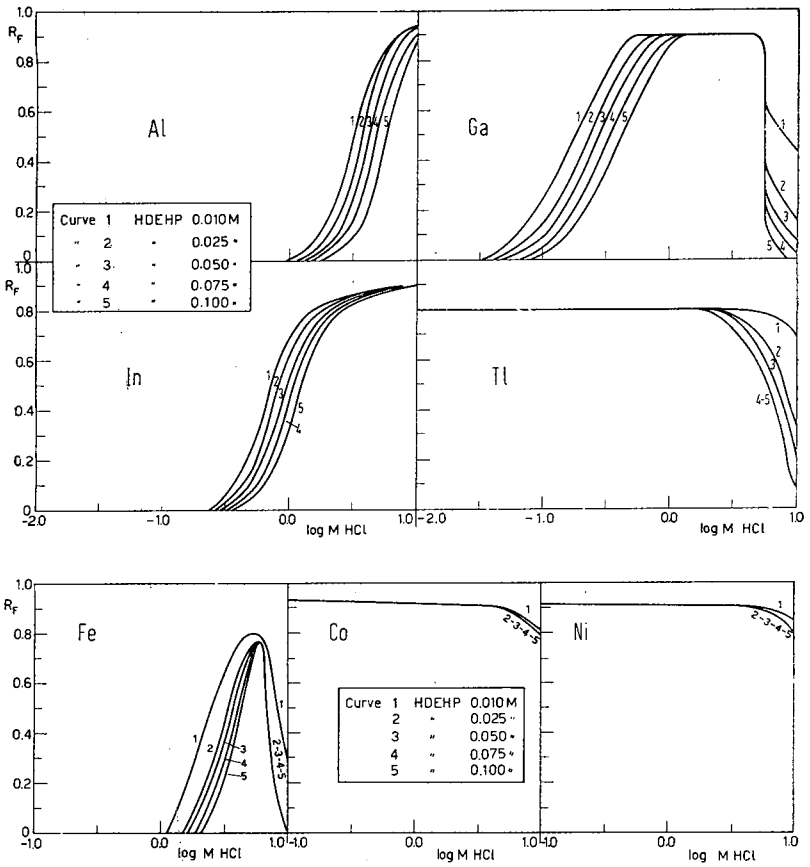

Fig. 1. R_F values of aluminium, gallium, indium, thallium and the transition metals of the iron group plotted *vs.* log *M* HCl. Paper treated with HDEHP.

From the equilibrium constant K of that reaction and from Eqn. (1), the following relationship may be obtained:

$$\log\left(\frac{1}{R_F} - 1\right) = c \log [\text{HDEHP}]_{\text{eff}} - b \log a_{\text{H}^+} + \text{const.} \qquad (2)$$

where $[\text{HDEHP}]_{\text{eff}}$ is the effective HDEHP concentration on the paper[3,4], a_{H^+} is the activity of the hydrogen ion and "const." stands for $\log K + \log k$. In Eqn. 2 the activity a_{H^+} can be used for the hydrogen ion concentration since solutions of concentrated hydrochloric acid are involved. The hydrogen ion activities were calculated from the H^+ molarities by taking as activity coefficients the mean activity coefficients for hydrochloric acid (at the corresponding molalities) as reported by ROBINSON AND STOKES[6].

For a given element, plots of $\log (1/R_F - 1)$ against either $\log [\text{HDEHP}]_{\text{eff}}$ or $\log a_{\text{H}^+}$ yield the values of the coefficients c and b respectively, which appear in Eqn. 2, thus giving information on the extraction mechanism.

TABLE II

R_F VALUES FOR GALLIUM, INDIUM AND IRON ELUTED WITH HCl, AS FUNCTIONS OF THE HDEHP CONCENTRATION IN THE SOLUTION USED TO TREAT THE PAPER

Operating temperature 23 ± 1°.

HCl molarity	*Cation*	R_F							
		HDEHP molarity							
		0.010	*0.015*	*0.030*	*0.040*	*0.050*	*0.060*	*0.080*	*0.100*
0.2 *M*	Ga^{3+}	0.55	0.42	0.34	0.28	0.25	0.21	0.19	0.13
0.5 *M*	In^{3+}	0.26	0.24	0.18	0.15	0.14	0.09	0.10	0.09
2.3 *M*	Fe^{3+}	0.34	0.25	0.18	0.12	0.11	0.09	—	—

To obtain additional data on the role of HDEHP in this mechanism, simultaneous elutions were carried out with paper treated with HDEHP at eight different concentrations. The experimental R_F values for gallium, indium and iron are collected in Table II.

Aluminium, gallium, indium and thallium

Figs. 2, 3 and 4 show the plots of log $(1/R_F - 1)$ against the logarithm of the hydrogen ion activity in the eluting solution, for Al, Ga and In, respectively.

The plots of log $(1/R_F - 1)$ against log $k\,[\mathrm{HDEHP}]_{\mathrm{eff}}$[3,4] for these elements are shown in Fig. 5. The developing solutions were 4 *M* HCl for Al, 0.2 *M* for Ga and 0.5 *M* for In. For such plots the R_F values derived from Fig. 1 and Table II were used.

No data are reported for aluminium in Table II because this element generally gives rise to the formation of tailed spots. It is known from the literature[7] that in a liquid–liquid system aluminium is very slowly extracted by HDEHP and equilibrium

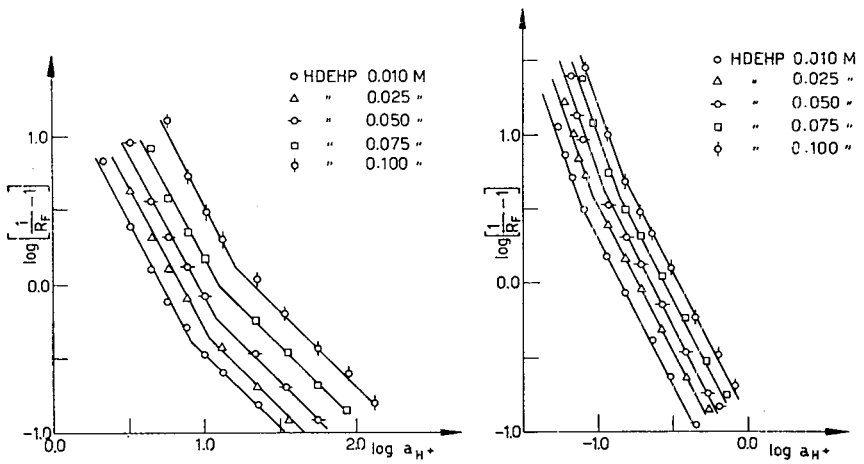

Fig. 2. Plot of log $(1/R_F - 1)$ *vs.* log a_{H^+} for aluminium. Paper treated with HDEHP at various concentrations. Slopes —2 and —1.

Fig. 3. Plot of log $(1/R_F - 1)$ *vs.* log a_{H^+} for gallium. Paper treated with HDEHP at various concentrations. Slopes —3 and —2.

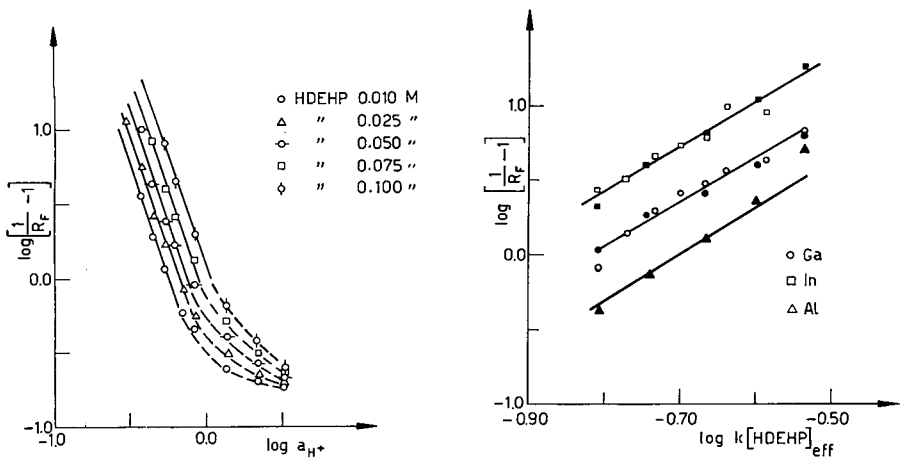

Fig. 4. Plot of log $(1/R_F - 1)$ *vs.* log a_{H^+} for indium. Paper treated with HDEHP at various concentrations. Slopes —3 and —3.

Fig. 5. Plot of log $(1/R_F - 1)$ *vs.* log $k[\text{HDEHP}]_{eff}$ for gallium, indium and aluminium eluted with 0.2 *M* HCl, 0.5 *M* HCl and 4 *M* HCl, respectively. Data from Table II (open symbols) and data from plots of Figs. 2 to 4 (closed symbols). Slope +3.

is only reached after a long contact time. This is responsible for irregular spots in reversed-phase chromatography with HCl up to 7 *M*; only after a very large number of experiments was it possible to obtain reliable mean R_F values for use in the curves reported in Fig. 1. For that reason results obtained for aluminium with simultaneous elutions of papers treated with various HDEHP concentrations were not reliable.

No plots were made for thallium, since, as can be seen in Fig. 1, the R_F values do not show any increase with the HCl concentration within the range investigated.

From the slopes of curves in Fig. 2 the value of 2 was calculated for coefficient *b* for aluminium up to a concentration of 4 *M* HCl ($\log a_{H^+} = -0.9$) which changes to 1 above that concentration. Gallium in Fig. 3 has a slope of —3 just up to about 0.1 *M* HCl ($\log a_{H^+} = -1.10$), which becomes —2 above that value. Similar behaviour is shown by indium (Fig. 4), which changes slope from —3 to —2 at about 1 *M* HCl.

From the considerations referred to above, such values which are derived from chromatographic data should agree with those from liquid–liquid extraction. Although few data are available in the literature on the extraction behaviour of these elements by HDEHP, a significant comparison can be made. KIMURA[8] reports a *b* value of 3 for liquid–liquid extraction of aluminium, gallium and indium by 50 % HDEHP–toluene from HCl solutions up to 1 *M*. In addition a nearly constant value of E_a° with the acidity was found for Tl^{3+}. Except for aluminium these results are consistent with those derived from chromatography, as reported above.

A further comparison can be made with respect to coefficient *c*, which correlates $\log E_a^\circ$, or $\log (1/R_F - 1)$, with log [HDEHP]. From the slope of the plots in Fig. 5 a value of 3 is obtained for aluminium, gallium and indium. Such a value agrees with that obtained by KIMURA[9] for indium, whilst a slope of 2 is reported for gallium by the same author. This result is probably due to the high HDEHP concentration used

for extraction. Data for aluminium and thallium are not reported. In HDEHP extraction of aluminium from sulphate solutions BLAKE *et al.*[7] have found $b = 3$ and $c = 3$.

It will be seen from the results given above that the value of coefficient b is often lower than 3, which is the ionic charge of the metallic species supposedly involved in the chemical reaction. Such a reduction of the value b can be explained by assuming that the adduct with the extractant is not formed by the bare cation. As already discussed in the case of the alkali metals and alkaline earths[3,4], the cation is likely to pass into the organic phase surrounded by a number of molecules of the anionic species which is present in the aqueous phase. This tendency is generally greater the higher the concentration of the anion, thus coefficient b differs from 3 when this concentration increases. Bonding of anions to the cation is very probable in the case of the three elements considered here, because they are known to form chloride complexes with various degrees of complexion in the range of the HCl concentration used in our experiments. If the chloride complex reaches the structure of an anion, as in the case of Tl^{3+}, which forms $TlCl_4^-$, even when the HCl concentration is lower than 0.1 M[10], the extraction by HDEHP, which under such conditions is mainly a cation exchanger, becomes very poor. Therefore the R_F values of that element would be consistently high even with dilute HCl. Actually a higher retention than was expected from these considerations was found, since an $R_F = 0.80$ instead of 1.00 resulted. But experiments performed with untreated paper showed that such an R_F value is solely due to the effect of the cellulose.

In addition to considering the behaviour of thallium, it can be concluded that aluminium, gallium and indium are retained by HDEHP on paper through a mechanism which involves the bare ion when the chloride concentration is low. At higher Cl^- concentrations the ionic species retained should be a complex of the cation with one chloride ion. In all cases, three dimeric molecules of HDEHP bind the cation, probably by means of a mechanism similar to that already suggested for the alkaline earths[4,11].

Iron, cobalt and nickel

As expected from literature data on liquid–liquid extraction[8], nickel and cobalt ran with the front and therefore no study on the retention mechanism was possible.

In Figs. 6 and 7 $\log (1/R_F - 1)$ for iron is plotted against $\log a_{H^+}$ and $\log k$ $[HDEHP]_{eff}$, respectively.

Iron, like aluminium, has a long equilibration time in liquid–liquid extraction with HDEHP[7]. Therefore iron, although to a less extent than with aluminium, had some tailing of the spots which affected the reproducibility of results. It was thought that because of this long equilibration time in extraction, the R_F values for iron would be affected by the elution speed. Thus, experiments were carried out with ascending chromatography by putting the drops to be eluted at different heights above the surface of the eluent solution, and developing until the front line reached the same distance from the point at which the spot had been applied. Chromatograms were obtained having the same front run and a longer elution time the farther the respective start line was from the liquid surface.

Results showed that among the seven elements considered in this work, only the R_F values of iron, and perhaps also of aluminium, are slightly dependent on

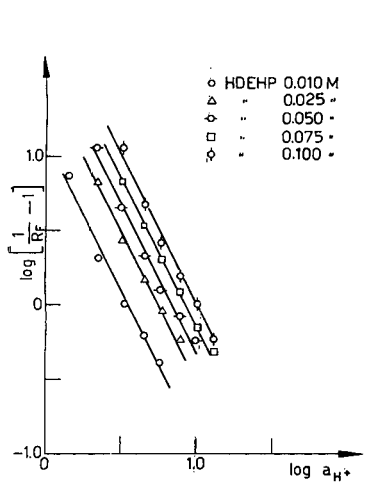

Fig. 6. Plot of log $(1/R_F - 1)$ *vs.* log a_{H^+} for iron. Paper treated with HDEHP at various concentrations. Slope −2.

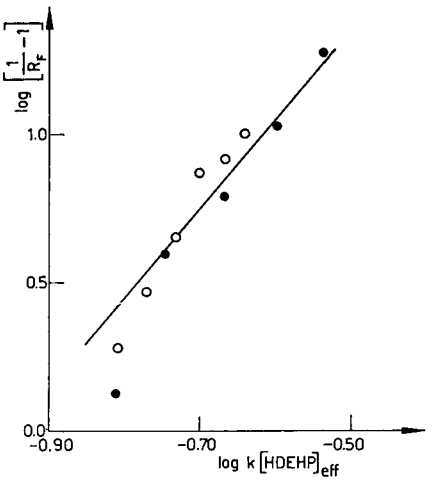

Fig. 7. Plot of log $(1/R_F - 1)$ *vs.* log $k[HDEHP]_{eff}$ for iron. Data from Table II (open symbols) and data from plots of Fig. 6 (closed symbols). Slope + 3.

elution time. Although these results show that equilibrium is not actually reached during development, we considered that the plots in Figs. 5 and 6 were in fact useful in revealing information on the extraction mechanism. In comparison, data reported by KIMURA[8] on the extraction of iron by HDEHP from chloride solutions show that although the extraction coefficients are lower the shorter the shaking time, their behaviour toward acid concentration is similar with any fixed shaking time.

From Figs. 6 and 7 the values of the reaction coefficients b and c appear to be 2 and 3, respectively. The value of 3 for coefficient c agrees with results reported by BAES AND BAKER[12] for liquid–liquid extraction of iron with HDEHP from perchlorate solutions. Extraction of iron by HDEHP from chloride solutions is reported bu KIMURA[8,9] to give $b = 3$ and $c = 2$, which is not in agreement with chromatographic data. This disagreement may be explained by assuming that the chromatography was carried out using higher HCl concentrations than were used in extraction experiments. A value of b lower than 3 should therefore result from the formation of chloride complexes such as was discussed for aluminium, gallium and indium. It should be noted that iron has a considerable tendency to form chloride complexes, and in the range of acidity considered might retain one chloride ion in the molecule of the adduct which extracts into the organic phase.

From Fig. 1 it can be seen that some elements show an increase of R_F with HCl molarity followed by an appreciable decrease when the HCl concentration becomes high. Although extensive discussion is deferred to a further publication, some observations are made here. The fact that the adsorption of those elements increases is not attributable to a cation exchange mechanism through the $DEHP^-$ radical, since such elements are supposed to be in the form of anionic chloride complexes, but rather to a sort of bonding with the oxygen atom of the P=O groups of the extractant. This bonding occurs normally with tributyl phosphate. Such a mechanism, which

takes place when the ion begins to dehydrate because of the very low activity of water in the solution, is responsible not only for the rapid decrease of R_F values of Ga^{3+} and Fe^{3+}, but is also responsible for the slow decrease of R_F in the case of In^{3+}, Co^{2+}, Ni^{2+} and of the alkaline earths.

Studies are being carried out to decide whether the peculiar decrease of R_F values of thallium is due to the same cause or to another one, as for example reduction of the ion from the trivalent state to the univalent one, which is complexed by chloride to a minor extent.

Application to chromatographic separations

From the experimental results described above, it appears that many interesting separations are feasible for aluminium, gallium, indium, thallium and the transition metals of the iron group. In addition some other ions, which were investigated in previous publications[3,4], can be considered. The complete separation of Al–Ga–In and Tl has already been published[1]. Other separations obtained are listed in Table III,

TABLE III

ASCENDING CHROMATOGRAMS OBTAINED WITH 3 × 40 CM PAPER STRIPS TREATED WITH HDEHP
Operating temperature 23 ± 1°.

No.	*Separation of*	*Run (cm)*	*HDEHP molarity*	*Eluent*	R_F
1	Be–Fe–Al–Sr	28.3	0.100	7.5 *M* HCl	Be = 0.00; Fe = 0.12; Al = 0.48; Sr = 0.85
2	Ti–Ga–Fe–Tl–U–Ba–Ca–Ni	29.2	0.050	8 *M* HCl	Ti = 0.00; Ga = 0.03; Fe = 0.10; Tl = 0.17; U = 0.34; Ba = 0.56; Ca = 0.76; Ni = 0.96
3	Zr–Fe–Y–In–Ni	33.1	0.025	2 *M* HCl	Zr = 0.00; Fe = 0.04; Y = 0.37; In = 0.83; Ni = 0.96
4	Ti–Fe–Tl–U–Ba–Sr	29.5	0.100	8 *M* HCl	Ti = 0.00; Fe = 0.09; Tl = 0.16; U = 0.22; Ba = 0.56; Sr = 0.64
5*	Ga–Fe–Ba–Mn	29.5	0.100	7 *M* HCl	Ga = 0.10; Fe = 0.41; Ba = 0.68; Mn = 0.98
6*	Y–Ga	31.5	0.100	1.5 *M* HCl	Y = 0.08; Ga = 0.92
7*	Ga–Y	29.8	0.100	7 *M* HCl	Ga = 0.05; Y = 0.84

* Operating temperature 28°.

together with the concentration of HDEHP in cyclohexane used to treat the paper and the hydrochloric acid concentration of the eluent. The length of the run to the solvent front of each chromatogram is also given. Strips 40 × 3 cm of Whatman No. 1 paper were used and were cut perpendicular to the machine direction. Paper was treated with HDEHP in the same way as CRL/1 sheets used in fundamental work. Diagrams of the separations quoted in Table III are reported in Fig. 8.

SUMMARY

Reversed-phase chromatography of aluminium, gallium, indium, thallium and the transition metals of the iron group on paper treated with di-(2-ethylhexyl) orthophosphoric acid (HDEHP) has been investigated, using hydrochloric acid as eluent in a range of concentrations from 10 *M* to $1 \cdot 10^{-4}$ *M*. The quantity $(1/R_F - 1)$

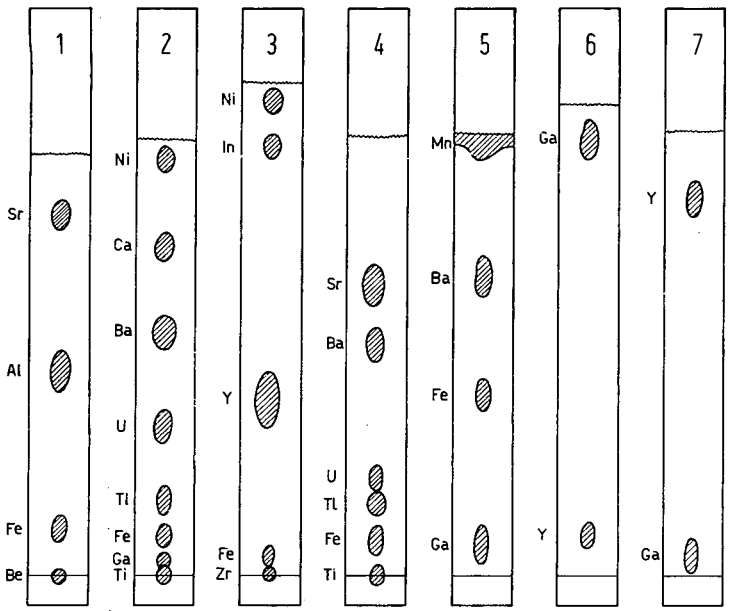

Fig. 8. Diagrams of the separations quoted in Table III.

has been related to the hydrogen ion activity in the mobile phase and to the effective HDEHP concentration on the paper.

The chromatographic behaviour has been correlated with that in liquid–liquid extraction systems. Chromatographic separations of the above elements from each other and from additional cations such as barium, calcium, titanium, strontium, manganese, yttrium, zirconium and uranium were performed.

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GEMISCHTLIGANDKOMPLEXE

IV. TRENNUNG STEREOISOMERER KOMPLEXIONEN DES Co(III) MIT HILFE IONOPHORETISCHER METHODEN

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In einer Arbeit über Trennung, Isolierung und Untersuchung von Gemischtligandkomplexen der Platinelemente mit Hilfe hochspannungsionophoretischer Methoden versuchten BLASIUS UND PREETZ[1-4] erstmalig, eventuell auftretende Stereoisomere papierionophoretisch zu isolieren. Die erzielten und durch spektrophotometrische Methoden ausgewerteten Ergebnisse liessen jedoch keine allgemeingültigen Aussagen zu.

Die vorliegende Arbeit behandelt speziell das Problem der ionophoretischen Trennung stereoisomerer Komplexionen, und zwar mit und ohne Trägermedium.

Um sichere und genaue Angaben machen zu können, wird von den reinen *cis*- bzw. *trans*-Verbindungen des Co(III) ausgegangen, die in der Literatur bekannt und schon rein hergestellt worden sind.

Die Gegenüberstellung von Papierionophorese und trägerfreier Ionophorese dient der Klärung der Frage nach dem Einfluss des Trägermediums auf die Beweglichkeitsunterschiede der stereoisomeren Komplexionen im elektrischen Feld.

Ionophoretische Trennungen von stereoisomeren Komplexionen sind, soweit uns bekannt, noch nicht beschrieben worden. Bis 1958 wurden ausschliesslich Methoden, die auf der unterschiedlichen Löslichkeit entsprechender Salze beruhen, zur Trennung herangezogen. Ab 1958 folgten einige chromatographische Arbeiten[5-8]. Bei der Papierchromatographie[5] lagen die R_F-Werte für die *cis*-Komplexionen immer höher als für die *trans*-Komplexionen. Mit Hilfe der Dünnschichtchromatographie[8] wurden die stereoisomeren Kationen $[Co\ en_2\ Cl_2]^+$ getrennt. Hier wanderte das *trans*-Kation schneller als das *cis*-Kation. Als wesentlich für die Trennung wurde die Polarität des Laufmittels und nicht die Löslichkeit der Verbindungen angesehen.

ARBEITSBEDINGUNGEN

Für die Hochspannungspapierionophorese diente eine diskontinuierlich arbeitende analytische Kammer[3] der Fa. Marggraf, Berlin.

Für die trägerfreie Ionophorese (Methode der wandernden Grenzflächen) wurde das in Fig. 1 dargestellte Gerät benutzt.

Die beiden Schenkel des U-Rohres sind mit Leitelektrolyt gefüllt, während in dem längeren mittleren Rohr eine gleichkonzentrierte Lösung des Gemisches der

Stereoisomeren eingebracht wird. Durch Öffnen des Verbindungshahnes wird vorsichtig eine Grenzschicht in dem U-Rohr erzeugt. Sehr gut haben sich ausserdem U-förmige dünnwandige Rohre ohne Ansatz mit einem Durchmesser von 4 mm bewährt. Die Lösung der *cis-trans*-Isomeren wird hier mit einer Injektionsspritze oder Kapillare vorsichtig an den Boden des Rohres gebracht. Als Elektroden dienen 2 Platindrähte von 1.5 mm Durchmesser. Als Thermostat bietet sich das Flüssigkeitsbad des Kühlaggregats der analytischen Kammer an.

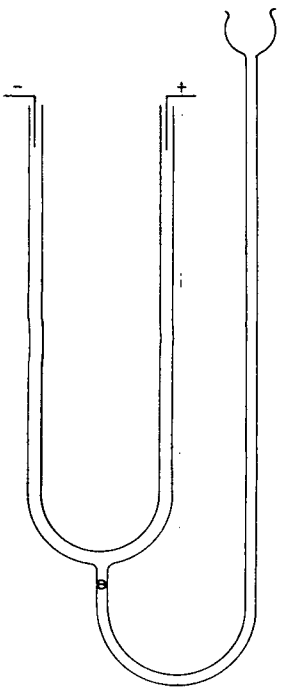

Fig. 1. Gerät für trägerfreie Ionophorese.

Zur Aufnahme der Absorptionskurven wurde das selbstregistrierende Spektralphotometer DK 2 der Fa. Beckmann verwendet. Der eingestellte Wellenlängenbereich lag zwischen 300–670 nm.

Folgende Co(III)-Komplexe wurden nach Literaturangaben rein hergestellt:

trans[9,10]- bzw. *cis*[11]- $[Co\ en_2\ Cl_2]Cl$ (grün bzw. violett),
trans[12]- bzw. *cis*[12]- $[Co\ en_2\ Br_2]Br$ (gelbgrün bzw. grauviolett),
trans[11]- bzw. *cis*[11]- $[Co\ en_2\ (NCS)\ Cl]Cl$ (blauviolett bzw. rot),
trans[13]- bzw. *cis*[13]- $[Co\ en_2\ (NO_2)_2]NO_2$ (gelb),
trans[11]- bzw. *cis*[11]- $[Co\ en_2\ (NH_3)\ Cl]Cl_2$ (rot), und
trans[11]- bzw. *cis*[11]- $[Co\ en_2\ (NH_3)_2]Br_3$ (gelb).

Ein grosser Nachteil der meisten Co(III)-Komplexe ist ihre Instabilität in wässrigen Lösungen, die in vielen Fällen zu Ligandenaustausch z.B. gegen H_2O führt. Es gelang jedoch durch Arbeiten, bei 0°C diese Schwierigkeit zu überwinden.

Zur Prüfung der Verbindungen auf Reinheit diente ein Vergleich der Lagen der Extremwerte in den Absorptionsspektren mit den Angaben aus der Literatur[14]. Die Absorptionsspektren sind bei 0°C aufgenommen worden. Bei dieser Temperatur sind alle untersuchten Verbindungen, wie aus der Literatur an Hand von Leitfähigkeitsmessungen[10,14] zu entnehmen ist, gegen Ligandenaustausch stabil.

HOCHSPANNUNGSPAPIERIONOPHORESE

Von den sechs Gemischen stereoisomerer Komplexionen lassen sich vier einwandfrei trennen.

Der verwendete Acetatpuffer war 0.3 *M* an CH_3COOH und 0.2 *M* an CH_3COONa. Die Spannung betrug 3 kV, die Versuchstemperatur durchweg 0°C. Bei Verwendung von 3 Streifen von 5 cm Breite und 100 cm Länge stellte sich bei den oben erwähnten Bedingungen die Stromstärke auf *ca.* 70 mA ein. Um möglichst lange Wanderungsstrecken zu erzielen und gleichzeitig störende Sogeffekte auszuschalten, erfolgte die Auftragung 20 cm vom Anodenraum entfernt. Zur Erhaltung scharfer Banden war es notwendig, störende Diffusionseffekte zu unterdrücken. Die Konzentration der Auftragelösung wurde deshalb so gewählt, dass sie einerseits kleiner als die des Elektrolyten war und andererseits gross genug, um kräftige Banden zu erzeugen. Als günstigster Bereich für die Auswertung und den Vergleich der Trenneffekte ergab sich eine Konzentration von 0.01 *M*; aufgetragen wurden immer 0.005 ml Analysenlösung.

Um bei den Arbeiten die Möglichkeit eines Ligandenaustausches gegen H_2O auszuschliessen, wurden die Auftragelösungen jedesmal vor dem Versuchsbeginn frisch mit eiskaltem Wasser hergestellt.

Zur Beschränkung eventuell auftretender Inhomogenitäten des elektrischen Feldes oder der Kühlung auf ein Minimum wurden sowohl die reinen Lösungen als auch die Mischung auf einen einzigen Streifen aufgetragen. Dabei lag die reine *trans*-Verbindung oben, die reine *cis*-Verbindung unten und die Mischung in der Mitte. Die nach der Trennung durch farbige Zonen gekennzeichneten Pherogrammstreifen wurden anschliessend durch Ammonsulfidlösung gezogen, wobei sich schwarze CoS-Zonen mit schärferen Umrissen bildeten (Fig. 2). Die Unterschiede in den Wanderungsstrecken verschiedener Isomerenpaare sind jeweils auf die gleiche absolute Wegstrecke von 40 cm (Abstand Startpunkt–*trans*-Zone) bezogen worden.

Zur spektrophotometrischen Auswertung der Zonen wurde wie folgt verfahren:

Eine Mischlösung der Isomeren (0.1 *M* an jeder Komponente) wurde an 3 Stellen im Abstand von 5 cm auf einen 15 cm breiten Streifen aufgetragen (0.03 ml) und der Elektrophorese unterworfen. Zur Eluierung wurden die einzelnen farbigen Zonen (je drei) aus dem Pherogramm ausgeschnitten, zerkleinert und in ein nach unten zu verjüngtes Glasrohr gestampft. Anschliessend wurden langsam etwa 2 ml Eiswasser durch die Rohre gepresst und von den erhaltenen Lösungen sofort die Absorptionskurven aufgenommen.

[Co en$_2$ Cl$_2$]Cl

Bei diesem Isomerengemisch zeigte sich nach Durchlaufen einer Wanderungsstrecke von 40 cm eine gute Trennung. Nach einer Elektrophoresedauer von 3 St. lagen die einzelnen Zonen der Mischung *ca.* 1.5 cm auseinander, wobei die grüne Zone

des *trans*-Kations der violetten des *cis*-Kations voraneilte. Dieser Wert stellt, wie auch bei allen anderen Komplexionen, einen Mittelwert aus 6 Einzelmessungen dar.

Fig. 2 zeigt als Beispiel das Pherogramm der getrennten Stereoisomeren. Analoge Pherogramme erhält man auch für die später beschriebenen Ionengemische. Das *trans*-Kation bildet immer die voraneilende Zone.

Fig. 2. Pherogramm der Stereoisomeren von $[Co\ en_2\ Cl_2]Cl$.

Die Absorptionsspektren der eluierten Zonen stimmten wie auch bei den folgenden Versuchen mit denen der reinen Verbindungen überein.

$[Co\ en_2\ Br_2]Br$

Nach 40 cm langer Wanderungsstrecke in *ca.* 3 Std. und 20 Min. lag eine Trennung der Zonen vor. Sie waren *ca.* 1.3 cm voneinander entfernt.

$[Co\ en_2\ (NCS)\ Cl]Cl$

Die Isomeren dieser Verbindung zeigten einen grösseren Unterschied in den Wanderungsstrecken als die beiden anderen. Der Abstand der einzelnen Zonen betrug nach 40 cm Wanderungsstrecke *ca.* 1.8 cm.

$[Co\ en_2\ (NO_2)_2]NO_2$

Auch hier trennte sich die aufgetragene Mischung in 2 Zonen. Ihr Abstand betrug *ca.* 2 cm bei 40 cm Wanderungsstrecke. Da beide Isomere die gleiche Farbe und Absorptionsspektren aufweisen, ist die Trennung dadurch bewiesen, dass die Lage der Zonen mit denen der oben und unten liegenden *trans*- bzw. *cis*-Verbindung identisch ist.

$[Co\ en_2\ (NH_3)\ Cl]Cl_2$

Auch bei einer Wanderungsstrecke von 100 cm, die durch Zurückverlegen des Streifenendes mit den Komplexen an die Kathode und Einsetzen eines neuen Streifens erzielt wurde, zeigte sich keine Trennung. Die Bande hatte sich während dieser langen Elektrophoresedauer lediglich infolge Diffusion verbreitert.

$[Co\ en_2\ (NH_3)_2]Br_3$

Auch hier fand, wie bei den vorher erwähnten Komplexionen, keine Trennung statt. Trotz Gebrauch eines dritten Streifens wurde kein Effekt sichtbar.

Orientierende Untersuchungen über die Abhängigkeit der Trenneffekte von der Elektrolytkonzentration wurden nur mit dem Isomerengemisch $[Co\ en_2\ Cl_2]Cl$ durchgeführt.

Folgende Konzentrationen kamen zur Anwendung:

(a) 0.3 *M* CH_3COOH und 0.2 *M* CH_3COONa
(b) 0.6 *M* CH_3COOH und 0.4 *M* CH_3COONa
(c) 0.9 *M* CH_3COOH und 0.6 *M* CH_3COONa.

Mit steigenden Konzentrationen musste mit verringerter Spannung und längerer Elektrophoresedauer gearbeitet werden, um wieder gleiche Wanderungsstrecken zu erzielen. Bei Festlegung einer 20 cm langen Strecke und einer Mischungskonzentration von 0.01 m wurden für die Trennabstände folgende Mittelwerte erhalten: (a) 7 mm, (b) 9 mm und (c) 10.5 mm. Der Unterschied der Wanderungsstrecken der stereoisomeren Komplexionen nimmt demnach mit steigender Elektrolytkonzentration zu.

IONOPHORESE OHNE TRÄGERMEDIUM

Für eine trägerfreie Ionophorese sind geringere Feldstärken ausreichend, da die Reibungswiderstände bedeutend kleinere Werte annehmen als bei der Papierionophorese. Hier treten grössere Wanderungsgeschwindigkeiten auf, wodurch eine Trennung möglicherweise erst nach Zurücklegung einer langen Wanderungsstrecke erfolgt. Das wiederum kann zu störenden Diffusionserscheinungen führen. Alle diese Fragen lassen sich nur durch wiederholende Versuche klären, aus denen sich die optimalen Bedingungen in apparativer und verfahrensmässiger Hinsicht ergeben.

Als optimale Versuchsbedingung wurden ermittelt:

Feldstärke 10 V/cm (Spannung 220 V, Elektrodenabstand 22 cm) und Temperatur 0°C. Um die Diffusion weitgehend zu unterdrücken, wurde darauf geachtet, dass die Ionenstärke der zu trennenden Gemische ungefähr gleich der der Fremdionen im Leitelektrolyten war. Somit traten keine zusätzlichen Potentialgefälle im Elektrolyten auf.

Das U-förmige Elektrolysegefäss (Fig. 1) wird mit Leitelektrolyt bis etwa auf $^2/_3$ der Schenkelhöhe gefüllt, in das als Thermostat dienende Kühlbad getaucht und auf 0°C abgekühlt. Das Isomerengemisch löst man in dem gleichen Leitelektrolyten, wobei die Konzentration an jeder Komponente 0.1 *M* ist. Zur Erhöhung der Dichte und um ein Vermischen zu verhindern, wird der Analysenlösung 10 Gew.-% Zucker oder Harnstoff zugesetzt. Dieses Gemisch lässt man vorsichtig bis auf etwa $^1/_3$ der Schenkelhöhe einfliessen.

Eine intensive Kühlung sorgt für die Abführung der Joulschen Wärme und damit für eine Verschärfung der Trennzonen. Als Modellsubstanz diente das Isomerengemisch [Co en_2 Cl_2]Cl.

Trennung mit Acetatpuffer als Leitelektrolyt

Mit dem Acetatpuffer (0.2 *M* CH_3COONa und 0.3 *M* CH_3COOH) als Leitelektrolyt und einer Elektrolysedauer von 10 Min. zeigte sich kein sehr befriedigendes Ergebnis. Eine unterschiedliche Wanderungsgeschwindigkeit der Isomeren war zwar zu bemerken, was man an der Verschiebung der Fronten erkennen konnte. Es traten eine grüne Zone, eine bläuliche Mischzone und eine violette Zone auf. Jedoch waren die Farbzonen nicht sehr scharf.

Trennung mit $AlCl_3 \cdot 6\ H_2O$-Zusatz

Die Trennungen liessen sich durch Steigerung der Leitelektrolytkonzentrationen besonders durch Zusatz von $AlCl_3 \cdot 6\ H_2O$ verbessern. Der hier benutzte Leitelektrolyt war 0.2 *M* an CH_3COONa, 0.3 *M* an CH_3COOH und 0.2 *M* an $AlCl_3 \cdot 6\ H_2O$. Im Kathodenraum bildete sich am oberen Ende der Berührungszone Leitelektrolyt–Analysenlösung eine grüne Zone des schneller wandernden *trans*-Komplexes, im

Anodenraum an entsprechender Stelle eine solche des violetten *cis*-Komplexes.

Zur Identifizierung wurden die grüne und die violette Zone mit der Injektionsspritze vorsichtig abgesaugt und ihre Absorptionsspektren aufgenommen. Diese waren mit denjenigen der reinen Verbindungen identisch.

Der Trenneffekt ist von der $AlCl_3$-Konzentration abhängig. Es steigen auch hier wieder die Unterschiede in den Beweglichkeiten der Isomeren mit Zunahme der Elektrolytkonzentration.

Es wurde wie vorher beschrieben verfahren. Als Leitelektrolyt dienten wässrige Lösungen von $AlCl_3 \cdot 6\ H_2O$ und CH_3COOH, die 0.1 *M*, 0.2 *M*, 0.3 *M*, 0.4 *M*, 0.5 *M* an jeder der beiden Komponenten waren. Nach 20 Min. wurde die Zonenlänge (= Unterschiede der Wanderungsstrecken) der violetten und der grünen Zone gemessen. Fig. 3 gibt das Ergebnis graphisch wieder.

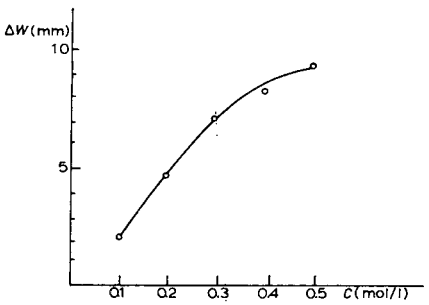

Fig. 3. Abhängigkeit des Trenneffektes von der $AlCl_3$-Konzentration. ΔW = Unterschied der Wanderung = Zonenlänge.

DISKUSSION DER ERGEBNISSE

Eine ionophoretische Trennung zweier Ionen lässt sich realisieren[1,2], wenn sie sich zumindest in einer der folgenden Grössen unterscheiden: (a) Ionenladung, (b) effektiver Ionenradius oder (c) Masse. Die Grössen (b) und (c) können durch unterschiedliche Solvathüllen verändert werden.

Die anorganischen stereoisomeren Komplexionen besitzen die gleiche Ladung und gleiche Masse. Zeigen diese Ionen eine unterschiedliche Wanderung im elektrischen Feld, so wird man vorerst annehmen, dass eine unterschiedliche Solvathülle für diese Erscheinung verantwortlich ist. Diese Frage muss jedoch gesondert untersucht werden.

Die vorliegende Arbeit lässt universelle Aussagen über die ionophoretische Trennbarkeit stereoisomerer Komplexionen nicht zu. Vielmehr hängt die Trennung von den individuellen Eigenschaften der einzelnen Komplexionen ab. Beim Wechsel der konfigurationsbestimmenden Liganden dürften die im Komplexion hervorgerufenen Polaritätsänderungen eine Rolle spielen. So sind *cis-trans*-Komplexionen, in denen durch den Konfigurationswechsel ein gewisser unterschiedlicher Dipolcharakter hervorgerufen wird, trennbar.

Möglicherweise ruft die Änderung des Dipolmomentes durch seinen Einfluss auf die Verteilung der umgebenden Ionenwolke einen unterschiedlichen Grad der Wechselwirkung hervor. Dadurch kann die abbremsende Kraft, die dem wandernden Ion entgegengesetzt ist, verschiedene Werte annehmen.

Diese Annahme stützt sich auf folgende experimentelle Ergebnisse:

(a) Die Wanderungsgeschwindigkeit der stereoisomeren Ionen ist nicht nur auf Trägermedien, sondern auch in trägerfreiem Medium unterschiedlich. Der Trenneffekt ist somit nicht auf Einflüsse des Trägermediums wie unterschiedliche Adsorptionskräfte oder dergleichen, sondern auf ein unterschiedliches Verhalten im elektrischen Feld zurückzuführen.

(b) Der Trenneffekt der stereoisomeren Ionen nimmt mit zunehmender Elektronegativität der die Konfiguration bestimmenden Gruppen zu. So bewirkt das elektronegativere Chlor als konfigurationsbestimmender Ligand einen besseren Trenneffekt als Brom. Ferner tritt beim *cis-trans* $[Co\ en_2\ (NO_2)_2]^+$ ein grosser Trenneffekt auf, während *cis-trans* $[Co\ en_2\ (NH_3)_2]^{3+}$ nicht getrennt wird. Der Stickstoff im NH_3 weist gegenüber dem NH_2 im en-Ring nur einen geringen Unterschied in der Elektronegativität auf.

Ebenso wie das *cis-trans*-$[Co\ en_2\ (NH_3)_2]^{3+}$ lässt sich das *cis-trans*-$[Co\ en_2\ (NH_3)\ Cl]^{2+}$ nicht trennen, da solche Komplexionen in bezug auf ihre Ladungsverteilung mit $[Co\ (NH_3)_5\ Cl]^{2+}$ zu vergleichen sind und in dieser Hinsicht keine oder nur sehr geringe konfigurativ bedingte Änderung in der Dipoleigenschaft aufweisen.

(c) Der Trenneffekt ist von der Art und der Konzentration der Ionen abhängig, die die entgegengesetzte Ladung tragen und damit die Ionenwolke bilden. Wie das Kapitel Hochspannungspapierionophorese zeigt, werden die Trenneffekte mit steigender Elektrolytkonzentration grösser. Auch der Zusatz von $AlCl_3 \cdot 6\ H_2O$ bzw. die Erhöhung seiner Konzentration führte bei der trägerfreien Ionophorese zur Verbesserung der Trennung. Möglicherweise ist diese Verbesserung auf die Tatsache zurückzuführen, dass ein solcher Zusatz dafür sorgt, dass die Ionen, die die Ionenwolke bilden (in diesem Falle negative), im Überschuss vorhanden sind. $AlCl_3$ liefert 3 Chloridionen, während die elektrostatische Anziehungskraft des Al^{3+} durch die Hydratisierung mit 6 Molekülen Wasser abgeschirmt ist.

DANK

Für die Bereitstellung von Mitteln und Apparaten danken wir der Deutschen Forschungsgemeinschaft und dem Bundesministerium für Wissenschaftliche Forschung.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden eine Anzahl stereoisomerer Komplexionen des Co(III) sowohl mit Hilfe der Hochspannungspapierionophorese als auch mit der trägerfreien Ionophorese einwandfrei getrennt. Die Identität der isomeren Komplexionen wurde durch Aufnahme der Absorptionsspektren sichergestellt.

Die ionophoretische Trennung stereoisomerer Komplexionen hängt von ihren individuellen Eigenschaften ab.

Beim Wechsel der konfigurationsbestimmenden Liganden dürften die im Komplexion hervorgerufenen Polaritätsänderungen eine Rolle spielen.

SUMMARY

In the above work a number of stereoisomers of complex-ions of Co(III) were separated by means of high-voltage paper ionophoresis and also by means of carrier-free ionophoresis. The identity of the complex-ions was determined through their absorption spectra.

The ionophoretic separation of complex-ion stereoisomers depends on their individual properties.

At the exchange of the configuration-determining ligands, the polarity alteration brought about in the complex may play a role.

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Short Communication

Chromatographic techniques using liquid ion-exchangers

Recently much attention has been given to the use of liquid ion-exchangers in chromatography (paper and column). Though the first publications[1,2] were devoted to the use of high-molecular-weight amines, recent developments all concern di-(2-ethylhexyl) hydrogen phosphate (HDEHP) and related compounds (for reviews *cf.* refs. 3 and 4), while special emphasis has been placed upon the separation of rare earth mixtures.

The interesting separations recorded in the rapidly increasing number of publications in this branch of chemistry and, moreover, the correlation of results so obtained with those from extraction procedures and ion-exchange techniques (*cf.* the work of KRAUS AND NELSON[5]) have led us to investigate further the possible applications of chromatographic techniques using amines, the more so since most attention has hitherto been focused on the rather expensive tri-*n*-octylamine. This preliminary report describes a method in which the use of liquid ion-exchangers is successfully combined with a simple form of thin-layer chromatography.

The first experiments were carried out using Amberlite LA-1 (weakly basic secondary amine; N-dodecenyl-(trialkylmethyl)-amine) and HCl, with silica gel (Fluka, Type DO (no binder)) as a support. The amine is converted into its HCl salt by equilibrating a solution in chloroform for 10 min in a separatory funnel with 3 vol. of HCl of appropriate normality (see below); the organic solution is separated, filtered on paper and dried for some hours over sodium sulphate. The amine·HCl solution so obtained is mixed with silica gel (silica gel–chloroform, 1:2, v/v) and the resulting suspension is agitated for some time.

Thin-layer plates are prepared by dipping ordinary microscope slides (2.5 cm × 7.5 cm) into the silica gel–chloroform mixture for approx. 5 sec. On leaving the glass plates for some minutes in the air, in order to evaporate off the chloroform, a thin film of amine-impregnated silica gel adheres to the slides. Using cotton wool, a margin of 1–2 mm is made along the edges of the slides in order to avoid undesirable contact with the eluting agent.

The thin-layer plates so prepared are spotted with the solution to be investigated using a 1-mm wide strip of filter paper; the resulting spot ought to have a diameter of 1–2 mm. When the spots are air-dry the thin-layer plates are put into small vessels (Hellendahl staining jars) containing the eluting agent (4–6 plates per vessel) and the chromatogram is developed for 10 min using the ascending technique. Subsequently, the plates are dried in the air and the spots are visualized using conventional techniques. The ions mentioned in Fig. 1 were detected using either H_2S gas [Pb(II), Bi(III), Hg(II) and Sb(III)], dipping with dithiooxamide in chloroform [Ni(II), Co(II) and Cu(II)] or spraying with 8-hydroxyquinoline in 80% ethanol [Al(III), Fe(III), Mn(II), Zn(II) and Cd(II)].

Preliminary investigations showed the use of 0.10 *M* amine·HCl solutions in

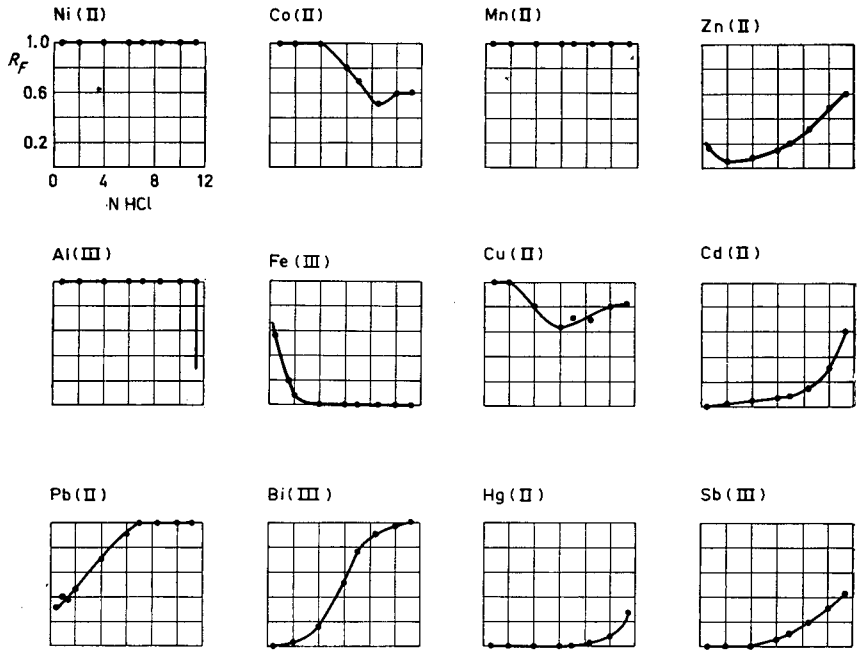

Fig. 1. R_F-spectra found for some cations in thin-layer chromatography using silica gel impregnated with Amberlite LA-1·HCl as support and HCl of varying concentration as eluting agent.

chloroform to give excellent results. Experiments were carried out using 0.5–11.5 *N* HCl as eluting agent. At first, the amine was equilibrated with HCl of the same normality as that used as eluting agent, but it was shown that equilibration with 2 *N* HCl in all cases — irrespective of the normality of the eluting agent — made hardly any difference.

R_F-spectra for some ions are shown in Fig. 1. It is clearly seen that the results obtained — which were fairly reproducible — exhibit good agreement with the ion-exchange data of KRAUS AND NELSON[5] and also with the results obtained in extrac-

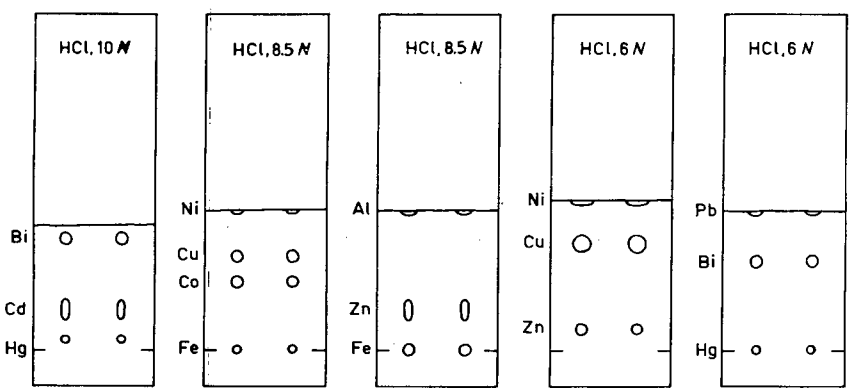

Fig. 2. Examples of qualitative separations obtained using the thin-layer technique described in this paper. For experimental details see text and figure.

tion procedures, as cited in the reviews by GREEN[4] and by COLEMAN[6]. Our data show, moreover, that (i) many qualitative separations are easily carried out using the inexpensive amine Amberlite LA-1: some examples are given in Fig. 2; (ii) the rapidity of the separation method outlined above makes it superior to most other methods of qualitative analysis so that detailed investigation would certainly be of interest, together with its possible quantitative application (*cf.* ref. 2).

The use of the rapid thin-layer technique described here is, moreover, important in view of the reliable predictions that can sometimes be made about the results of ion-exchange or extraction procedures under comparable circumstances.

Investigations are now in progress on the use of other supports, amines and complexing agents. Attention has already been given to the use of paper-chromatographic procedures analogous to those outlined by CERRAI AND TESTA[1,3]. It was found that good results are obtained when using 0.15 *M* Amberlite LA-1·HCl solutions in chloroform or benzene; Whatman No. 1 paper was used throughout. Schleicher and Schüll paper No. 2043a gave comparable results, while Schleicher and Schüll 2040a and 2045a were somewhat less satisfactory. In general, the results are comparable to those given above for the thin-layer experiments. Excellent separations can be accomplished in 3–4 h.

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Notes

Thin-layer chromatographic method for the separation of rare earths

Although thin-layer chromatography (TLC) has proved to be an excellent and rapid separation technique in organic and biochemical analysis, it has found little use in inorganic separations. The technique offers many unique advantages, the most important being its rapidity and the possibility to use corrosive reagents and impregnations. The separation of some adjacent rare earths (R.E.) with a rapid chromatographic technique is interesting in view of radiochemical studies. The use of TLC

was not put forward yet, until an article from PIERCE[1] appeared during the course of our work.

It appears from the literature that the best separation factors between adjacent R.E.'s are obtained with di-(2-ethylhexyl) phosphoric acid (HDEHP). This acid, originally used as an extracting agent[2], has already been applied in chromatography column- and paper partition).

All these techniques used reversed phase chromatography[3–6]. This is also the case with the above mentioned TLC separation. The only attempt to use HDEHP as a mobile phase is due to WINCHESTER (personal communication). We have tried the normal phase method by impregnating silica gel with buffers or acids and eluting with HDEHP.

Preparation of thin layers

Silica gel H was used, to avoid the presence of calcium sulphate, which can possibly give rise to difficulties, as its solubility in an aqueous phase is far from negligible.

Plates prepared without a binding agent offered, however, low mechanical resistance. Better results were obtained by adding 6 % soluble starch as a binder. The starch was sieved before mixing with the silica gel, purified according to SEILER[7]. The mixture was then sieved again through a 350 mesh sieve. To obtain 20 plates of 5 × 20 cm, 30 g of this mixture were slurried with 57 ml water and shaken vigorously for 3 min. The layers were then prepared with the Shandon applicator (thickness of layer 250 μ) and air-dried at room temperature overnight.

To adjust the pH of the stationary phase, the silica gel was slurried with an adequate buffer. Impregnation by acids was achieved by developing the plates first with the acid in question ($HClO_4$ was preferable to HCl since it gave more reproducible results) and again drying overnight.

Development

Development was achieved by the ascending method in Shandon development chambers lined with filter paper. The mobile phase (HDEHP, with a controlled purity better than 99.5 %) was preequilibrated with the stationary phase and separated from it by centrifugation. A solution in carbon tetrachloride was used.

Detection of rare earths and determination of R_F values

To detect the R.E.'s, radioactive tracers were used. Most of these were prepared by irradiation of silica ampoules containing about 200 μl of a solution of the nitrates in water (5 mg element/10 ml) in the BR-1 reactor. Some isotopes were obtained from "The Radiochemical Centre", Amersham (^{91}Y) or the S.C.K., Mol (^{144}Ce, ^{153}Gd). The tracers were detected on the plates with a self built Geiger-Müller scanner coupled to a recorder. The R_F's were determined from the resulting graph. In some cases an autoradiographic technique was applied using Structurix Röntgenfilm D7, Gevaert. In this last case the R_F values were obtained by densitometry.

Results

Since HDEHP is itself strongly adsorbed on the silica gel layers a second front s formed, the first front being due to carbon tetrachloride. All the R_F values were

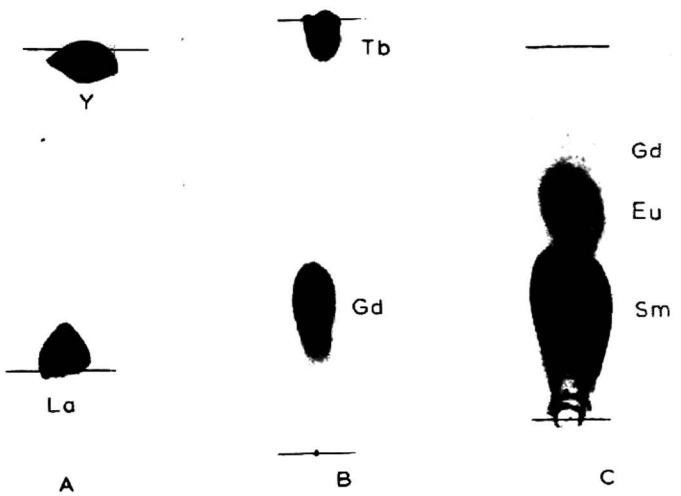

Fig. 1. Autoradiography of rare earth separations. (A) La–Y; stationary phase: buffer of pH 1.08. (B) Gd–Tb; stationary phase: 0.4 *M* $HClO_4$. (C) Sm–Eu–Gd; stationary phase: 0.3 *M* $HClO_4$.

determined with the HDEHP as the reference front. It was necessary to use 1 *M* HDEHP since in the case of a 0.1 *M* solution, the second front lies 9 cm under the first front when this has moved 12 cm. For 1 *M* HDEHP under the same conditions the second front lies only 1 cm behind the first.

The application of buffers as stationary phase is very limited since at high pH (2.23) all the R.E.'s have large distribution constants and thus migrate with the

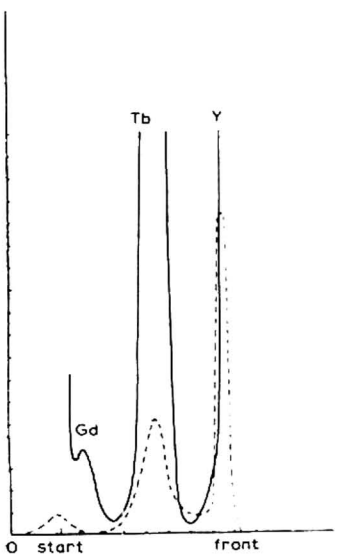

Fig. 2. Separation of Gd–Tb–Y. ——— densitometric analysis. – – – – – Geiger-Müller scanning.

front. Only La has an R_F of 0.30 and can be easily separated from the rest. With a KCl–HCl buffer (pH 1.08) La has an R_F of 0.05, Ce 0.55, whereas Nd and the others migrate with the front. The separation of Y and La is shown in Fig. 1A. To separate the heavier R.E.'s lower pH's in the stationary phase are required which is obtained by impregnation with acids. In this way Eu and Sm can be separated completely with 0.2 *M* $HClO_4$. The separation Y–Tb–Gd (Y front, Tb 0.57, Gd 0.16) was achieved with 0.4 *M* $HClO_4$ as the stationary phase and is shown in Fig. 2. All these separations, however, suffer from the fact that a residual activity of nearly 5 % is irreversibly fixed on the spotting place. Since spotting of the R.E.'s was done in an aqueous solution, it was assumed that this technique disturbed the pH of the stationary phase. The R.E.'s were therefore first extracted into HDEHP and then spotted in the organic phase. This technique allowed good separations of Tb–Gd (Fig. 1B), Eu–Gd and Eu–Gd–Sm (Fig. 1C) within at most 70 min.

Although as yet only the group Tb, Gd, Eu and Sm has been examined thoroughly and at most three R.E.'s have been separated, it is reasonable to expect that this method should be valuable to separate any combination of three adjacent R.E.'s and, in favourable circumstances, even five on one plate. This can be concluded from the fact that for example Eu, Sm and Gd (Fig. 1C) have R_F's of 0.29, 0.54 and 0.73, respectively. Tb should have an R_F of approx. 1 while the R_F of Nd would be approx. 0.

Work is now in progress to examine other R.E. separations with this technique.

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Gas chromatography of inositols as their trimethylsilyl derivatives

Gas chromatography is now a standard technique in carbohydrate structure determination. Owing to their non-volatile nature, however, carbohydrates must be first converted into suitable derivatives. Methoxy and acetoxy derivatives of mono- and oligosaccharides have been analyzed successfully by gas chromatography. More recently, a trimethylsilylation technique[1] was introduced for the same purpose, and the range of applicability of gas chromatography of carbohydrates was expanded greatly. Compounds as large as a tetrasaccharide have been analyzed by gas chro-

matography after trimethylsilylation, and even sugar phosphates have been chromatographed by this technique[2].

During our work on myoinositol mannosides of mycobacteria, we found that trimethylsilylation was useful not only in volatilizing the sample compounds, but also in improving separation. For example, methyl 2,3,4-tri-O-methyl-α-D-mannoside and methyl 3,4,6-tri-O-methyl-α-D-mannoside could not be separated from each other on a neopentylglycol succinate column or any other column we have tried[3]. After introduction into the mannosides of the trimethylsilyl group at the 6- and 2-positions, respectively, the compounds could be separated with ease. In a similar fashion, better separation of penta-O-methyl-*myo*-inositol isomers was achieved after trimethylsilylation[4].

In this report is described the separation of inositol isomers by gas chromatography as their trimethylsilyl derivatives. The method seems to be advantageous to that described by KRZEMINSKI AND ANGYAL[5], in which acetoxy derivatives of inositols were used.

Experimental

Gas chromatography. The apparatus used was the Aerograph Hy-Fi model A-600B, furnished with hydrogen flame detector and Brown-Honeywell Class 15 recorder. The following stationary phases were coated on Anakrom ABS (100–110 mesh, Analabs, Inc., Hamden, Conn.), in the percentage indicated: (a) QF-1, 5%; (b) SE-30, 3%; (c) Carbowax 20 M, 10%; and (d) diethyleneglycol succinate, 10%. The columns were 5 ft. × $^1/_8$ in. (O.D.) in size. The flow rates of nitrogen and hydrogen were both 20 ml/min.

Preparation of samples. Samples were trimethylsilylated according to SWEELEY *et al.*[1]. Since inositols are not easily soluble in pyridine, the mixture was warmed to bring about complete solution. Although trimethylsilylation proceeded to some extent with the inositol in suspension, prolonged shaking (30–60 min), or warming after the addition of the reagents, was necessary for complete reaction. After the reaction, the mixture was centrifuged and portions of the supernatant were injected for analysis.

TABLE I

RETENTION TIME (MINUTES) OF TRIMETHYLSILYL DERIVATIVES OF INOSITOLS

Inositol isomer	*Column and temperature*				
	QF-1	*QF-3*	*SE-30*	*Carbowax*	*Diethylene glycol succinate*
	(150°)	(160°)	(200°)	(160°)	(146°)
allo	9.9	5.4	7.3	4.7	6.4
neo	10.4	5.9	7.5	5.0	7.7
muco	11.4	6.3	8.0	5.8	7.3
DL	13.3	7.2	9.4	7.0	9.2
scyllo	18.4	9.6	11.8	11.2	12.3
epi	20.0	11.6	11.3	10.0	13.1
myo	24.3	12.7	14.7	14.5	18.4
cis	24.5	12.7	12.8	—	—

Results and discussion

The retention times of the trimethylsilyl derivatives of inositol isomers are listed in Table I. The order of their appearance, regardless of the stationary phase used, is as follows: *allo*, (*neo–muco*), DL, (*scyllo–epi*), and (*myo–cis*). The order within the parentheses is sometimes reversed, however, depending on the stationary phase. This order is considerably different from that of the acetoxy derivatives of inositol isomers as reported by KRZEMINSKI AND ANGYAL[5], but as in that case there is no apparent correlation between retention time and configuration.

The best results were obtained with the QF-1 column. A typical separation on this column is illustrated in Fig. 1. In this system, all but the *myo–cis* pair of inositols could be separated, either partially or completely.

The present system has some advantages over the method employing acetoxy derivatives. Trimethylsilyl derivatives of inositols can be prepared quickly and the reaction mixture can be injected directly for analysis. The time needed for separation is much less than that needed for the acetoxy derivatives. Since all of the naturally occurring inositols (*neo*, DL, *scyllo*, and *myo*) can be separated completely in this system, it is suitable for their quantitative determination.

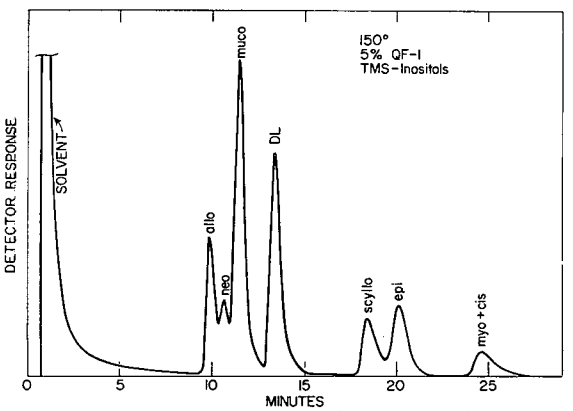

Fig. 1. A gas chromatography tracing representing the separation of trimethylsilyl derivatives of the inositols on a QF-1 column at 150°.

Acknowledgements

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Quantitative separation of cymene isomers by gas chromatography

Separation of *o*-, *m*- and *p*-xylenes has been reported[1]; also 2,6- and 2,7-dimethylnaphthalenes have been separated[2], using a Bentone-SE 52 column. During the course of an investigation in this laboratory it became necessary not only to detect the three isomers of cymene (isopropyltoluene), but also to analyse them quantitatively. The most closely related work published to date is that of VAN DER STRICHT AND VAN RYSSELBERGE[3], who by using a Bentone-34 column were able to separate the three isomers, but not sufficiently for quantitative work.

The work of the above-mentioned workers was taken as a starting point, and columns (6 m × 5 mm) packed with Bentone-34, SE-30, and didecyl phthalate (DDP) and mixtures thereof on 60/80 chromosorb W were tried. A PE-116 apparatus* (Thermister detector) was used, and the method of preparing the packing was similar to that of MORTIMER AND GENT[1]. Columns with the following different stationary phases were tried:

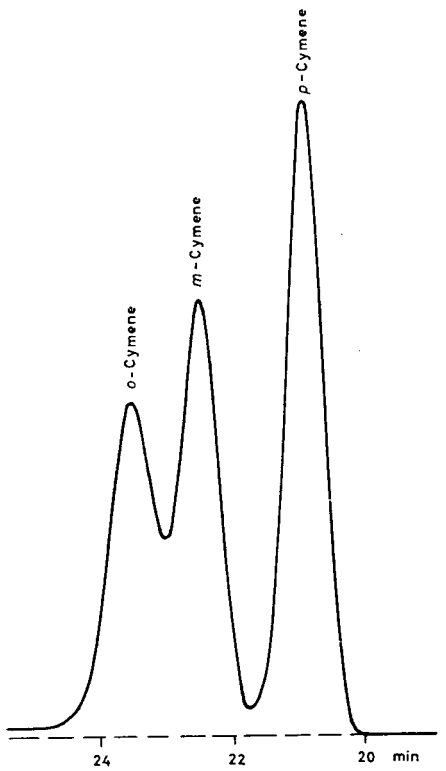

Fig. 1.

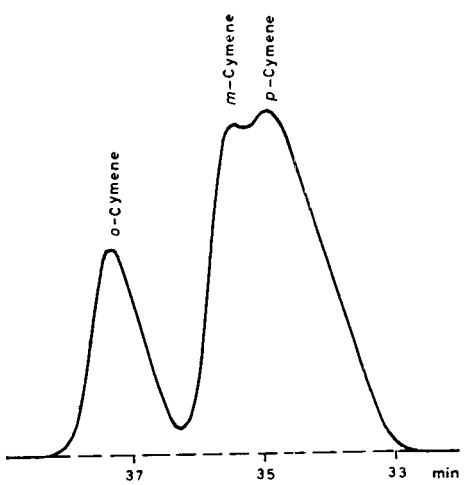

Fig. 2.

Fig. 1. Column packing: 5% SE 30–15% Bentone-34; column temperature: 161°; carrier gas: hydrogen; inlet pressure: 2.5 kg/sq.cm; sample size: 4 μl.

Fig. 2. Column packing: 10% DDP–20% Bentone-34; column temperature: 144°; carrier gas: hydrogen; inlet pressure: 2.5 kg/sq.cm; sample size: 4 μl.

* Perkin Elmer Fractometer Model 116.

(1) 20 % SE 30.
(2) 5 % SE 30–15 % Bentone-34.
(3) 15 % DDP– 5 % Bentone-34.
(4) 10 % DDP–15 % Bentone-34.
(5) 10 % DDP–20 % Bentone-34.

Results obtained with columns 2 and 5 were best. As can be seen from Fig. 1, stationary phase 2 gives a satisfactory separation of the *meta* and *para* isomers, but there is tailing between *ortho* and *meta*. Fig. 2 where column 5 is used shows that the tailing between *ortho*- and *meta*-cymene has been eliminated, but there is a poor separation of *para* from *meta*. To overcome this difficulty the two columns (total length 12 m) were used in series with each other, and the chromatogram obtained is shown in Fig. 3.

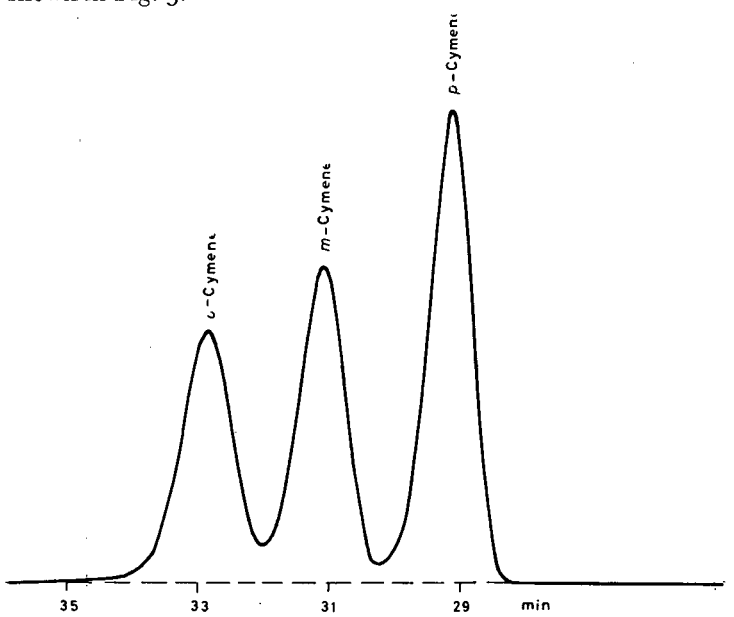

Fig. 3. Column packing: (I) 5 % SE 30–15 % Bentone-34; (II) 10 % DDP–20 % Bentone-34; column temperature: 155°; carrier gas: hydrogen; inlet pressure: 2.5 kg/sq.cm; sample size: 6 μl.

In addition a single column was tried combining the three stationary phases as follows: 5 % SE 30–10 % DDP–20 % Bentone-34. The separation was not as good as the one obtained by using the two columns in series, possibly because of the shorter length of this column.

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Gas–liquid chromatography of plant glycosides

Methods for separating and identifying microquantities of plant glycosides are of great importance for work in phytochemistry, pharmacognosy and chemotaxonomy. Thin-layer chromatography, which is most extensively used, does not possess the high sensitivity that is often desirable.

The successful separation of naturally occurring compounds such as aglycones by means of gas–liquid chromatography has been reported by several authors[1–7], but does not appear to have been achieved with plant glycosides. Recently SWEELEY and his coworkers[6] were able to separate the two plant glycosides aesculin and phlorizin on a gas chromatograph.

Gas chromatographic separation of seventeen plant glycosides, which are treated with hexamethyldisilazane and trimethylchlorosilane, has now been carried out on column containing SE-30 silicone rubber (0.75 %) on Chromosorb W (80–100 mesh). This paper describes the results obtained with a representative variety of simple phenolic, coumarin, isocoumarin, isoflavone, anthraquinone, cyanogenetic, isothiocyanate and monoterpene glycosides.

Experimental

Materials. Many of the plant glycosides used were available in this laboratory, but the author is very much indebted to Prof. H. INOUE for the monotropein, catalposide and arbutin derivatives, to Dr. TAKIDO for emodinglucoside, and to Mr. M. MATSUO for sinigrin. Hexamethyldisilazane was obtained from Peninsular Chemresearch, U.S.A. Trimethylchlorosilane was obtained from Applied Science Laboratories, Inc. Solvents were reagent grade.

The column packing 0.75 % of SE-30 silicone rubber on 80–100 mesh Chromosorb W was obtained from Applied Science Laboratories, Inc., U.S.A.

Procedure. The standard conditions for trimethylsilylation used were as follows: (a) 10 mg of plant glycoside was treated with 1 ml of anhydrous pyridine, 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. (b) When sufficient material was not available, 1 mg of plant glycoside was treated with 0.1 ml of anhydrous pyridine, 0.1 ml of hexamethyldisilazane, 0.05 ml of trimethylchlorosilane, or (c) 1 mg of plant glycoside was dissolved in 0.2 ml of anhydrous tetrahydrofuran and treated with 0.4 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane.

The reaction was carried out in a glass-stoppered vial. The mixture resulting from (a) and (b) was shaken vigorously for about 30 sec, and was then allowed to stand for 10 min. In the case of (c), the reaction mixture was left standing overnight at room temperature.

0.1 to 1 μl of the resulting mixtures were used for injection into the gas chromatograph.

Gas chromatography. A Shimazu Model GC-1B gas chromatograph equipped with a hydrogen flame ionization detector was used in this work. The column containing 0.75 % SE-30 silicone rubber on Chromosorb W (80–100 mesh) consisted of stainless steel U tubes, 2.25 m in length and having an inner diameter of 4 mm. The trimethylsilyl ethers of plant glycosides were introduced with a Hamilton microliter syringe.

Results and discussion

A number of plant glycosides gave single sharp peaks which appears to indicate the absence of decomposition or, if any, a little decomposition such as that with sinigrin. The gas chromatograms and retention times are shown in Table I and Fig. 1. No difference in retention times could be observed when either pyridine or tetrahydrofuran was used as the solvent for trimethylsilylation.

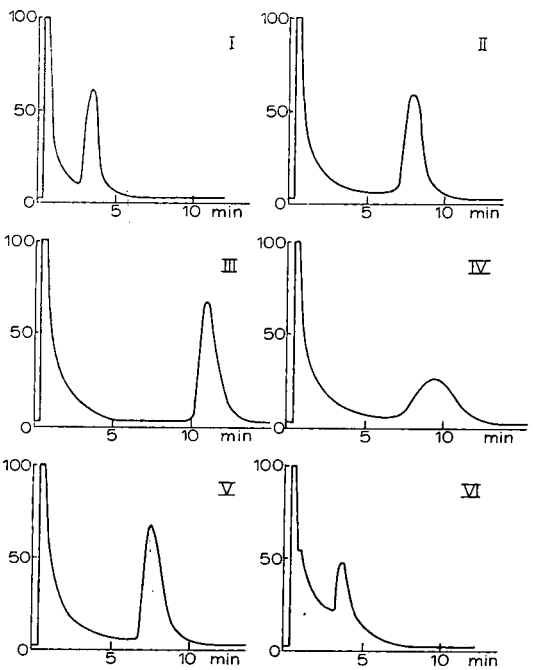

Fig. 1. Gas chromatograms of plant glycosides as the trimethylsilyl ethers. I = salicin (t_R = 3.7); II = fraxin (8.5); III = bergenin (11.0); IV = amygdalin (9.0); V = paeoniflorin (7.4); VI = sinigrin (3.4). For the operating conditions see the footnote to Table I.

Five simple phenolic glycosides were gas chromatographed. Arbutin, glucoside of hydroquinone, occurs widely in Ericaceae (*Arctostaphylos**) and other families. Homoarbutin has been isolated from Pirolaceae (*Pirola*). Arbutin can be easily separated from homoarbutin and isohomoarbutin methyl ethers used as reference compounds. Salicin, phenolic glucoside of salicyl alcohol, was first found in Salicaceae (*Salix*). Gaultherin, glucoside of salicylic acid methyl ester, was found in Ericaceae (*Gaultheria*). Salicin and gaultherin both gave good peaks. Aesculin, 6-glucoside of aesculetin, has been reported in Hippocastanaceae (*Aesculus*), fraxin, 8-glucoside of fraxetin, has been found in Oleaceae (*Fraxinus*), and daphnin, 7-glucoside of daphnetin, was distributed in Thymeleaceae (*Daphne*). The three coumarin glycosides mentioned above can be more easily separated in this way than by thin-layer chromatography[8]. Bergenin is one of the isocoumarin derivatives and its

* The name in parentheses shows the representative genus, from which plant glycoside has been isolated.

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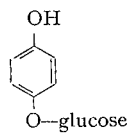
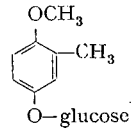
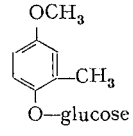
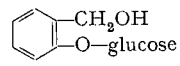
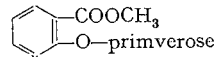
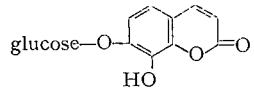
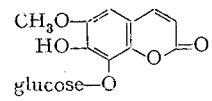
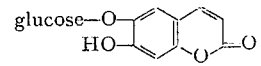
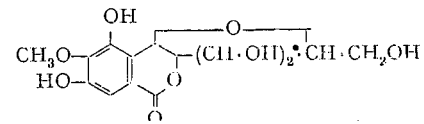
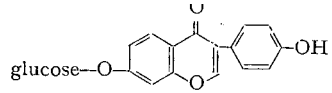
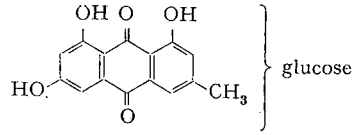
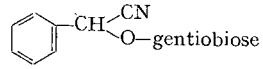
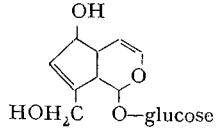
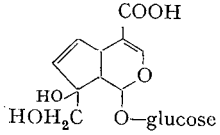
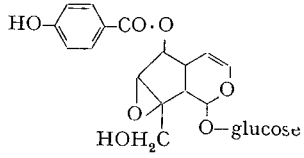
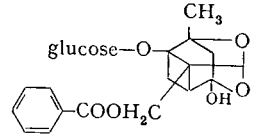
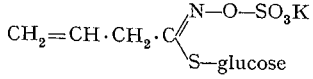
TABLE I

RETENTION TIMES IN MINUTES OF PLANT GLYCOSIDES

Group	*Compound*	*Structure*	*Column temperature*		
			188°	*203°*	*243°*
Simple phenols	Arbutin		8.2	4.2	1.2[a]
	Homoarbutin methyl ether		7.6	3.5	—
	Isohomoarbutin methyl ether		7.0	3.3	—
	Salicin		—	3.7	1.3[a]
	Gaultherin		—	5.8	—
Coumarin glycosides	Daphnin		—	7.4	3.0[a]
	Fraxin		—	8.5	3.3[a]
	Aesculin		—	10.5	3.9[a]
Isocoumarin glycosides	Bergenin		11.0	11.0	2.8[a]

			—	—	14.2
Anthraquinone glycosides	Emodin glucoside		—	—	8.0
Cyanogenetic glycosides	Amygdalin		—	—	9.0
Other glycosides	Aucubin			2.9	1.7
	Monotropein		—	7.6	2.6
	Catalposide			26.9[b]	11.1[m], 7.4[s]
	Paeoniflorin			24.9[b]	7.4
	Sinigrin		3.4[m], 1.8[s]	1.9[m], 1.1[s]	—

Conditions: stainless steel column 2.25 m long, 4 mm I.D.; packing 0.75% SE-30 on Chromosorb W (80–100 mesh); N_2 flow rate 122.4 ml/min at column temp. 188°, 119 ml/min at 203°, 102 ml/min at 243°; a = 110.5 ml/min at 243°. Detector temp. 265°; flash heater temp. 305°.
m = main peak; s = shoulder or minor peak; b = broad peak.

peak indicated approximately 500 theoretical plates. Daidzin, 7-glucoside of daidzein and emodin glucoside isolated from Leguminosae have got well-defined peaks. Amygdalin, one of the cyanogenetic glycosides, appears to be most stable to heat. Monoterpene glycosides, such as aucubin from Cornaceae (*Aucuba*), monotropein from Pirolaceae (*Monotropa*), catalposide from Bignoniaceae (*Catalpa*) and paeoniflorin from Paeoniaceae (*Paeonia*) gave good peaks whose retention times increase in proportion as the number of C-atoms in the compounds increases as shown below: aucubin (C_{15}, $t_R = 1.7$), monotropein (C_{16}, $t_R = 2.6$) and catalposide (C_{22}, $t_R = 11.1$). The gas chromatogram of sinigrin showed a major peak with an additional minor peak. At a column temperature of 175° three peaks, namely $t_R = 6.35$ (main), 4.8 (minor), 3.1 (minor), were observed.

Because of its great sensitivity and resolving power, it is suggested that this method will be useful for the analysis of medicinal plants and their constituents.

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The importance of column material in the gas chromatography of isocyanides

In gas chromatography, interferences caused by the material of the tube itself have received little notice until recently. For the analysis of chlorinated pesticides, quartz[1] or glass[2] tubes have been recommended instead of metal; glass is preferred for phosphorus compounds[3]. Glass is also safer for steroid analysis although metal may be used in a properly designed system[4]. On the other hand, all-glass is the only way to handle some pyrrolizidine alkaloids[5]. In these cases it appears that the metal surface catalyses decomposition.

We have observed that with aliphatic isocyanides glass tubing is satisfactory

but metal is not. Our results appear to explain the difficulty experienced by LIFSHITZ, CARROLL AND BAUER[6] when attempting quantitative analyses of methyl cyanide–isocyanide mixtures.

Using a Perkin-Elmer model 154 gas chromatograph with 1 m metal columns (carrier gas, helium; stationary phase, "Reoplex 400"; temperature, 90°), we obtained unsatisfactory chromatograms for methyl, ethyl, propyl and butyl isocyanides which varied with the use of different inert supports.

On acid-washed but otherwise untreated material (such as "Embacel"), no peak at all was seen for 0.5 μl samples. Many such samples (up to twenty) were injected at intervals equal to the expected retention time without observing a measurable peak. Injection of 2–5 μl samples gave badly tailed peaks at varying times after the expected retention time.

On "Chromosorb W-HMDS" (a hexamethyldisilazane-treated support) 0.5 μl samples produced small peaks at the correct retention time. When further samples were injected, the peak height increased. A total of approximately 4 μl (introduced in small or large injections) was required before the peak height of a 0.5 μl sample became constant. If a column subjected to such a series of samples were left with carrier gas passing through it overnight, a repetition of this behaviour was observed again next day. This suggests that the effect is due to a reversible adsorption. Similar effects were observed with both stainless steel and copper tubes.

Satisfactory chromatograms for sub-microgram quantities of alkyl isocyanides had been previously obtained using a Pye Argon chromatograph (4 ft. glass tube). When a glass tube was used in the Perkin Elmer instrument with either "Embacel" or "Chromosorb W-HMDS" as support, normal peaks were obtained with one 0.5 μl injection.

The improvement in performance of the metal tubes using a silanized support could be due to the partial coating of the metal wall by HMDS from the treated support. This could mean that metal tubes, if internally precoated with a suitable material, would give chromatograms comparable with those obtained using glass.

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A sampling device for viscous and solid materials for gas chromatography*

A number of devices have been reported for introducing samples of solids and difficult-to-handle materials into a gas chromatograph[1–4]. We have constructed a simple sampling device which can be used in conjunction with an independently heated injection port for sampling viscous materials and solids for gas chromatographic analysis. This device has a number of advantages. It is as easy to use as any

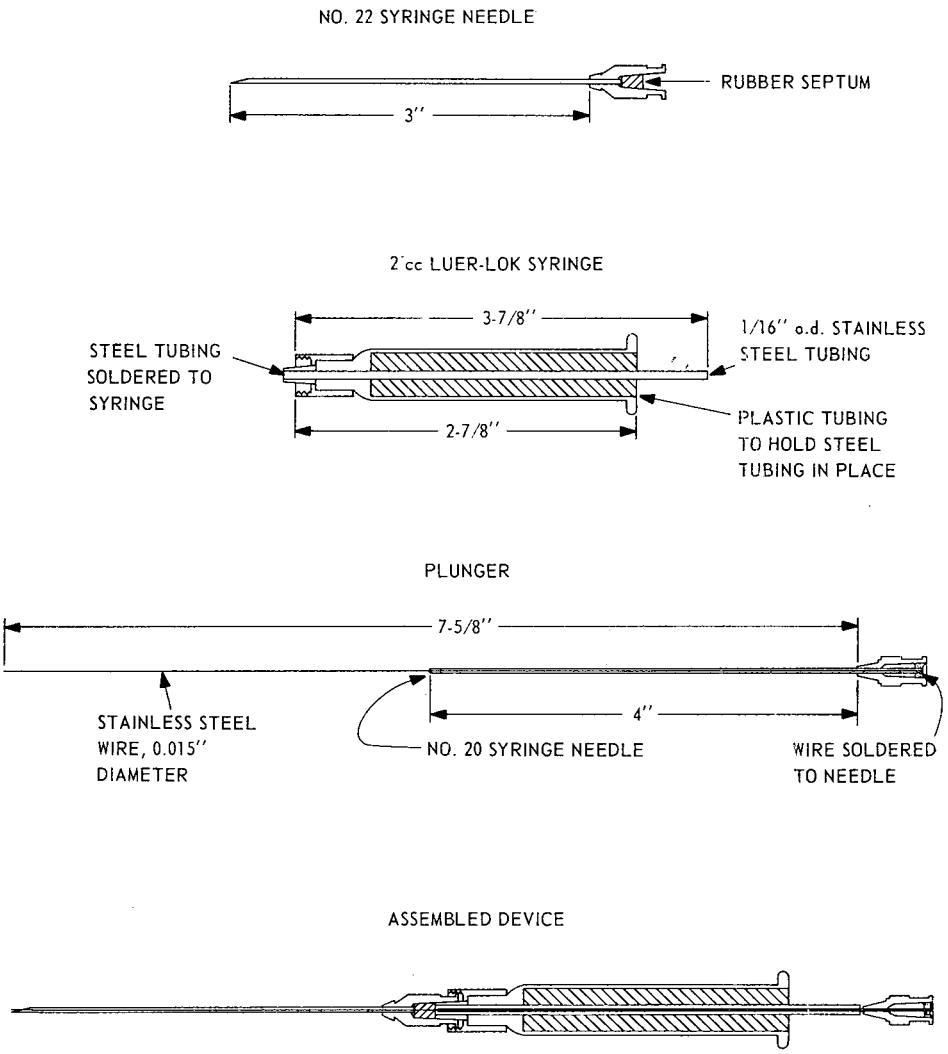

Fig. 1. Sampling device for viscous and solid materials.

* This work was supported in part by the U.S. Atomic Energy Commission, Contract No. AT(40-1)-2490.

other type syringe; it is not necessary to modify the gas chromatographic unit provided the injection port is heated independently; it is not necessary to dissolve the sample in a volatile solvent followed by evaporation of the solvent as the solution is applied to a plunger or trough[1,2]; the loss of volatile components can be minimized by taking samples through a rubber septum; and the flow of carrier gas is not interrupted. The device is shown in Fig. 1. It can be easily constructed from readily available materials with no instructions needed other than the details given in Fig. 1. Variations in the dimensions can be made to give a device with either a smaller or larger capacity. With a No. 22 syringe needle, samples up to about 10 λ can be taken. In practice, the sampling technique is to heat the sample so that it becomes less viscous or melts. After drawing a sample into the needle, the needle is immediately injected into the heated port. If necessary, a short period of time is allowed for the needle to heat up before the sample is ejected. In our work, samples of the molten material have been taken through a rubber septum so as to minimize any loss of the more volatile components present in small amounts. For quantitative work, internal standards have been used. A convenient manner for taking samples when only a small quantity of solid is available is to melt the sample in an ordinary melting point tube. The needle portion should be heated to approximately the same temperature before sampling.

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A multi-purpose device for the collection of fractions separated by gas chromatography

The collection of fractions emerging from a gas chromatographic column has been the subject of several publications in the past few years. Effective trapping of these fractions is necessary for their further identification, which can be done by re-running them on other columns, by spectrometric analysis, etc. Generally the collecting devices described are designed in such a way as to meet the demands associated with only one of the above methods for further identification.

In this note we wish to describe a multi-purpose device for the collection of fractions separated by gas chromatography. It is suitable both for the re-running of fractions on other columns and for their further identification by I.R., U.V. or mass spectrometry. In Fig. 1 a photograph is given of this device. It consists of a socket joint ($^{12}/_{1}$) to which a capillary (1 mm I.D.) is attached. The capillary is bent to give it a zig-zag shape. It ends in a glass tube of 4 mm I.D., which makes an angle of

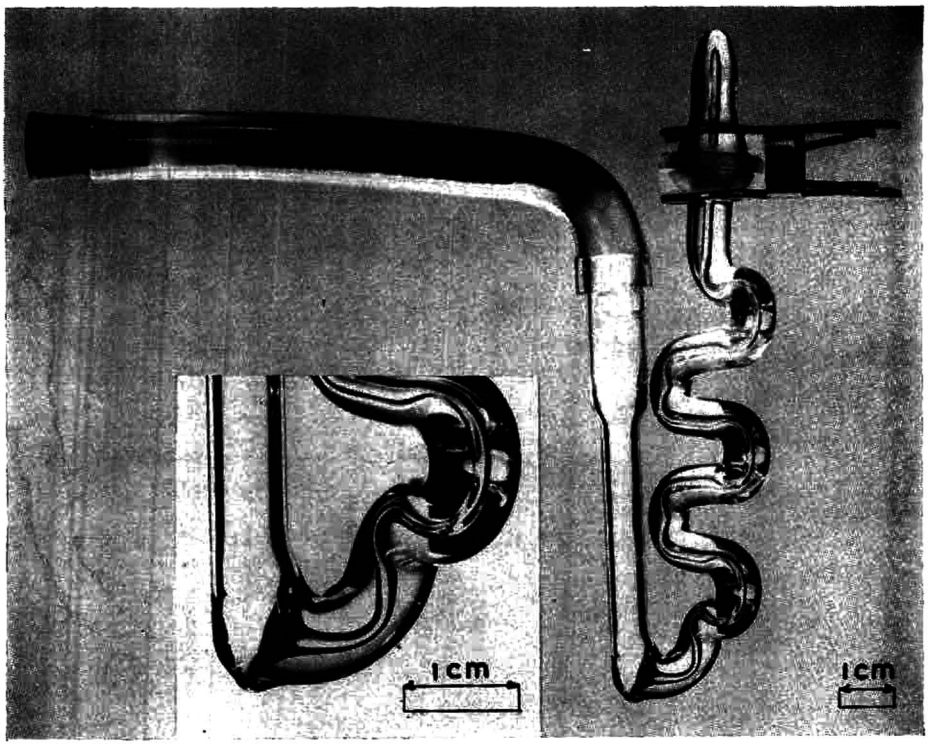

Fig. 1. Device for the collection of fractions separated by gas chromatography. It should be noted that the imaginary plane through the capillary is perpendicular to the plane through the glass tube and the drying tube.

approximately 90° with the capillary. At the junction between the capillary and the glass tube (see the enlarged detail in the photograph) the interior of the trap has a special shape: the bottom of the glass tube is pulled out to a very small cone which enables the almost quantitative removal of liquid from the trap by means of a syringe inserted through the glass tube. It should be noted that the imaginary plane through the capillary is perpendicular to the plane through the glass tube and the drying tube.

When using the trap, it is first of all fitted to a piece of polyethylene tubing filled with molecular sieve pellets or some other drying agent, kept in place with two pieces of cotton wool. It is flushed with pure dry nitrogen, after which the ends of the trap are closed with a ball joint and a rubber stopper respectively. Now the trap is ready to be cooled to the desired temperature in a Dewar flask which is usually filled with liquid nitrogen. As soon as a peak appears, the stoppers are removed and the trap is immediately connected with its socket to a ball joint attached to the outlet of the gas chromatography apparatus.

After collection of a fraction, the trap is closed again at both ends, after which it is brought to room temperature. The drying tube is removed and small portions (approximately 3 μl) of a suitable solvent are introduced by means of a micro-syringe via the top of the capillary. By carefully closing and opening the glass tube at the other side of the trap (which is easily done with a finger pressed on this opening) the

velocity of the solvent portions washing the capillary is easily regulated. The successive portions of solvent are collected with another micro-syringe which is inserted into the trap via the glass tube. The tip of the needle should be kept at the bottom of the conical end of the glass tube in order to remove all the solvent from the trap. The removal of a fraction out of the trap can be carried out very effectively in this way; in model experiments we found losses to be less than 1 %.

It is clear that fractions which are finally collected in this way in a micro-syringe (with a suitable solvent) can be used for further gas chromatographic analysis, for spectrometric studies as well as any other method for further identification.

The collecting efficiency for peaks emerging from a gas chromatographic column was tested with a number of aldehydes. The losses were determined by re-running the collected fractions on the gas chromatograph and measuring the surface area of the peaks. Results are given in Table I. It can be seen that the collecting

TABLE I

DETERMINATION OF THE COLLECTING EFFICIENCY OF THE DESIGNED TRAP FOR A NUMBER OF ALDEHYDES

Sample	*Amount applied* (μl)	*Trapping efficiency* (%)
n-Nonanal	2	80
n-Hexanal	2	80
n-Nonanal	0.2	85
n-Hexanal	0.2	80
n-Hexanal	0.05	83

efficiency is 80 % or more. (This figure includes the loss of less than 1 % which is due to the manipulations for removing a fraction from the trap.) Even samples as small as 0.05 μl could be collected efficiently.

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Einfache Volumenermittlung von Dosiermikropipetten

Als Probengeber für Flüssigkeiten werden bei vielen Gaschromatographen Mikropipetten benutzt (z.B. magnetisch gesteuerte Mikropipetten des Pye Argon-Chromatographen). Solche einfache Probeneinführung hat viele Vorteile. Man kann sehr geringe Mengen (bis zu 20 μg) Substanz bis auf die Oberfläche der Säulenfüllung dosieren, was die Leistungsfähigkeit der gaschromatographischen Methode bei analytischer Anwendung günstig erhöht. Sowohl die Menge der eingeführten Substanz, als auch die Art der Probeneinführung kann dabei mit genügender Genauigkeit reproduzierbar gehalten werden, was wieder die quantitative Auswertung der Chromatogramme günstig beeinflusst.

Die Glaspipetten können einfach im Labor angefertigt werden. Es sind offene, annähernd 10 mm lange Glaskapillaren von variablem inneren Durchmesser zwischen 0.05 und 0.12 mm, Aussendurchmesser um 0.5 mm.

Eine Volumenermittlung solcher Mikropipetten in üblichem Labor ist mit gewissen Schwierigkeiten verbunden. Die auf gewöhnliche Weise durchgeführte Quecksilberkalibrierung ist mühevoll und beansprucht eine Präzisionsmikrowaage, weil das Gesamtvolumen solcher Mikropipetten 0.02–0.10 μl beträgt. Eine gaschromatographische Vergleichsmethode der Volumenermittlung setzt schon eine geeichte Kalibrierungsmikropipette voraus.

Auf einfache Weise kann man das Volumen solcher Glasmikropipetten spektrofotometrisch feststellen. Prinzipiell wird dabei die hohe molare Extinktion einiger organischer, flüssiger Substanzen ausgenützt.

Wir haben zu diesem Zweck rektifiziertes Pseudojonon ($\varepsilon = 21.800$)[1] verwendet. Es ist aber klar, dass jede Substanz von genügend hoher molarer Extinktion benutzt werden kann.

Praktische Ausführung der Volumenermittlung

Durch Kapillarwirkungen wird Pseudojonon von sich selbst in die Mikropipette, welche in einen weichen Gummistöpsel am Ende eines Glasröhrchens eingesteckt ist, eingefüllt (Fig. 1). Das Äussere der Pipette wird sorgfältig gesäubert und danach auf ein Glasstäbchen, das in einen Messkolben von 10 ml Inhalt getaucht ist, durch ausblasen entleert. Auf gleiche Art und Weise wird dann die Mikropipette dreimal mit Äthylalkohol durchgespült und durch Abspülen des Glasstäbchens mit Äthanol der Messkolben bis zur Marke gefüllt.

Bei Mikropipetten von etwa 0.1 μl Inhalt wird die Extinktion in einer Schichtdicke von 0.5 cm und bei der Wellenlänge von 291 nm[1] gemessen*. Im Falle eines kleineren Volumens der Mikropipette wird eine grössere Schichtdicke gewählt. Den Leerwert stellt dabei die Extinktion der gleichen Schicht von Äthanol dar. Ebenso wird die Extinktion einer Äthanollösung von Pseudojonon bekannter Konzentration (etwa 0.5 mg/100 ml) bestimmt.

Die Berechnung des Pipettenvolumens ergibt sich aus der leicht ableitbaren Beziehung:

$$V = \frac{E_P \cdot c_V \cdot d_V \cdot V'}{100 \cdot \rho \cdot E_V \cdot d_P} \text{ (ml)}$$

* Alle Messungen durchgeführt mit dem Universal Spektrofotometer VSU 1 (Zeiss, Jena).

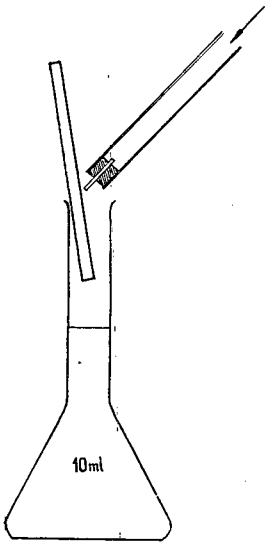

Fig. 1.

in der

V = Volumen der Mikropipette in ml,
E_P = Extinktion der Probelösung,
c_V = Konzentration der Vergleichslösung in g/100 ml,
d_V = Schichtdicke der Vergleichslösung in cm,
V' = Volumen des Messkolbens in ml,
ρ = spezifisches Gewicht der Testsubstanz,
E_V = Extinktion der Vergleichslösung,
d_P = Schichtdicke der Probelösung in cm.

Bei acht durchgeführten Volumenermittlungen einer ca. 0.10 μl Mikropipette wurden folgende Messwerte ermittelt:

0.109 0.108 0.110 0.111 0.112 0.110 0.111 0.113

was den Ist-Wert von 0.1105 $\pm$ 0.0016 μl darstellt.

Es ist ersichtlich, dass diese Methode ausreichend genaue Volumenermittlung ermöglicht.

Bei verhältnismässig kurzem Zeitaufwand ermöglicht sie auch eine höhere Anzahl der Mikropipetten im Vergleich mit derselben Standardlösung zu kalibrieren.

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Nachweis von Sterinen mit Perchlorsäure–Naphthochinon-Reagens auf Kieselgelschichten*

Das sehr empfindliche Phosphormolybdänsäure-Reagens zur Sichtbarmachung von Lipoiden hat bei der Auftrennung von ungereinigten oder teilweise gereinigten Lipoidextrakten den Nachteil, dass es relativ unspezifisch ist und keine selektiven Farbreaktionen mit einzelnen Verbindungsklassen zeigt[1]. Antimon(III)chlorid, das vielfach zum Nachweis von Steroiden herangezogen wird[2–6] und dessen Nachweisgrenze für Cholesterin im entwickelten Chromatogramm in derselben Grössenordnung liegt wie die des Phosphormolybdänsäure-Reagenses, gibt auch eine positive Reaktion mit anderen fettlöslichen Verbindungen, besonders mit Carotinoiden und Vitamin A[7,8]. Die in der Dünnschicht-Chromatographie zur Anwendung kommenden anorganischen Adsorbentien machen auch die Verwendung aggressiver Reagentien mit anschliessendem Erhitzen zur Identifizierung von Substanzen möglich. Auf dem Steroidgebiet sind ausser dem LIEBERMANN-BURCHARD-Reagens 70%ige Perchlorsäure oder 98%ige Schwefelsäure[9], 50%ige Schwefelsäure[10], Kaliumbichromat–Schwefelsäure[11] und Chlorsulfonsäure–Eisessig[5,6,12,13] zum Nachweis verwendet worden. Die Empfindlichkeit der Farbreaktion dieser Reagentien ist jedoch nur für Essigsäureanhydrid–Schwefelsäure und Chlorsulfonsäure–Eisessig ausserordentlich gross.

In diesem Laboratorium wurde zum Nachweis von Sterinen in Dünnschicht-Chromatogrammen mit Erfolg das von ADAMS[14] für histologische Zwecke entwickelte Perchlorsäure–Naphthochinon-Reagens verwendet. Dieses Reagens zeigt zwar wie das Vanillin–Schwefelsäure-Reagens mit Sterinen im U.V.-Licht keine Fluorescenz, hat aber den Vorteil:

(a) dass es mit 3β-Hydroxy-5-en- und 3β-Hydroxy-5,7-dien-Sterinen und ihren Estern eine spezifische Blaufärbung von starker Intensität ergibt,

(b) dass die Reaktion ausserordentlich empfindlich ist,

(c) dass andere in Lipoidextrakten auftretende Verbindungen diese Blaufärbung nicht zeigen,

(d) dass der Farbton für längere Zeit beständig ist, wenn die Platten in einem Exsikkator über einem Trockenmittel aufbewahrt werden,

(e) und dass das Reagens selbst für einige Wochen haltbar ist.

Fig. 1 zeigt an Hand von Cholesterin einen Vergleich der Empfindlichkeit des Perchlorsäure–Naphthochinon-Reagenses mit der gebräuchlicher empfindlicher Sprühreagentien für Steroide. Wie zu erkennen ist, liegt die Nachweisgrenze von Cholesterin mit Perchlorsäure–Naphthochinon-Reagens (III) im entwickelten Chromatogramm bei 0.03 μg. Diese hohe Empfindlichkeit wird von 40% Orthophosphorsäure nur bei Nachbehandlung mit Phosphormolybdänsäure (I) erreicht. TSCHESCHE UND SNATZKE[13] geben als Nachweisgrenze für Cholesterin mit Chlorsulfonsäure–Eisessig 0.025 μg an. Der Nachweis von Cholesterin mit den anderen untersuchten Reagentien scheint etwas weniger empfindlich zu sein. Für Antimon(III)chlorid–Eisessig (II) liegt die Nachweisgrenze mit 0.05 μg in demselben Bereich, den auch SNATZKE[6] für das Anfärbeverfahren von Cholesterin mit Antimon(III)chlorid und

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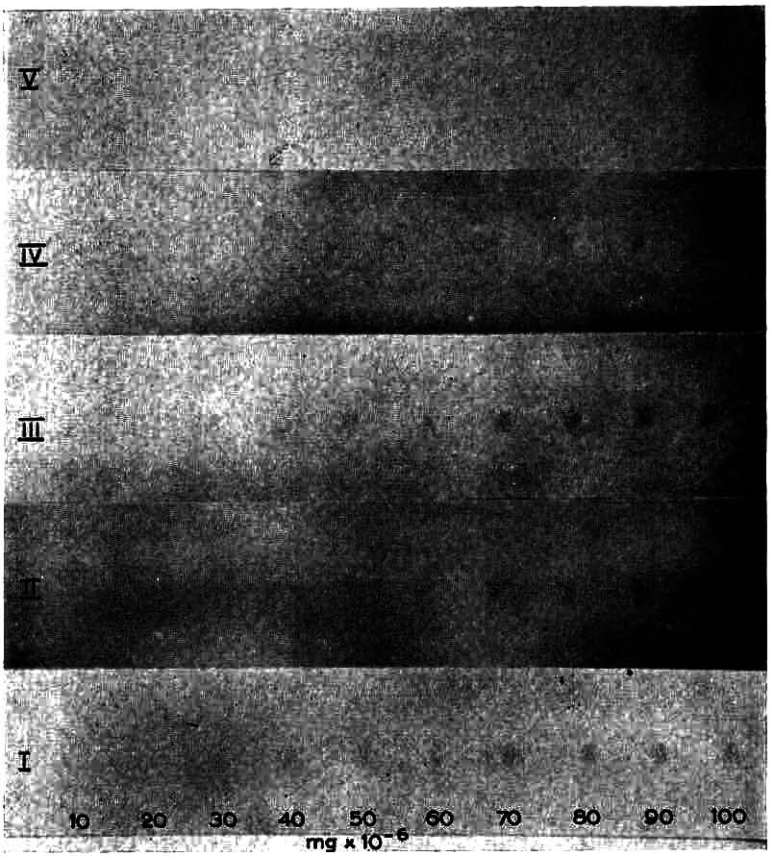

Fig. 1. Nachweisgrenze verschiedener Sprühreagentien für Cholesterin im entwickelten Chromatogramm. Adsorbens: Aktive Kieselgel G-Schichten nach den Standardbedingungen bereitet. Fliessmittel: Chloroform–Aceton (80:20). Laufzeit: 30 Min. R_F-Wert: 0.53. Anfärbung: I = 40% Orthophosphorsäure, nachbehandelt mit 1.5% alkoholischer Phosphormolybdänsäure; II = Antimon(III)chlorid–Eisessig; III = Perchlorsäure–Naphthochinon-Reagens; IV = Vanillin–Schwefelsäure; V = Essigsäureanhydrid–Schwefelsäure.

Antimon(V)chlorid in Chloroform feststellte. BENNETT UND HEFTMANN[10] geben als Nachweisgrenze für Sterine mit 50%iger Schwefelsäure 0.1 μg, DAVÍDEK UND BLATTNA[9] für Vitamin D_2 mit 70%iger Perchlorsäure 3 μg an.

Auf Grund seiner Spezifität und grossen Empfindlichkeit gelang es, mit dem Perchlorsäure–Naphthochinon-Reagens geringe Mengen Sterin in Lipoidextrakten, die in dem Lösungsmittelgemisch von WAGNER *et al.*[15,16] chromatographiert worden waren, einwandfrei zu identifizieren. In diesem Fliessmittel wandern die schwach polaren Sterine dicht unter der Lösungsmittelfront. Der R_F-Wert betrug im Mittel aus 21 Trennungen 0.92. Von den mit der Front wandernden Neutralfetten liessen sich die Sterinflecken sicher unterscheiden, da Triglyceride mit dem Perchlorsäure–Naphthochinon-Reagens eine Braunfärbung ergeben. Natürliche Lecithine und Kephaline zeigen gelbe bis braune Farbtöne, während synthetisches β,γ-Dipalmitoyl-L-α-lecithin (Sigma Chemical Company) völlig farblos bleibt. Auch bei Auftrennung

von Lipoidextrakten in weniger polaren Fliessmitteln erscheinen auf mit dem Perchlorsäure–Naphthochinon-Reagens besprühten Platten lediglich die Sterine als blaue Flecken. Freie Fettsäuren bleiben farblos oder zeigen eine schwache Gelb- bis Braunfärbung und auch Glyceride erscheinen gelb bis braun.

Methodik

Herstellung des Reagenses. 100 mg 1,2-Naphthochinon-2-sulfonsäure werden in 100 ml eines Gemisches Äthanol 60% Perchlorsäure–40% Formaldehyd–Wasser (2:1:0.1:0.9, V/V) gelöst.

Anwendung. Nach dem Chromatographieren werden die Platten getrocknet, um das Fliessmittel aus den Schichten zu entfernen. Dann wird mit dem Reagens besprüht bis die Schichten gleichmässig durchfeuchtet sind. Durch anschliessendes Erhitzen bei 70–80° werden die Sterine sichtbar gemacht. Während des Erhitzens beobachtet man die Farbtöne. Die Sterinflecken erscheinen zuerst rosa. Bei weiterem Erhitzen vertieft sich der Farbton und geht allmählich in blassblau und dunkelblau über. Wird zu lange erhitzt, werden die Flecken braunschwarz. Die Geschwindigkeit der Farbentwicklung ist temperaturabhängig. Je höher die Temperatur ist, um so schneller verläuft die Entwicklung. Temperaturen über 100° sollten möglichst ver-

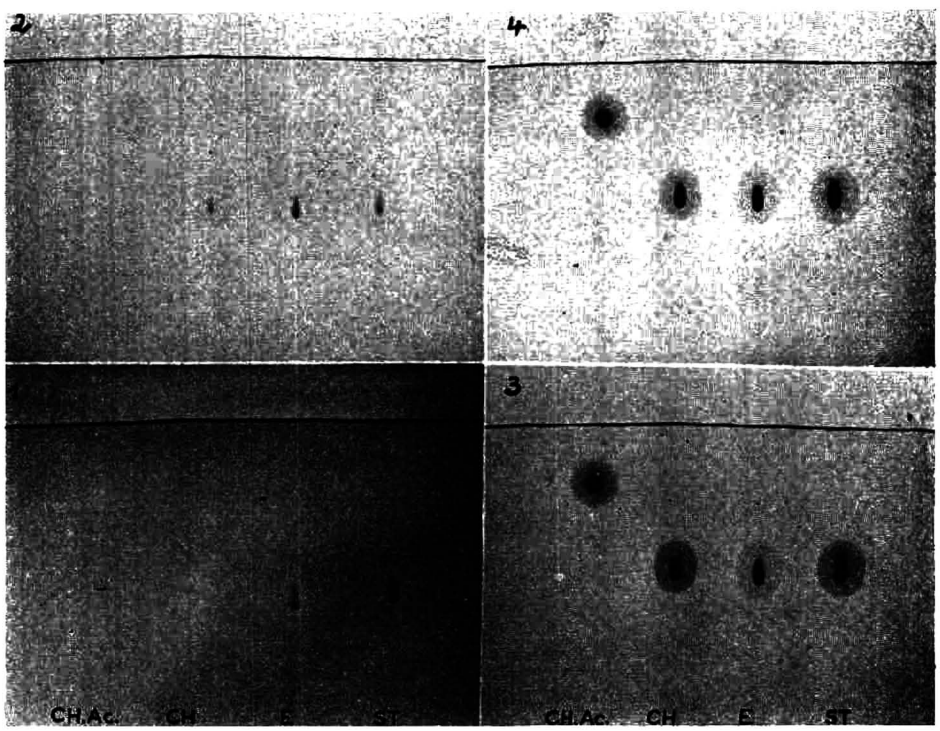

Fig. 2. Farbentwicklung verschiedener Sterine mit Perchlorsäure–Naphthochinon-Reagens im entwickelten Chromatogramm. Adsorbens: Aktive Kieselgel G-Schicht nach den Standardbedingungen bereitet. Fliessmittel: Chloroform–Aceton (80:20). Laufzeit: 30 Min. Aufgetragen: je 50 µg CH. Ac. = Cholesterylacetat (R_F = 0.81); CH. = Cholesterin (R_F = 0.52); E. = Ergosterin (R_F = 0.51); ST. = Stigmasterin (R_F = 0.52).

TABELLE I

FARBENTWICKLUNG VERSCHIEDENER STERINE MIT PERCHLORSÄURE–NAPHTHOCHINON-REAGENS

Sterin	*Nach 15 Min.*	*Nach 30 Min.*	*Nach 45 Min.*	*Nach 60 Min.*
Ergosterin	tief rosa, bereits schwache Blaufärbung	tief blau	braunschwarz	tief braunschwarz
Stigmasterin	rosa	tief rosa, beginnende schwache Blaufärbung	blau	tief blau
Cholesterin	schwach rosa	rosa	tief rosa mit blauer Färbung	blau
Cholesterylacetat	kaum sichtbar	schwach rosa	tief rosa mit schwacher Blaufärbung	blassblau

mieden werden, da sonst der Übergang der Färbung in braunschwarz so schnell verläuft, dass die rosa und blauen Farbtöne kaum noch registriert werden können.

Fig. 2 veranschaulicht die Farbentwicklung von drei Sterinen und einem Sterinester mit Perchlorsäure–Naphthochinon-Reagens in einem Chromatogramm, das nach dem Besprühen bei 60° erhitzt und in Zeitabständen von 15 Min. photographiert wurde (Tabelle I).

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The use of chromatographic procedures in the synthesis of ^{14}C-labelled phosphodiesters

L-Serine ethanolamine phosphodiester (L-SEP) and L-threonine ethanolamine phosphodiester (L-TEP) are natural phosphates which have been recently crystallized from the tissues of many animal species[1–7]. Much attention has been paid to L-SEP and L-TEP, since it is thought they may be involved in some biosynthetic reactions of phospholipid metabolism[2,4–6], and have in addition a clear evolutionary role.

A method of organic synthesis for ^{14}C-labelled L-SEP and L-TEP has been described earlier by us[8,9] and we now wish to report briefly on the use of the ion-exchange chromatography procedures and thin-layer chromatography techniques which have enabled us to obtain pure crystalline material and to control easily and quickly the process of separation and isolation of the labelled phosphodiesters.

Experimental and results

The synthesis of crude ^{14}C-L-serine ethanolamine phosphate and ^{14}C-L-threonine ethanolamine phosphate has been reported elsewhere[8,9]. The hydrogenolysis of the neutral phosphate ester mixture was carried out as previously described[8] and subjected to separation and purification as follows.

After removal of the catalyst and addition of suitable amounts of 10% NaOH to give a pH of 1.8–2.0, the mixture was concentrated and analyzed at this stage by thin-layer chromatography and radioautography where the single clearly defined components of the phosphate ester mixture were checked. Desalting was then carried out through a column (2.05 × 9.2 cm) of Dowex 50 W × 4 (H^+ form, 200–400 mesh), until the conductivity of the eluate had almost disappeared; the adsorbed material was then eluted with 2 *N* aqueous ammonia, which was removed by evaporation in a small rotary evaporator. The concentrated eluate was again tested by thin-layer chromatography and radioautography.

The eluate was now transferred to a column (2.25 cm × 123 cm) of finely graded[10] Dowex 50 W resin (15–40 μ particle size diameter), NH_4^+ form, and allowed to pass through at a rate of about 30 ml/h, with water as eluant. A complete separation of the various labelled and non-labelled compounds was achieved with only one treatment (Fig. 1). Rapid screening of alternate fractions by thin-layer techniques revealed the chromatographic purity of each peak. With regard to the synthesis of L-TEP, a satisfactory but incomplete separation of the mixture was obtained by this procedure (Fig. 2), although this phosphodiester was well separated from the other components.

The final steps of the purification process were the evaporation nearly to dryness of the relevant fractions, the crystallization of the dry residue and the further purification of the crystalline material, as described elsewhere[4,5,8,9,11].

At the end of the process the labelled crystalline L-SEP and L-TEP showed the same physical and chemical properties (melting point, optical rotation, infrared spectra, hydrolysis products, R_F values and chemical composition) as the unlabelled corresponding products synthesized previously[3–5,11]. In addition, other labelled compounds have been isolated, purified and characterized by this procedure, *e.g.* L-serine-L-serine phosphodiester (SSP) (Fig. 1), which is a by-product in the synthesis of L-SEP, and what is also most probably L-threonine-L-threonine phosphodiester (TTP) (Fig. 2),

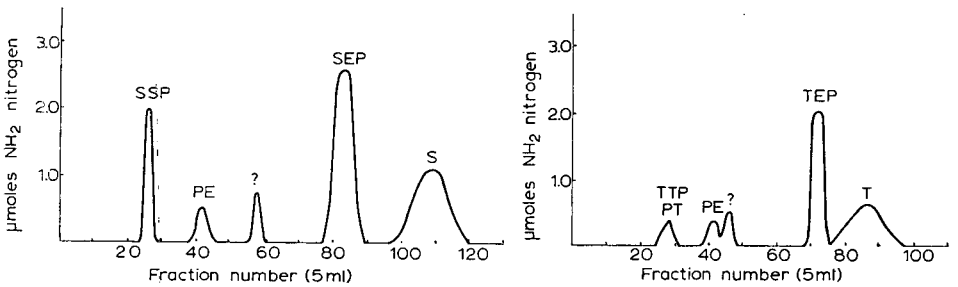

Fig. 1. Synthesis of L-SEP. Ion-exchange chromatogram on a Dowex 50 W resin column, NH_4^+ form, of the reaction mixture after desalting. SSP = L-serine-L-serine phosphodiester; PE = phosphorylethanolamine; SEP = L-serine ethanolamine phosphodiester; S = serine.

Fig. 2. Synthesis of L-TEP. Ion-exchange chromatogram on a Dowex 50 W resin column, NH_4^+ form, of the reaction mixture after desalting. TTP = L-threonine-L-threonine phosphodiester; PT = phosphorylthreonine; PE = phosphorylethanolamine; TEP = L-threonine ethanolamine phosphodiester; T = threonine.

which is presumably a by-product in the synthesis of L-TEP. SSP was obtained as a dry powdery product after numerous evaporations of the homogeneous chromatographic fractions, but TTP was contaminated with some traces of phosphorylthreonine even after the final purification steps.

The chromatographic procedures briefly outlined in this note provide a simple and rapid way of purifying labelled synthetic L-SEP, L-TEP, L-SSP and L-TTP from their reaction mixtures. The yields of these phosphodiesters were found to be satisfactory.

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Beitrag zur Verwendung extrem kleinvolumiger Trennkammern in der Dünnschichtchromatographie*

Bei der Entwicklung einer neuen Bestimmungsmethode für Test steron haben wir für die Abtrennung von Steroidhydrazonen aus Urinextrakten neben der Entwicklungskammer von Desaga (Heidelberg) auch die Sandwich-Kammer (Camag, Muttenz/Schweiz, vgl. Zit. 1) benutzt. Der Lauf in der Sandwich-Kammer erwies sich nach wenigen Zentimetern für unsere Zwecke trotz der Sättigung der Gegenplatte mit dem System Chloroform–Aceton (9:1) als nicht befriedigend. Eine Verbesserung, die darin besteht, dass das nach JÄNCHEN[1] vorbereitete Plattenpaar vor dem Lauf an drei Seiten jeweils 0.5 cm tief in geschmolzenes Wachs getaucht wurde, erwies sich als brauchbar. Wir konnten bei der Isolierung des Testosteronhydrazons durch Einsatz von 20 × 35 cm grossen Platten die bei Verwendung der üblichen Desaga Technik (20 × 20 cm) notwendige zweite Chromatographie weglassen. Wir möchten noch erwähnen, dass die Platten nicht geklammert zu werden brauchen. Sofort nach dem Lauf empfiehlt es sich, zur Erhaltung des scharfen Trenneffektes, die Platten voneinander zu lösen.

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Separation of carbohydrates on borate-impregnated silica gel G plates

Thin-layer chromatography has been used extensively in the separation of saccharides and their derivatives[1–9]. The separation of D-glucose from D-galactose in the presence of other sugars has, nevertheless, remained a problem. According to STAHL AND KALTENBACH[1], many sugars separate better on kieselgur than on silica gel; however, on kieselgur the separation of D-glucose from D-galactose is unsatisfactory.

Silica gel G plates prepared with 0.1 *N* boric acid solution have been used in thin-layer chromatography for separating a limited number of saccharides[2–4]. We have found that a good separation of D-glucose from D-galactose as well as of other carbohydrates can be easily achieved on 0.02 *M* borate buffer (pH 8.0) impregnated silica gel G plates using a mixture of 1-butanol–acetic acid–water (5:4:1) as the developing system.

Experimental

Reagents. Borate buffer, 0.02 *M*, pH 8.0: 100 ml 0.02 *M* boric acid solution (pH 5.9) and 3.0 ml of 0.02 *M* sodium tetraborate solution (pH 9.3). Reagent grade powders from Matheson, Coleman and Bell Chemical Company.

α-Naphthol spray reagent: 10.5 ml α-naphthol (15 % solution in 95 % ethanol w/v), 6.5 ml concentrated sulfuric acid, 40.5 ml 95 % ethanol and 4.0 ml water.

Procedure. Thirty grams of silica gel G (Merck) was manually shaken in a closed jar with 60 ml of borate buffer 60–90 sec. Glass plates (20 × 20 cm) were coated with the slurry to a 0.25–0.275 mm thickness using a Desaga applicator. Good separations were also achieved on plates prepared by the method of LEES AND DEMURIA[10]. The plates were kept at room temperature until they set and were then dried for 30 min at 100°. They were stored at room temperature and activated for 30 min at 100° before use.

A Hamilton microsyringe (Hamilton Company, Inc., Whittier, Calif.) was used to spot the samples, and a warm stream of air from a hair dryer or heat gun was used to dry the spots. The application is carried out by spotting about 0.5 ml, drying, and repeating this procedure until the desired volume is put on the plate. Spots of 1–50 μl were applied.

The plates were allowed to develop by ascending chromatography to a height of 10 cm in closed glass tanks containing 1-butanol–acetic acid–water (5:4:1) as the solvent system. The average development time at 20° was 60–70 min (it was unnecessary to have a saturated atmosphere in the tank). The plates were dried at 100° for 10–15 min, cooled to room temperature, and either returned to the solvent tank for a second run or sprayed with the α-naphthol solution.

The sprayed plate was heated for 3–6 min at 100°. Most of the sugars appeared as blue spots on a light tan background. Deoxyribose gave a gray spot and rhamnose an orange spot. The colors were stable at room temperature for 2–3 days; the only noticeable change was a darkening of the spots on standing.

Results and discussion

Tables I–VI give the R_F values and colors of the separated sugars. The method was effective for saccharide mixtures where the individual sugar concentration varied from 1 to 30 μg.

TABLE I

SEPARATION OF SACCHARIDES SHOWING R_F VALUES FOR FIRST AND SECOND DEVELOPMENT IN THE SAME SOLVENT

Saccharide	R_F*	R_F**	*Color*
Stachyose	0.05	0.15	Blue
Raffinose	0.10	0.25	Blue
Sucrose	0.30	0.45	Blue
D-Galactose	0.35	0.55	Blue
D-Glucose	0.40	0.65	Blue
D-Xylose	0.50	0.75	Blue
L-Rhamnose	0.55	0.80	Orange
Phenyl-β-D-glucopyranoside	0.65	0.95	Blue

* Developed once in 1-butanol–acetic acid–water (5:4:1).
** Developed twice in the above system with intermittent drying.

TABLE II

SEPARATION OF D-MANNOSE FROM D-XYLOSE IN THE PRESENCE OF OTHER SACCHARIDES

Saccharide	R_F	*Color*
Stachyose	0.05	Blue
Raffinose	0.10	Blue
Sucrose	0.30	Blue
D-Mannose	0.40	Blue
D-Xylose	0.50	Blue
Phenyl-β-D-glucopyranoside	0.65	Blue

TABLE III

SEPARATION OF RIBOSE FROM DEOXYRIBOSE IN THE PRESENCE OF OTHER SACCHARIDES

Saccharide	R_F	*Color*
Stachyose	0.05	Blue
Raffinose	0.10	Blue
Maltose	0.30	Blue
Ribose	0.40	Blue
Deoxyribose	0.50	Gray
Phenyl-β-D-glucopyranoside	0.65	Blue

TABLE IV

SEPARATION OF D-FRUCTOSE FROM D-XYLOSE IN THE PRESENCE OF OTHER SACCHARIDES

Saccharide	R_F	*Color*
Stachyose	0.05	Blue
Raffinose	0.10	Blue
Sucrose	0.30	Blue
D-Fructose	0.40	Blue
D-Xylose	0.50	Blue

TABLE V

SEPARATION OF LACTOSE FROM MALTOSE IN THE PRESENCE OF D-ARABINOSE AND D-XYLOSE

Saccharide	R_F*	*Color*
Lactose	0.35	Blue
Maltose	0.45	Blue
D-Arabinose	0.65	Blue
D-Xylose	0.73	Blue

* Developed twice in 1-butanol–acetic acid–water (5:4:1).

TABLE VI

SEPARATION OF OLIGOSACCHARIDES IN THE PRESENCE OF D-GALACTOSE AND D-MANNOSE

Saccharide	R_F*	*Color*
Maltoheptaose	0.00	Blue
Maltopentaose	0.10	Blue
Maltotetraose	0.15	Blue
Maltotriose	0.25	Blue
Maltose	0.40	Blue
D-Galactose	0.52	Blue
D-Mannose	0.60	Blue

* Developed twice in 1-butanol–acetic acid–water (5:4:1).

The results in Table I show a separation where the concentration of individual sugars in the mixture varied from 2.5 to 12.5 μg. The higher R_F values in this table were obtained when the plate was run twice in the same solvent with intermittent drying.

The separation of the given saccharides was best accomplished on 0.02 *M* borate (pH 8.0) impregnated plates. Silica gel G in water or a mixture of silica gel G and kieselgur (1:1) impregnated with borate buffer (0.02 *M*, pH 8.0) gave poorer separations. The results were also unsatisfactory when pH 8.5 (0.02 *M*) borate buffer or unactivated plates were used.

An optimum separation of D-glucose from D-galactose was achieved by running the plate twice to the 10 cm mark in 1-butanol–acetic acid–water (5:4:1) with intermittent drying. This entire operation takes 2–3 h whereas a separation of D-glucose from D-galactose in a saccharide mixture may take days if it is to be done by paper chromatography.

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An artefact in the chromatographic analysis of 2,4-dinitrophenylhydrazones of keto acids

The present study arose from a chance observation during our work on the production of keto acids by incubated brain homogenates from normal and ethanol intoxicated rats (Fig. 1). Because an unknown substance *x*, which separated from the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid, seemed to be related to the effects of ethanol, we decided to collect it in amounts sufficient for identification. The complete separation of substance *x* was achieved when the lid of the chromatographic vessel was left slightly ajar toward the end of the run for the evaporation of the solvent. The unknown substance was not detected in the preparations from other sources (urine, blood, liver and muscle). Even in different experiments with brain the

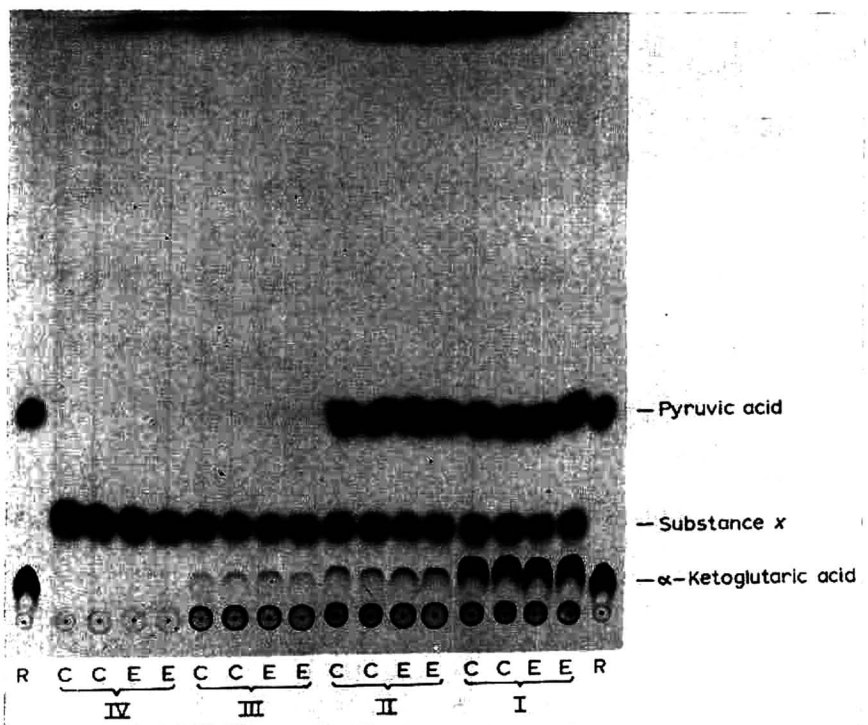

Fig. 1. Thin-layer chromatogram of 2,4-dinitrophenylhydrazones of acid carbonyl compounds of brain homogenates, incubated under various conditions (photographed in U.V. light). E = samples from ethanol intoxicated rats; C = samples from control rats; R = references; I = homogenates incubated for 60 min at + 37° in the presence of glucose and glutamic acid; II = in the presence of glucose; III = without glucose or glutamic acid; IV = not incubated at all. Otherwise the incubation medium was that of DiPietro and Weinhouse[1] with slight modifications. The preparation and isolation of the acid hydrazones were based on the work by Seligson and Shapiro[3]. The plates were coated with "Silica gel G according to Stahl" (E. Merck AG, Darmstadt, No. 7731). The powder (30 g) was suspended in 60 ml of propionic acid–water mixture (1:2, v/v), spread with the applicator to a 0.2 mm nominal thickness, and heated at + 110° for 30 min. The solvent system consisted of petroleum ether–ethyl formate–propionic acid (65 ml:35 ml:0.1 moles)[2]. The run lasted at room temperature for 3–4 h. When the front had reached the top of the plate, the lid was left slightly ajar.

amount of substance x was variable, sometimes almost negligible, and it was unstable at room temperature.

The spot containing the unknown substance x was scraped off from several chromatograms, and eluted with water, ethanol or dioxane–phosphate buffer[2] for rechromatography.

The unknown substance was rather pale yellow, and it was best observed in ultraviolet light. It had two absorption maxima: the first at 278 mμ, with no shift in N HCl, 10 % Na_2CO_3 (w/v) and 5 % NaOH (w/v), the second absorption maximum was at 340 mμ in HCl, at 410 mμ in Na_2CO_3 and at 400 mμ in NaOH. In contrast to the 2,4-dinitrophenylhydrazones of the keto acids, the absorption was not increased in NaOH by comparison with the colour in Na_2CO_3.

The 2,4-dinitrophenylhydrazones of keto acids yield on reduction the corresponding amino acids[4,5]. Therefore several attempts were made to reduce the unknown substance x to identifiable products. Treatment with tin and hydrogen chloride gas[6] rapidly yielded a ninhydrin-positive product but only ammonia could be identified. Hydrogenation in the presence of Raney nickel or of Adams' catalyst produced ninhydrin-positive substances from the 2,4-dinitrophenylhydrazone of α-ketoglutarate (glutamic acid with Raney nickel, and glutamic and γ-aminobutyric acid with Adam's catalyst) and from the 2,4-dinitrophenylhydrazone of pyruvate (alanine), but

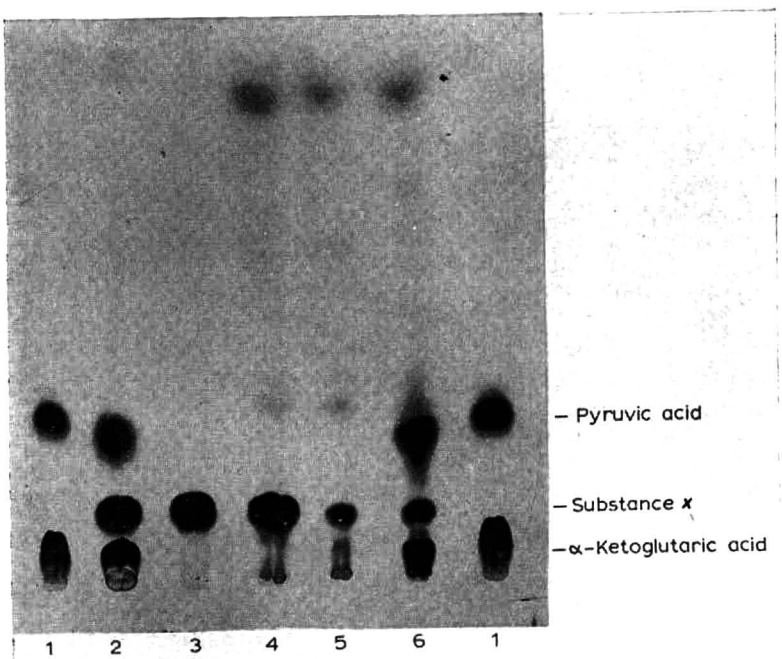

Fig. 2. Thin-layer chromatogram of mixed samples including the substance x. Conditions were the same as explained in the legend of Fig. 1. 1 = reference containing 2,4-dinitrophenylhydrazones of pyruvic acid and α-ketoglutaric acid; 2 = reference mixed with substance x derived from 2,4-dinitrophenylhydrazine itself; 3 = substance x derived from 2,4-dinitrophenylhydrazine; 4 = substance x derived from 2,4-dinitrophenylhydrazine mixed with substance x from brain homogenate; 5 = substance x from brain homogenate; 6 = substance x from brain homogenate mixed with reference.

none from the unknown substance in spite of several attempts under different conditions. 2,4-Dinitrophenylhydrazine itself gave ninhydrin-positive substances (presumably ammonia and aniline) on reduction with tin and hydrochloric acid.

When 2,4-dinitrophenylhydrazine was treated similarly to the 2,4-dinitrophenylhydrazones of keto acids, *i.e.*, by repeated extractions with ethyl acetate, sodium carbonate solution and (after acidification) again with ethyl acetate, the final product yielded the unknown substance *x* on chromatography (Fig. 2, sample 3). The spectrum (in the range 320–700 mμ) of the substance *x*, which had been derived from pure 2,4-dinitrophenylhydrazine, resembled that of the original substance *x*. When 2,4-dinitrophenylhydrazine was dissolved in *N* HCl, 10% Na_2CO_3, 5% NaOH, dioxane–phosphate buffer or ethyl acetate, the absorption maxima of substance *x* were not observed, even after storage of the solutions.

We concluded that the substance *x* was an artefact arising from the 2,4-dinitrophenylhydrazine during the manipulations and therefore further work for the identification was abandoned.

It remains to be explained, why in the first experiments less of the unknown substance *x* was formed on incubation of the brain homogenates from the ethanol intoxicated rats. The presence of substances in the homogenates which affect the formation of this artefact from the hydrazine during the repeated extractions must be assumed.

The question also arises, whether this artefact has to be accounted for in the routine chromatographic analyses of 2,4-dinitrophenylhydrazones of keto acids. If the artefact appears as a clearly separated spot, it might be confused with the 2,4-dinitrophenylhydrazones of other acid carbonyl compounds. However, as a rule it does not separate from the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid. The colour yield of the unknown substance in NaOH solution is comparatively low, but in an unfavourable case it may be noticeable. For accurate analyses of α-ketoglutaric acid as its 2,4-dinitrophenylhydrazone, the possibility of this artefact should be kept in mind.

We wish to thank Miss LEENA LAUKALA for her skillful technical assistance.

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Urea clathrates of fatty acids in thin-layer and paper chromatography

Clathrate formation has been suggested as a method of fractionation in chromatography and CASON *et al.*[1] succeeded in separating straight chain from branched chain fatty acids by means of urea columns. Though clathrate formation cannot be taken as a general method of fractionation, its application to thin-layer and paper chromatography of fatty acids may be of certain interest and provide a quick evaluation of the nature of an unknown acid and achieve separations which may simplify the determinations of complex mixtures of various acids.

The investigation has been carried out by means of cellulose papers impregnated with urea and with urea–calcium sulphate thin layers. The former were strips of cellulose paper (Whatman No. 3) impregnated with a 20 % methanolic solution of urea and dried under normal atmospheric conditions, and the latter glass plates coated with a mixture of urea and calcium sulphate. Urea thin layers cannot be obtained unless a binding material such as calcium sulphate is used. The binding agent (25 g) was mixed in a mortar with 60 ml urea solution to make a paste which was then spread on glass plates. Uniform and smooth layers were obtained when 20 to 40 % urea was used with the above amount of calcium sulphate. The plates were dried at room temperature.

The chromatograms were run at room temperature by the ascending technique. After the eluant had reached a certain height, the strips were taken out of the chromatographic vessel and the acids detected as yellow spots on a blue background by spraying with alcoholic bromocresol purple and exposing for a short time to ammonia vapours.

A large number of acids were investigated. Identical results were obtained with the impregnated papers and the chromatoplates. The main features of the investigation are summarized below:

1. The fatty acids up to C_{10} moved towards the front. Similar behaviour was observed when the clathrate forming material (urea) was not used.

2. Fatty acids with a number of carbon atoms higher than C_{16} did not travel at all and stayed at the starting point. Lauric and myristic acids were found near the starting point.

3. *Trans* and *cis* fatty acids could be separated as the former were clathrated easily while the latter did not enter the clathrate channels with ease. This was observed using a mixture of elaidic and oleic acids which yielded two spots, one for elaidic acid at the starting point and the other for oleic acid travelling almost with the front. Other unsaturated fatty acids (linoleic, linolenic, etc.) behaved in the same way.

4. Oxyacids, with short or long carbon chains, were not clathrated, *e.g.* ricinoleic, citric, tartaric acids etc. travelled with the front.

5. The introduction of a certain group in an organic molecule did not affect the efficiency of clathrate formation, no matter what kind of functional group, *e.g.* hydroxyl, carbonyl or halogen, was present. The longer the C chain the better the clathration. If there was branching or a cyclic ring at the end of an aliphatic chain the formation of the clathrate depended on the chain length and unsaturation.

Chromatography thus provides another method of clathrate formation besides the already existing conventional solution techniques, in which clathrates are prepared by addition of a small amount of the compound to be clathrated to a saturated

methanolic solution of urea. When there was no clathrate formation, the guest molecule remained a free-moving sample and moved along with the front. Clathrate formation is not considered to be a stepwise equilibrium reaction but a molecular addition between the host and the guest molecules where the shape and size of the components are the determining factors.

The results clearly show that oleic and the lower saturated fatty acids could be separated from other saturated fatty acids, at room temperature, by taking advantage of the preferential formation of urea clathrates by the higher saturated components of the mixture. Separation of fatty acid is mainly concerned with chain length and unsaturation, while branched chains are seldom involved. The difference in clathrate formation is particularly pronounced with acids up to capric acid and from lauric to acids with longer chains.

It is interesting to note the different behaviour of oleic and elaidic acids. The *cis* double bond seems to cause a slight distortion in the long hydrocarbon chain and thus increases the diameter to a point where the fatty acid will not easily enter the host molecule as a planar molecule without considerable bending. Thus, this slightly greater spatial requirement in oleic acid differentiates it from other fatty acids in clathrate formation.

We suggest that the urea clathrate formation (or in general, this clathrate technique) may be successfully employed for fractionation of mixtures of organic acids as it gives a fairly sharp separation into two classes of acids.

We also investigated fatty acids using a 20 % methanolic solution of thiourea. This clathrating agent shows a similar selectivity, reacting easily with cyclohexane or branched chain molecules but not with straight chain molecules. The fatty acids moved to the top of the thin layers and paper chromatostrips, and no clathrate formation was observed. This permits comparison of urea and thiourea for fractionations of the same compounds.

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Thin-layer chromatography of aromatic acids

Despite the fact that they should lend themselves readily to analysis by thin-layer chromatography, aromatic acids have largely been ignored in the many recent applications of this popular technique. A few scattered reports have appeared[1–5] mentioning aromatic acids in connection with other substances but no attempt has been made to develop a method specifically for the acids themselves.

This communication describes a simple procedure applicable to a variety of aromatic acids. Readily available materials are employed and no special apparatus beyond ordinary chromatographic equipment is required.

Experimental

Commercially available, reagent grade solvents and aromatic acids were used without purification. Glass plates 10 × 20 cm were covered with a 0.3 mm thick coating of silicagel (Swiss type D-5 with calcium sulfate)* using the "Camag" apparatus*. The plates were air dried overnight and stored in a desiccator. Ether solutions containing 3–100 μg of each component of the mixture were spotted 2.5 cm from the lower end of the plate. The chromatograms were developed for 10 cm in an ascending manner using either benzene–pyridine (85:15) or *n*-heptane–glacial acetic acid (95:5) as the mobile phase. A development time of about 80 min was required in an

TABLE I

R_B* VALUES OF VARIOUS AROMATIC ACIDS

Acid	R_B *in solvent system***	
	A	*B*
p-Toluic	1.22	
m-Toluic	1.19	
o-Toluic	1.18	
Benzoic	1.00	1.00
Cinnamic	0.97	0.75
Phenylacetic	0.65	
p-Hydroxybenzoic	0.60	
m-Hydroxybenzoic	0.50	
o-Hydroxybenzoic (salicylic)	0.37	
Isophthalic	0.17	
Terephthalic	0.14	
Phthalic	0.06	

* $R_B = \frac{\text{migration distance of acid}}{\text{migration distance of benzoic acid}}$; R_F of benzoic acid in solvent system A: 0.60–0.68; in solvent system B: 0.25.

** A = benzene–pyridine (85:15, v/v); B = *n*-heptane–glacial acetic acid (95:5, v/v).

unsaturated chamber. Excess solvent was removed from the plates by air drying for 3 h or by heating to 115° for 1 h. The acid spots were visualized by spraying with a 1% solution of bromocresol green in 90% alcohol (yellow spots on a green background).

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Discussion

Chromatographic data for various aromatic acids are summarized in Table I. Migration rates are given as R_B values (where R_B is the migration distance of the acid divided by the migration distance of benzoic acid) rather than as R_F values. The R_F values for benzoic acid averaged about 0.65 in solvent system A and 0.25 in solvent system B. A somewhat better separation of benzoic and cinnamic acids was observed in *n*-heptane–acetic acid (solvent system B) than in benzene–pyridine. However, the latter system is more generally useful for separating a large number of acids.

Considerably sharper separations of substances with similar R_B values were obtained without solvent saturation of the chromatographic chamber. However, under conditions of saturation, development time was reduced by nearly 50 % and the slight "edge effect" was eliminated. R_F values were generally reduced by 1/3 by saturating the chamber. The values given are those obtained in an unsaturated atmosphere.

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Auftrennung von Veratrum-Alkaloiden durch Dünnschichtchromatographie

(Herrn Prof. Dr. Ing. Dr. med. Helmut Niemer zum 65. Geburtstag gewidmet).

Die seit alten Zeiten schon in der Volksmedizin verwendete Veratrum-Droge ist seit 1945 als hypotensives Mittel wieder in die Therapie eingeführt worden. Da die Droge jedoch aus mehreren Alkaloiden besteht, treten demzufolge unsichere therapeutische Ergebnisse und Nebenwirkungen auf, sodass eine Methode zur schnellen Auftrennung und Bestimmung kleinerer und grösserer Mengen ihrer Inhaltsstoffe auch von pharmakologischem Interesse ist.

Besonders in Pflanzen der Liliaceen- (Fritillaria, Schoenocaulon, Veratrum, Zygadenus) und Solanaceen-Gattungen (Lycopersicum, Solanum) sind polycyclische Aminoalkohole enthalten, deren Kohlenstoffgerüst an das der Sterine erinnert, wobei die Seitenkette unter Stickstoffeinbau zu einem Heterocyclus umgewandelt ist.

Diese *Sterin-Alkaloide* liegen in der Pflanze als freie Aminoalkohole vor, zum Teil auch als deren Glykoside oder Ester. Hinsichtlich der Aminoalkohole lassen sich 4 Gruppen bilden, die man sich formal von einem aus Cholestanol durch Kondensation mit Ammoniak gebildeten Molekül ableiten kann: *Solanidin*, *Solasodin*, *Veratramin* und *Cevin*.

Die im "Veratrin"* enthaltenen Hauptkomponenten Cevadin und Veratridin sind Ester-Alkaloide und leiten sich von dem Alkamin *Veracevin* vom Cevin-Typ ab, dessen sauerstofffreies Grundgerüst das hexacyclische Cevan darstellt.

Die am C-3 β-ständige Hydroxylgruppe im Veracevin (= Protocevin) ist mit Angelicasäure bzw. mit Veratrumsäure verestert, im Cevacin mit Essigsäure. Die Chemie dieser Sterin-Alkaloide ist vor allem von KUPCHAN[1] und seinen Mitarbeitern aufgeklärt worden.

Den umständlichen Reinigungsverfahren der fraktionierten Fällung[2,3] oder der Gegenstromverteilung[4] hat die Methode der Dünnschichtchromatographie die Einfachheit und Schnelligkeit voraus, mit der Trennungen im Mikromasstab wie auch zu präparativen Zwecken durchgeführt werden können.

Während Kieselgur als Sorptionsmittel zur Trennung der Veratrum-Alkaloide ungeeignet ist, gelingt diese vorzüglich auf voreluiertem aktiviertem Kieselgel HF_{254} (Merck). Die Zonen zeichnen sich durch eine zum Teil intensive Fluoreszenz bzw. Absorption im U.V.-Licht (350 mμ, 254 mμ) aus.

Als geeignetes Laufmittel erwiesen sich Gemische aus Cyclohexan und Diäthylamin. Besonders zur Auftrennung grösserer Substanzmengen ist eine wiederholte Entwicklung der gleichen Dünnschichtplatte notwendig, um bei nahe zusammenliegenden R_F-Werten einen besseren Trenneffekt zu erreichen. Die R_F-Werte zeigen eine Abhängigkeit vom Aktivitätszustand der Sorptionsschicht, sowie bei mehrmaliger Entwicklung auch von der Belüftungszeit der Dünnschichtplatten zwischen den einzelnen Entwicklungen. Bei kurzer Belüftung (unvollständige Entfernung des Diäthylamin) verschieben sich die R_F-Werte zu höheren Werten.

Experimenteller Teil

Zur präparativen Auftrennung wurden 2 g Veratrin (Merck) in 8 ml Chloroform gelöst und strichförmig auf 2 Platten (20 × 20 cm) mit einer 2 mm dicken Sorptionsschicht aus Kieselgel HF_{254} aufgetragen, welches vorher zur Abtrennung von Verunreinigungen in einer Soxhlet-Apparatur mit Chloroform und anschliessend mit Essigester extrahiert worden war (Sorptionsschichten von mehr als 0.5 mm Dicke werden 12 Stdn. luftgetrocknet und erst anschliessend bei etwa 105° aktiviert). Beide Platten wurden dreimal im System Cyclohexan–Diäthylamin (180 + 20) entwickelt, nach jeder Entwicklung ausreichend belüftet und anschliessend die 3 im U.V.-Licht bei 254 mμ dunkel erscheinenden Zonen (Zone 1 am Start, Zone 2 mit R_F 0.2 und Zone 3 mit R_F 0.5) abgeschabt, mit Chloroform eluiert und die jeweiligen Eluate eingedunstet. Die Rückstände der 3 Fraktionen ergaben bei der Rechromatographie auf je 2 Platten (1 mm Kieselgel HF_{254}) im gleichen Laufmittel nach dreimaliger Entwicklung bereits einheitliche Banden. Lediglich aus der Zone 1 wurde bei der Rechromatographie noch eine geringe Menge der 2. Zone abgetrennt. Nach Elution

* Veratrin Merck, No. 8530, DAB. 6 ist ein aufgereinigtes Alkaloidgemisch aus *Sabadilla officinarum*.

und Trocknen im Hochvakuum (100°) wurden aus der Zone 1 286 mg, Zone 2 (Veratridin!) 448 mg und Zone 3 (Cevadin!) 880 mg erhalten.

Diskussion

Zur Trennung bzw. Identifizierung der durch Hydrolyse der Esteralkaloide bzw. Umlagerung aus Veracevin (= Protocevin) entstehenden Produkte Cevagenin und Cevin, sowie deren Essigsäureester[5] (vgl. Fig. 1) eignen sich die Systeme Cyclohexan–Diäthylamin (180:20) und Cyclohexan–Diäthylamin (140:60).

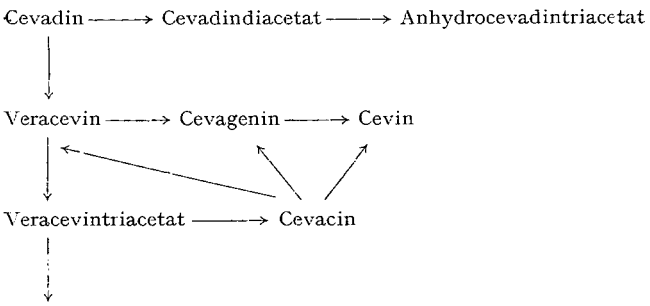

Fig. 1. Chemische Umwandlung einiger Veratrum-Alkaloide.

Wie aus der Tabelle I hervorgeht (die R_F-Werte gelten für frisch aktivierte Kieselgel HF_{254}-Platten, 250 μ dicke Sorptionsschicht) lassen sich die Ester-Alkaloide Veratridin, Cevacin und Cevadin im System (I) trennen, während zur Trennung der

TABELLE I

R_F-WERTE EINIGER VERATRUM-ALKALOIDE UND IHRER DERIVATE

Die in Klammern gesetzten R_F-Werte gelten für kurze Belüftung zwischen den einzelnen Entwicklungen.

		Laufmittel			
		Cyclohexan–Diäthylamin			*Cyclohexan–abs. Äthanol* (170:30) (*System III*)
		(180:20) (*System I*)	(140:60) (*System II*)		
		1 × *entwickelt*	3 × *entwickelt*	1 × *entwickelt*	2 × *entwickelt*
1	Veratridin	0.07	0.16 (0.28)	0.54	0.13
2	Cevadin	0.25	0.49 (0.68)	0.78	0.22
3	Veracevin	0.00	0.03	0.23	0.14
4	Cevagenin	0.00	0.03	0.26	0.05
5	Cevin	0.03	0.08	0.35	0.09
6	Cevacin	0.19	0.42 (0.54)	0.65	0.19
7	Anhydrocevagenin-triacetat	0.24	0.48 (0.66)	0.66	0.27
8	Anhydrocevadin-triacetat	0.45	0.81 (0.90)	0.84	0.29
9	Anhydroveracevin-tetraacetatperchlorat	0.35	0.67 (0.80)	0.75	0.13

Stereoisomere Veracevin (β-OH an C-3) und Cevin (α-OH an C-3) das System (II) herangezogen werden kann. Cevagenin ist in Chloroform schwerer löslich als Veracevin und Cevin.

Zur analytischen Beurteilung werden die Dünnschicht-Platten mit einer 25 %-igen Lösung von Trichloressigsäure in Chloroform kräftig besprüht, 10 bis 15 Min auf etwa 120° erhitzt und anschliessend unter der U.V.-Lampe bei 350 mμ betrachtet. Die Ester Veratridin, Cevadin und Cevacin fluoreszieren bläulich bis grünlich, die Alkamine Veracevin und Cevin gelb, Cevagenin gelbbraun und die Acetate der Anhydro-Verbindungen blau.

Wie die Tabelle I und die Fig. 2 zeigen, ist das System Cyclohexan–abs. Äthanol zur Auftrennung weniger geeignet. Die geringen Unterschiede in den R_F-Werten und die langgezogene Fleckenform erschweren die Identifizierung.

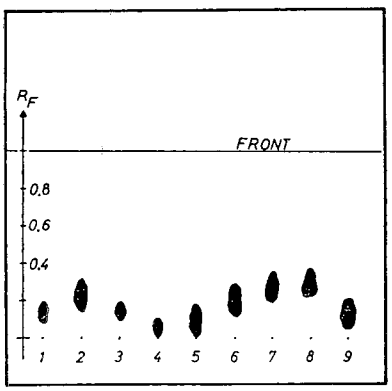

Fig. 2. Dünnschichtchromatographie von Veratrum-Alkaloiden im System III. (Die Ziffern unter den Startpunkten beziehen sich auf die Tabelle I.)

Dank

Herrn Prof. S. M. KUPCHAN* möchte ich an dieser Stelle für die freundliche Überlassung einiger Vergleichspräparate danken.

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Studies on *Catharanthus* alkaloids

III. Separation of vincaleukoblastine, leurocristine, leurosine and leurosidine by thin-layer chromatography

A report by BEER in 1955[1] that extracts from the Madagascan periwinkle, *Catharanthus roseus* (L) G. Don (*Vinca rosea* L.) elicit leukopenic activity, and a subsequent report on the isolation of the leukopenic and antineoplastic alkaloid vincaleukoblastine (VLB) from this plant by NOBLE, BEER AND CUTTS[2] have led to numerous subsequent reports from other laboratories on the separation of alkaloids from this plant. To date, a total of 59 alkaloids[2–30] have been reported isolated from *C. roseus*, four of which exert marked antineoplastic activity. Leurocristine, leurosine, and leurosidine, isolated by SVOBODA[22,23], in addition to VLB by NOBLE *et al.*[2], are reported to be active against a wide variety of neoplasms in experimental animals[2,31,32]. Further, leurocristine (vincristine) and vincaleukoblastine (vinblastine) are now available for routine use in the treatment of certain human neoplasms. Leurocristine (Oncovin®, Lilly) is employed for the treatment of acute lymphocytic and monocytic leukemias in children[31,33,34], whereas vincaleukoblastine (Velban®, Lilly) has been shown to be of value in treating Hodgkin's disease[31,35] and choriocarcinoma[31,36].

Certain studies now in progress in our laboratories require a method for identification and estimation of each of the four active, dimeric, antineoplastic alkaloids present in *C. roseus*. In particular, we are concerned with the development of a rapid, reproducible, and effective procedure for use in screening commercial samples of *C. roseus* for the presence of VLB, leurocristine, leurosine and leurosidine.

A report by JAKOVLJEVIC *et al.*[37] has shown that alumina plates prepared with 0.5 *N* lithium hydroxide, in conjunction with a 5 % absolute ethanol in acetonitrile (v/v) eluent, will effectively separate leurosidine (R_F 0.23) and leurocristine (R_F 0.51) by thin-layer chromatography. However, the same adsorbent matrix, together with a 30 % acetonitrile in benzene (v/v) eluent was required to separate leurosine (R_F 0.27) and VLB (R_F 0.36). These investigators did not demonstrate a method for separating all four active dimeric alkaloids from a single mixture.

Similarly, in a report by CONE *et al.*[38] in which adsorbent layers of silica prepared with water, silica prepared with 0.5 *N* KOH, or alumina were used, in conjunction with various combinations of seven different eluent systems, separation of VLB, leurocristine, leurosine and leurosidine from a mixture by means of thin-layer chromatography was not possible. No other reports concerned with this problem have been found in the literature.

At this time we wish to present a method found useful for separating and identifying the four closely related dimeric alkaloids (VLB, leurocristine, leurosine and leurosidine) by conventional Silica Gel G thin-layer chromatography, together with the useful alkaloid detecting ceric ammonium sulfate chromogenic spray reagent developed by JAKOVLJEVIC *et al.*[37], and first reported by CONE *et al.*[38] as a useful aid in differentiating certain of the *Catharanthus*-derived alkaloids.

Experimental

Preparation of plates. Silica Gel G plates were prepared in the usual manner with

a spreading applicator designed to produce a 250 μ matrix*. The prepared plates were activated by heating at 95–105° for 30 min in a circulating-air oven followed by cooling to room temperature in a desiccator over calcium sulfate. All plates were utilized within 72 h of activation, a precaution found necessary to insure reproducibility.

Sample preparation. The alkaloid samples, as free bases, were dissolved in benzene (2 mg/1 ml). Leurocristine, VLB, leurosine and leurosidine decompose as free bases under ordinary storage conditions and they are usually only available as the relatively stable sulfates. Each alkaloid (as sulfate) was accurately weighed, dissolved in a minimum volume of distilled water, made alkaline with NH_4OH, and extracted several times with benzene. The benzene extracts from each alkaloid were combined, dried over anhydrous sodium sulfate, filtered, and taken to dryness *in vacuo* using a flash evaporator set at 35°. Sufficient benzene was then added to each sample giving a concentration of 2 mg of alkaloid for each 1 ml of solvent (2 μg/1 μl).

The solutions of VLB and leurocristine free base (in benzene) were found to be stable for several weeks if kept frozen. However, leurosine and leurosidine as free base in benzene were found to decompose within two or three days, under the same storage conditions, to a point where interpretation of the major alkaloid component following chromatography of each sample was impossible.

Although 20 μg (10 μl of prepared sample) of each alkaloid could be readily detected with the ceric ammonium sulfate (CAS) reagent following chromatographic elution, 20 μl of each sample (40 μg of alkaloid) was found to be desirable because of the production of a more pronounced and stable chromogenic effect.

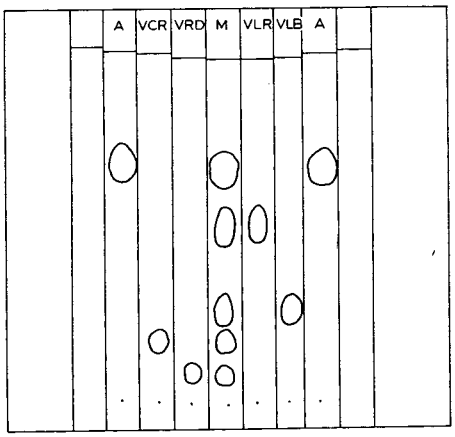
Uni-dimensional chromatography. To insure against sample swerving effects, the matrix on each plate was scored with a dissecting needle at parallel 10 mm intervals, in the same direction that the adsorbent matrix was originally spread on the plates, to give 20 separate channels. The two end channels were not utilized. Points for application of the samples were marked with a needle 15 mm from the bottom edge of the plate and centered within each channel. This general procedure has been previously described by McLAUGHLIN *et al.*[39] and appears to be beneficial in maintaining reproducibility.

Following removal of the plates from the desiccator and scoring the matrix as described, 20 μl of each alkaloid sample was applied by means of a micropipet to the point previously marked 15 mm from the bottom edge of the plate. A stream of air during application of the samples facilitated rapid evaporation of the solvent. Ajmalicine (10 μl = 20 μg) is also applied to the same spot as a reference alkaloid.

Each plate was then placed into an equilibrated (30 min) glass developing chamber ($8^1/_2$ in. × 4 in. × $8^1/_2$ in.), lined with eluent-moistened Whatman No. 1 filter paper and containing 100 ml of a chloroform–methanol (95:5) eluent. Freshly prepared eluent was used for each plate. Elution was allowed to continue until a solvent front of 100 mm (measured from the point of sample application) was attained. The plates, following a drying period in air of several minutes, were then sprayed lightly with the CAS reagent. The chromogenic reaction for each alkaloid was recorded and the separated alkaloid spots were outlined and the respective R_F values were calculated.

A typical separation of VLB, leurocristine, leurosine and leurosidine under

* The Desaga apparatus (Brinkmann Instruments, Inc., New York) was used to prepare 200 × 200 mm glass plates of the adsorbent matrix.



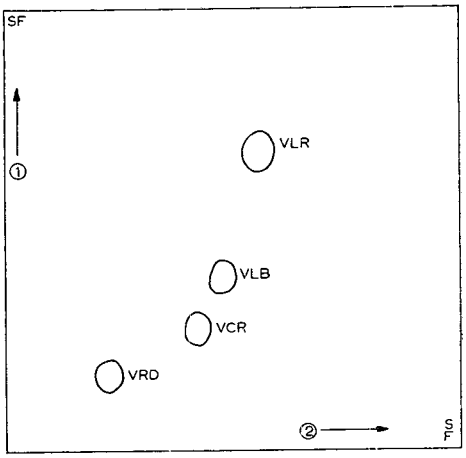


Fig. 1. A typical thin-layer chromatogram of vincaleukoblastine (VLB), leurocristine (VCR), leurosine (VLR), leurosidine (VRD) and ajmalicine (A), singly and in mixture (M). Matrix: Silica Gel G. Eluent: chloroform–methanol (95:5). Ascending development to a solvent front of 100 mm. Average development time 10–12 min.

Fig. 2. A typical two-dimensional thin-layer chromatogram of vincaleukoblastine (VLB), leurocristine (VCR), leurosine (VLR) and leurosidine (VRD). Matrix: Silica Gel G. Eluent in first direction: chloroform–methanol (95:5) to a solvent front (SF) of 120 mm. Average time of development 23–31 min. Eluent in second direction: methanol to a solvent front of 120 mm. Average development time 25–30 min.

these conditions is presented in Fig. 1. The mean R_F values for the alkaloids, together with their chromogenic behavior following application of the CAS reagent are presented in Table I.

Two-dimensional chromatography. Silica Gel G plates, prepared as previously described, were used for this procedure. Application of the alkaloid samples was made at the lower left corner of each plate at a point 15 mm from the bottom and 15 mm from the edge, in such a manner that the first direction of eluent flow would be the same as described for the uni-dimensional procedure.

TABLE I

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF VINCALEUKOBLASTINE, LEUROCRISTINE, LEUROSINE AND LEUROSIDINE *

Alkaloid	*R_F value* **	*Chromogenesis following CAS reagent*		
		Immediate	*After 15 min*	*After 1 h*
Leurosidine	0.06 ± 0.01	Orange-brown	Fades to tan	Tan
Leurocristine	0.16 ± 0.03	Blue	Light blue	Light blue
Vincaleukoblastine	0.24 ± 0.02	Orange-brown	Lavender	Light lavender
Leurosine	0.45 ± 0.03	Orange-brown	Yellow	Yellow
Ajmalicine (reference)	0.64 ± 0.03	Yellow-green	Yellow	Yellow

* On Silica Gel G plates (250 μ) using a chloroform–methanol (95:5) eluent. Development time was 10–12 min for 100 mm solvent front.

** Data were derived only from plates on which the reference alkaloid R_F value fell within a range of 0.60–0.68 and the solvent front (100 mm) was attained within 9–13 min.

Elution of each plate in an equilibrated (30 min) paper-lined chamber ($8^1/_2 \times 4 \times 8^1/_2$) with freshly prepared chloroform–methanol (95:5) was continued until a solvent front of 120 mm was attained. Each plate was then removed, air-dried, rotated 90°, and introduced into a second equilibrated chamber having the same dimensions and containing 100 ml of fresh methanol. Elution was continued until a 120 mm solvent front was again attained, following which the plate was removed, air-dried, and lightly sprayed with the CAS reagent (Fig. 2).

Results and discussion

The conditions described herein effectively and rapidly resolve a mixture of vincaleukoblastine, leurocristine, leurosine and leurosidine. It should be pointed out that fresh solvents must be used for each plate chromatographed since we have observed that plates chromatographed in previously used eluent will not lead to a separation of the two slower moving alkaloids leurosidine and leurocristine. Leurosidine, under these conditions, frequently remains at the point of sample application and leurocristine moves only slightly.

The alkaloid samples should be applied to the plates as quickly as possible after removal from the storage desiccator and elution should follow immediately. Plates prepared for more than 72 h, even when stored in a desiccator, were found to produce unreliable separations of the four alkaloids. As reported by JAKOVLJEVIC *et al.*[37], we have also observed a necessity for spraying the chromatograms with CAS reagent within 30–60 min following elution in order to insure reproducible chromogenic reactions.

With reference to the uni-dimensional thin-layer chromatographic procedure described, we have observed a high degree of R_F value reproducibility by considering as invalid all data derived from plates on which the reference alkaloid (ajmalicine) R_F value was determined to be outside the range of 0.60–0.68. Further, if the time required to attain a 100 mm solvent front was less than 9 or more than 13 min, R_F data derived from such plates were observed to be unreliable. No significant difference was noted in the R_F value of the alkaloids chromatographed singly or in mixture.

A measure of reliability concerning the relative position of the four alkaloids on chromatograms, following the two-dimensional procedure, was found to be in the time required to attain a 120 mm solvent front with each eluent. Considerable variation has been observed only on those plates in which a solvent front (120 mm) time falling outside the range of 23–31 min for chloroform–methanol (95:5) and 24–31 min for methanol, was noted.

In attempting to locate any one of the four dimeric antineoplastic alkaloids in crude mixtures, uni-dimensional thin-layer chromatography is of no value. However, it is possible to prepare a mixture of alkaloids from *C. roseus* by selective extraction that will contain the alkaloids vincaleukoblastine, leurocristine, leurosine, leurosidine, as well as others, but with a number of interfering alkaloids removed. Two-dimensional thin-layer chromatography of such a mixture, in the manner reported herein, separates the four active alkaloids from others which react to produce similar chromogenic reactions with the CAS reagent. It should be pointed out that leurosine, leurosidine, leurosivine, isoleurosine, neoleurosidine, VLB and desacetyl VLB all react similarly with the CAS reagent. Similarly, leurocristine and neoleurocristine produce identical colors with this reagent. Full details for a method that will separate

and allow identification of all 59 *Catharanthus roseus* alkaloids will be reported at a later date.

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Separation of 17-oxosteroid conjugates by thin-layer chromatography

In a recent communication KAY AND WARREN[1] have reported the extraction of urinary 17-oxosteroid conjugates as pyridinium salts and their electrophoretic separation.

The method to be described here uses thin-layer chromatography for the separation of these steroid conjugates and may be suitable for screening purposes in a survey of the pattern of 17-oxosteroid conjugates in normal and pathologic urines.

Silicagel GF 254 (Merck) containing an inorganic fluorescent dye and calcium sulphate as binding material was used as adsorbent.

A layer of 200 μ on microscope slides was prepared with the apparatus consisting of one glassplate 2.5 × 30 cm, thickness 4.45 mm, four microscope slides 2.5 × 7.5 cm, approximately 1.20 mm thick, and two 5.85 mm diameter rods. The rods are fixed to the glassplate with plasticine in order to hold the slides in place. The adsorbent, 10 g in 20 ml water, is poured on to the slides and spread with the 4 mm wide polished edge of a glassplate (2.5 × 20 cm).

The coated microscope slides are separated from each other with a scalpel blade, allowed to dry at laboratory temperature (18–23°) for 2 h and then heated in an electric oven at 105–110° for 1 h. They are kept in a desiccator over Silicagel until used.

An ethanolic extract of the urinary steroid conjugates, containing 8–10 μg in 10–20 μl is applied to the adsorbent layer with a Drummond disposable pipette 1.2 cm from the bottom edge of the slide and dried with a jet of N_2. The slide is placed in a small glass jar (7 × 9 cm) which is lined with filterpaper, covered with a glassplate and protected from draught by standing in a cardboard box.

The solvent system, chloroform–methanol–ammonia (19:1:0.2) is poured into the jar several hours before development starts to saturate the atmosphere. Time of development is approximately 7 min when the solvent front reaches about 6 cm from the origin. Under a Chromatolite Short Wave Lamp which has maximum radiation at 2536 Å the Silicagel GF 254 layer shows a yellow-green fluorescence while the steroids having a quenching effect appear as dark spots.

For quantitative work larger plates 10 × 20 cm or 20 × 20 cm, a glasstank of suitable size, and a layer of 400 μ can be used.

A separation of steroid pyridinium salts extracted from the urine of a normal male, age 35, shows 5 spots. Paper electrophoresis[1] resolved the extract into three zones only.

A parallel separation was performed of steroid pyridinium salts extracted from the urines of a normal male, age 35, and of a young female, age 23, afflicted with hirsutes for which no endocrine abnormality could be found. There is a striking difference in these two patterns.

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Limits of detection of some lipids in thin-layer chromatography

The use of thin-layer chromatography in lipid biochemistry is now widespread. In our laboratory we have found it convenient to employ thin-layer chromatography to monitor eluants of chromatographic columns or to determine the purity of compounds before use. It is therefore necessary to know the minimum quantities detectable in order to know the degree of contamination of the compounds tested. Accordingly, employing the three detection systems commonly used in our laboratory, we have undertaken experiments to determine the limits of detectability of various lipids.

Experimental

Thin-layer chromatographic plates, 250 μ thick, were made in the usual manner. We customarily dissolve 10 mg of Rhodamine 6G (National Aniline Division, Allied Chemical Corporation*) in the water used to make the slurry. The solution must be filtered before use. Silica Gel G (Brinkmann Instruments, Inc.*) and Silica Gel H (Brinkmann Instruments, Inc.*) work equally well in our laboratory.

The lipid compounds, with the exception of lecithin, were passed through silicic acid columns[1] before use. The compounds were weighed on an analytical balance and serial dilutions in benzene, chloroform or hexane were made, so that the proper quantity of compound (0.01–3 μg) might be applied to the plate in less than 5 μl of solvent.

The thin layers were developed with either a mixture of petroleum ether (b.p. 60–70°), diethyl ether and acetic acid (90:10:1, v/v)[2] or benzene and ethyl acetate (9:1, v/v) (for sterol and alcohol only)[3]. The spots were observed as follows: First, under ultraviolet illumination, which caused the Rhodamine to fluoresce pale green and the lipids to appear pink; secondly, after exposure to iodine vapors for 2–5 min, once again under ultraviolet light, the spots appearing intense blue; and thirdly, after spraying the plate with 50 % sulfuric acid and charring at 110°. In the last procedure the Rhodamine 6G is decolorized and does not mask the charring of the lipid.

Results

The results are summarized in Table I. It can be seen that the iodine in conjunction with the Rhodamine increases the sensitivity several fold. Experiments not recorded here have shown that iodine alone is not as sensitive as iodine–Rhodamine fluorescence. Unsaturation increases the sensitivity with the iodine–Rhodamine and with the charring. Some of the compounds did not char under our conditions (110°), although they do char at higher temperatures (200°); but we have not determined how leuco–Rhodamine reacts at the higher temperature.

Discussion

Most of the lipids have a lower limit of detection of about 0.1 μg. When 20 μg (a commonly used quantity) of compound is chromatographed, 0.1 μg represents 0.5 %. By increasing the load, a greater purity test can be obtained.

Although the Rhodamine–iodine detection is more sensitive than the charring

* Mention of a proprietary name or company does not necessarily imply endorsement by the U.S. Department of Agriculture.

TABLE I

MINIMUM AMOUNT (IN μg) OF COMPOUND DETECTED

Compound	*U.V.*	$I_2 + U.V.$	H_2SO_4
Saturated compounds			
Eicosane	3	0.1	0
Tristearin	1.2	0.7	0
Behenic acid	0.5	0.1	0
Hexadecanol	1	0.5	0.5
Cholestanol	0.2	0.05	0.1
Cholestan-3-one	1	0.1	0.5
Lecithin (dipalmitoyl)	0.5	0.01	0.5
Unsaturated compounds			
Squalene	1.8	0.02	0.2
Cholesteryl oleate	1	0.05	0.1
Cholesteryl stearate	1	0.05	0.2
Triolein	2	0.05	1
Oleic acid	0.5	0.05	0.2
Cholesterol	0.5	0.05	0.1
Δ^4-Cholesten-3-one	1	0.05	0.5

method, we often employ both methods to obtain additional useful information. The Δ^5-sterols and their esters give an intense purple color after 5–10 min of heating at 110° and can be readily distinguished from other lipids which only char. Also lanosterol, which turns a distinctive pink-black color, can be distinguished from some long-chain alcohols which run in almost the same position.

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Chromatography of dinitrophenyl amino acids on silica gel-impregnated glass paper*

The reaction of dinitrofluorobenzene (DNFB) with amino acids, peptides and proteins was the key to the solution of the primary structure of insulin[1]. This reaction has retained a position of importance in studies of the amino acid sequence and composition of proteins and peptides[2]. The dinitrophenyl (DNP) amino acid and peptide products of the reaction have been separated by distribution between solvents, electrophoresis, column chromatography on silica gel, and by paper chromatography; however, breakdown products of DNFB or of DNP-amino acids often complicate analysis of DNP-derivatives. Preliminary solvent extraction or time-consuming treatment to remove buffers or acid contaminants is generally necessary prior to chromatography. Most methods require hours for completion. The method presented here overcomes many of these difficulties.

Methods

Ascending chromatography is performed using glass paper impregnated with silica gel. Such paper may be prepared[3] or purchased from Applied Sciences, Inc. of State College, Pa. Standard DNP-derivatives of amino acids are easily prepared[2] or can be purchased from a number of supply houses. Standards (1 to 10 mmole/l) are dissolved in methanol or water. Spots 3 to 4 mm in diameter are applied to paper with melting point capillaries. Resolution is best when less than 2 μg of a specific derivative is applied.

R_F values of DNP-derivatives in three solvent systems are presented in Table I. Although R_F values obtained in a specific solvent system may vary slightly from those recorded in Table I, the relative positions of derivatives to each other are constant. Before analyzing an unknown one should adjust the composition of the solvent system until a standard derivative migrates with the desired R_F value. Any two or more compounds listed may be separated by a single chromatographic run or by two-dimensional chromatography. In general, the first dimension is used to separate an unknown into groups, and the second to isolate specific compounds within a group. The isooctane–chloroform–acetic acid solvent system is used primarily for the group separation; the ether systems are used in the second dimension for separations of derivatives within a group.

The isooctane–chloroform–acetic acid (100:100:10) solvent system separates the derivatives into five groups. Group I. Highly polar derivatives remain near the origin. These include DNP-dicarboxylic amino acids, their amides, hydroxy DNP-amino acids, etc. Group II. DNP-glycine, DNP-tryptophane and the di-DNP-derivatives of lysine and tyrosine cluster about R_F 0.25. Group III. Proline, phenylalanine, alanine and methionine derivatives move to the region of R_F 0.5. Group IV. DNP-leucine, DNP-isoleucine and DNP-valine have R_F values of about 0.7. Group V. DNFB and breakdown products of DNFB and DNP-amino acids such as dinitrophenol and dinitroaniline move with the solvent front.

Compounds in Groups I and II are separated by chromatographing in the first dimension in ether–acetic acid–water (100:3:3) and, in the second dimension, in

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TABLE I

R_F VALUES OF DINITROPHENYL AMINO ACIDS IN THREE SOLVENT SYSTEMS

Compound	*Isooctane system**			*Acid ether system***			*Alkaline ether system****		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Group I									
DNP-L-Arginine	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.14	0.78
Di-DNP-L-histidine	0.00	0.00	0.00	0.00	0.04	0.07	0.11	0.38	0.94
ε-DNP-L-Lysine	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.03	0.37
Di-S,N-DNP-L-cysteine	0.00	0.02	0.06	0.16	0.49	0.83	0.40	0.96	0.95
N,N'-Di-DNP-L-cystine	0.00	0.00	0.00	0.00	0.16	0.53	0.00	0.14	0.72
DNP-DL-Methionine sulfoxide	0.00	0.00	0.00	0.00	0.14	0.36	0.02	0.28	0.76
DNP-L-Serine	0.00	0.00	0.02	0.24	0.55	0.85	0.07	0.36	0.92
DNP-L-Threonine	0.00	0.02	0.08	0.28	0.65	0.90	0.13	0.49	0.97
DNP-L-Asparagine	0.00	0.00	0.00	0.02	0.32	0.58	0.00	0.24	0.71
DNP-L-Glutamine	0.00	0.00	0.00	0.03	0.35	0.58	0.01	0.17	0.82
DNP-L-Aspartic acid	0.00	0.00	0.04	0.04	0.42	0.72	0.00	0.00	0.02
DNP-DL-Glutamic acid	0.00	0.04	0.10	0.15	0.58	0.85	0.00	0.00	0.03
Group II									
Di-N,N'-DNP-L-lysine	0.02	0.17	0.29	0.35	0.77	Front	0.26	0.92	Front
Di-O,N-DNP-L-tyrosine	0.02	0.20	0.33	0.40	0.80	Front	0.37	0.99	Front
DNP-Glycine	0.06	0.25	0.31	0.51	0.83	Front	0.16	0.58	Front
DNP-L-Tryptophane	0.09	0.27	0.40	0.79	0.97	Front	0.29	0.86	Front
Group III									
DNP-L-Alanine	0.24	0.50	0.54	0.79	0.93	Front	0.17	0.68	Front
DNP-DL-Methionine	0.26	0.54	0.54	0.77	0.95	Front	0.36	0.95	Front
DNP-L-Phenylalanine	0.26	0.54	0.59	0.77	0.95	Front	0.39	0.97	Front
DNP-L-Proline	0.28	0.55	0.54	0.74	0.96	Front	0.26	0.77	Front
Group IV									
DNP-L-Valine	0.41	0.70	0.67	0.95	0.98	Front	0.39	0.96	Front
DNP-L-Isoleucine	0.50	0.75	0.74	0.97	0.98	Front	0.49	0.98	Front
DNP-L-Leucine	0.49	0.73	0.71	0.95	0.97	Front	0.56	0.97	Front

* Isooctane–chloroform–acetic acid. A = 100:50:5; B = 100:100:10; C = 100:150:5.

** Diethyl ether–acetic acid–water. A = 100:1:1; B = 100:3:3; C = 100:5:5.

*** Diethyl ether–methanol–7 *M* ammonium hydroxide. A = 100:6:2; B = 100:8:3; C = 100:10:4.

ether–methanol–7 *M* ammonium hydroxide (100:8:3). DNP-glutamine and asparagine are the most difficult to separate from each other. Compounds in Groups II, III and IV are separated by chromatography in the first dimension in isooctane–chloroform–acetic acid (100:100:10) and in the second dimension in ether–methanol–7 *M* ammonium hydroxide (100:6:2). DNP-phenylalanine and DNP-methionine are the most difficult compounds of Group IV to separate.

Discussion

Chromatography of DNP-derivatives on silica gel-impregnated glass paper overcomes many analytical problems. N-terminal amino acid identifications or the qualitative analysis of a mixture of DNP-amino acids in a submicrogram sample of an unknown can be carried out in less than one hour. Neither the reagents used in pre-

paring DNP-derivatives nor those used in hydrolyzing DNP-peptides hinder the resolving power of the glass paper technique. Upon completion of dinitrophenylation of a sample in bicarbonate or trimethylamine buffers, the reaction mixture may be spotted directly on the glass paper without preliminary extraction or purification of DNP-amino acids. Solutions of DNP-amino acids in 6 *N* HCl or in the various mixtures containing glacial acetic, formic and perchloric acids used in digesting DNP-peptides or DNP-proteins can be applied directly to the glass paper without seriously altering R_F values or quality of DNP-amino acid resolution. The three solvent systems presented here, an acidic, a basic and a non-aqueous acidic, offer wide selectivity and versatility in separating DNP-derivatives.

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The use of ninhydrin in the detection of cationic complexes of cobalt, nickel and chromium

In the course of the preparation[1–4] and purification of metal coordination compounds prior to the assessment of their biological properties[5], several methods of detection on paper and thin-layer chromatograms were tried. Of a number of reagents used, ninhydrin was found to give characteristic stains with the cationic complexes of cobalt, nickel and chromium.

All the complexes excepting compound No. 10 (Table I) were dissolved in water, applied to paper strips and developed by the descending method in a solvent system made up of *n*-butanol–acetic acid–water–pyridine (30:6:20:24, v/v). After staining with 0.2 % ninhydrin in acetone, the spots were visualised by heating the paper strips for 10–15 min at 80–90°. The water-labile compound No. 10 was suspended in acetone to which water was added drop by drop to get a fine suspension.

In spot tests carried out on strips of paper the cationic complexes listed in Table I reacted sensitively to ninhydrin, the range of detectability being 0.2–1.0 μg.

TABLE I

SPOT TESTS OF CATIONIC COMPLEXES OF COBALT, NICKEL AND CHROMIUM WITH NINHYDRIN

No.	*Compound*[a]	*Colour of spot*	*Sensitivity* (μg)
1	$[Co(en)_2Cl_2]Cl$	Deep brown-red	0.5
2	$[Co(en)_3]Cl_3$	Purple-red (brownish)	0.5
3	$[Cr(en)_3]_2(SO_4)_3$	Deep violet (bluish)	0.5
4	$[Co(NH_3)_6]Cl_3$	Orange-brown (purplish)	1.0
5	$[Ni(en)_3]Cl_2$	Very deep-violet	0.2
6	$[Co(NH_3)_5(H_2O)]Br_3$	Orange-brown (purplish)	1.0
8	$[Co_4(en)_6(OH)_6]Cl_6$	Dull red-brown	0.5
9	$[Cr_4(en)_6(OH)_6]Cl_6$	Deep violet-brown	0.5
10	$[Ni(NH_3)_6]Cl_2$	Light orange-yellow	1.0

[a] (en) = ethylenediamine.

Ethylenediamine-containing complexes of cobalt and nickel responded more intensely to the ninhydrin test than the corresponding ammonia-containing complexes of cobalt and nickel. The stains were stable for several months on paper chromatograms and 5–15 μg of the compounds, excepting compounds 8 and 9, were detectable without any trailing effect. Compounds 4 and 6 containing ammonia had, however, to be used in 20–35 μg amounts on account of the lower order of sensitivity. Compound No. 5, the nickel–ethylenediamine complex, was by far the most reactive of this series. Some typical results are summarised in Table II.

Presumably ninhydrin reveals the compounds studied by reacting with ammonia or ethylenediamine. In independent experiments it was found that ninhydrin

TABLE II

CHROMATOGRAPHIC DETECTION OF COBALT, NICKEL AND CHROMIUM AND THEIR CATIONIC COMPLEXES WITH NINHYDRIN

No.	*Compound*	*Shade*[a]	R_F *value*	*Amount taken* (μg)
1	$[Co(en)_2Cl_2]Cl$	Violet-brown (intense and deep shade)	0.33	8
2	$[Co(en)_3]Cl_3$	Pink-violet (deep shade)	0.21	8
3	$[Cr(en)_3]_2(So_4)_3$[b]	Violet (deep shade)	0.168	8
4	$[Co(NH_3)_6]Cl_3$	Distinct yellow (weaker and lighter shade)	0.165	15
5	$[Ni(en)_3]Cl_2$	Violet (very deep and intense)	0.21	5
6	$[Co(NH_3)_3(H_2O)]Br_3$	Yellow pinkish (very light shade as No. 4)	0.184	15
8	$[Co_4(en)_6(OH)_6]Cl_6$[c]	Pink-brown	0.20	8
9	$[Cr_4(en)_6(OH)_6]Cl_6$[c]	Violet-pink (intense and deep)	0.23	8
10	$CoCl_2$[d]	Pink-yellow	0.77	40
11	$NiCl_2$[d]	Yellow (very light shade)	0.78	40
12	$Cr_2(SO_4)_3 \cdot K_2SO_4$	Green-gray (very light and dull)	0.20	60

[a] The shades of the detected compounds on the chromatograms were in some cases slightly different from those of the spot tests.

[b] Compound 3 contained a minor impurity corresponding to an R_F value of 0.11 in a brown-violet shade.

[c] Compound 8 showed a weak streaking from the point of application up to the major zone while compound 9 gave a prominent streaking both below and above the major zone.

[d] The shades of the detected spots became very prominent on storage.

could react with simple salts of cobalt, nickel and chromium. These ions reacted with ninhydrin relatively less sensitively. It would seem, therefore, that the net colour produced by ninhydrin is the result of reaction with the nitrogen-containing compounds—ammonia or ethylenediamine—as well as direct reaction with the metals that constitute the complexes. The relative difference in the intensity and shade of stains produced by ethylenediamine-containing complexes and ammonia-containing complexes could be used for distinguishing these two groups of metal complexes. The presence of metals (Ni, Co, Cr.) may be responsible for the stability of the stains of all the metal complexes detected as against the transient stains given by ninhydrin with amino acids. In the latter case, stability can be conferred on the stains by incorporating metallic cations[6,7] into the ninhydrin reagent.

In view of the fact that ninhydrin can stain a number of nitrogenous and non-nitrogenous compounds as demonstrated by the present study as well as by a number of recent reports[8–14], the results of the use of ninhydrin for the specific detection of amino acids and amines have to be very cautiously interpreted.

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Separation of chlorinated cresols and chlorinated xylenols by paper chromatography

This work arose out of studies on the chlorination of tar acids from low temperature tars. Paper chromatography has been applied to the separation and identification of isomeric cresols[1], xylenols[2], chlorophenols[3] and chlorocresols[4]. Successful separation of chlorocresols and chloroxylenols in mixtures is now reported.

Materials

Whatman No. 1 (16 × 9 in.) filter was impregnated with methanol–formamide solution (30:100, v/v) and dried at room temperature for 15–30 min. Solutions (0.5–1 %) of each compound in acetone were spotted with a fine glass capillary (10–20 μg). Three solvent systems were used:

(A) Petroleum ether (80–100°) saturated with formic acid.

(B) Xylene saturated with formamide.

(C) The organic layer from a mixture of benzene–acetic acid–water (2:2:1 v/v).

The chlorocresols and chloroxylenols used for separation were chromatographically pure and obtained from Coalite and Chemical Products Ltd., Bolsover and Aldrich Chemical Company Inc., Wisconsin.

Detection of the spots. Phosphotungstomolybidic acid (Folin Denis reagent) was sprayed on the developed chromatogram. When exposed to ammonia, blue spots resulted.

TABLE I

R_F VALUES OF DIFFERENT CHLORINATED CRESOLS AND CHLORINATED XYLENOLS

Temperature of chromatography, 20 ± 2°. Time of run for all solvent systems, *ca.* 2.5 h.

No.	*Compounds*	*R_F values (× 100) in various solvent systems*			*Mixtures of phenols (indicated as their No. in column 1)*
		A	*B*	*C*	
1	*o*-Cresol	8	50	50	
2	6-Chloro-*o*-cresol	88	89	92	
3	4-Chloro-*o*-cresol	19	66	66	1–4
4	4,6-Dichloro-*o*-cresol	86	91	93	
5	*m*-Cresol	5	34	42	
6	6-Chloro-*m*-cresol	70	73	76	
7	4-Chloro-*m*-cresol	10	55	59	
8	4,6-Dichloro-*m*-cresol	62	84	84	5–9
9	2,4,6-Trichloro-*m*-cresol	73	90	94	
10	2,3-Dimethylphenol	20	62	67	
11	4-Chloro-2,3-dimethylphenol	26	73	79	10 and 11
12	2,5-Dimethylphenol	25	60	76	
13	4-Chloro-2,5-dimethylphenol	34	76	86	12 and 13
14	3,4-Dimethylphenol	8	44	56	
15	6-Chloro-3,4-dimethylphenol	64	79	85	14–16
16	2,6-Dichloro-3,4-dimethylphenol	79	92	94	
17	3,5-Dimethylphenol	11	59	63	17–19
18	4-Chloro-3,5-dimethylphenol	25	72	76	
19	2,4-Dichloro-3,5-dimethylphenol	78	91	92	
20	5-Ethyl-3-methylphenol	30	70	76	
21	4-Chloro-5-ethyl-3-methylphenol	34	81	89	20–22
22	2,4-Dichloro-5-ethyl-3-methylphenol	86	92	93	

Results and discussion

Table I lists the R_F values of the chlorocresols and chloroxylenols. The spots were free of tailing. 6-Chloro-*o*-cresol could not be separated from 4,6-dichloro-*o*-cresol with any of the solvent systems tried. Low R_F values were exhibited in solvent system A which has the lowest polarity. R_F values in solvents B and C were about the same. Generally the R_F values increased with increasing substitution in the nucleus; 6-chloro-*o*-cresol and 6-chloro-*m*-cresol showed comparatively high R_F values in solvent A. The R_F values for *o*-substituted compounds are higher than for the corresponding *m*-isomer, a result perhaps of the *ortho* effect[5]. Thus the $R_F \times 100$ values in solvent B were: *m*-cresol 34, *o*-cresol 50, 4-chloro-*m*-cresol 55, 6-chloro-*o*-cresol 73; 3,4-dimethylphenol 44, 6-chloro-3,4-dimethylphenol 79. The effect is more pronounced in the 2,6-disubstituted products as seen in the $R_F \times 100$ values for 4-chloro-*o*-cresol 66, 6-chloro-*o*-cresol 89, 6-chloro-3,4-dimethylphenol 79, and 2,6-dichloro-3,4-dimethylphenol 92. In these disubstituted products the relative order was generally maintained in all the three solvent systems.

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Papierchromatographische Analyse von Aerosolbestandteilen

Kennzeichnend für ein Aerosol sind seine Teilchengrösse und elektrische Ladung, Teilchenkonzentration und seine chemische Zusammensetzung. Zahlreiche Untersuchungen über Aerosole sind unter vorwiegend physikalischen Aspekten durchgeführt worden, wie die der Teilchengrösse, Teilchengrössenverteilung oder die der elektrischen Ladungsverhältnisse[1–3].

Um Kenntnis von der chemischen Struktur und dem quantitativen Verhältnis der einzelnen Komponenten eines komplexen Aerosols (Misch-aerosol) zu erhalten, ist eine chemische Analyse nach Abscheidung des Aerosols notwendig.

An Modellversuchen untersuchten wir die Möglichkeit einer unmittelbaren papierchromatographischen Analyse von homo- oder heterodispersen Misch-aerosolen, die mit den üblichen Methoden (Membranfilter, Cascade impector[1–6]) aufgefangen bezw. niedergeschlagen werden können. Es wurde von uns eine Farbstoffiösung, die aus gleichen Volumina Methylenblau (12 g/l) und Fuchsin (1.7 g/l) bestand, durch einen kleinen Ultraschallvernebler (*ca* 20 W), der eine sehr dichte und feinverteilte Aerosolerzeugung gestattet[7,8], in den Aerosolzustand übergeführt. Die gebildeten

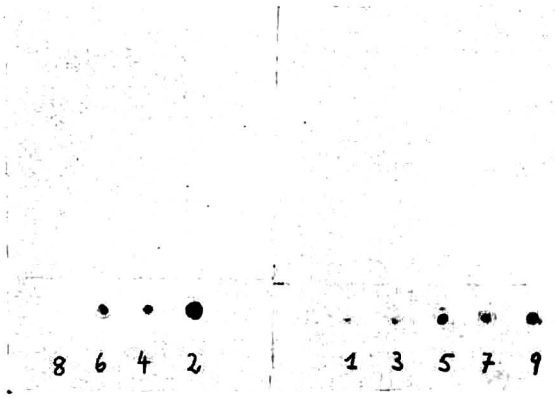

Fig. 1. Papierbögen mit den fraktionierten Abscheidungen der komplexen Aerosolpartikel im Teilchenanalysator nach DIRNAGL. Rechte Seite: Fraktionen 1, 3, 5, 7, 9; linke Seite: Fraktionen 2, 4, 6, 8.

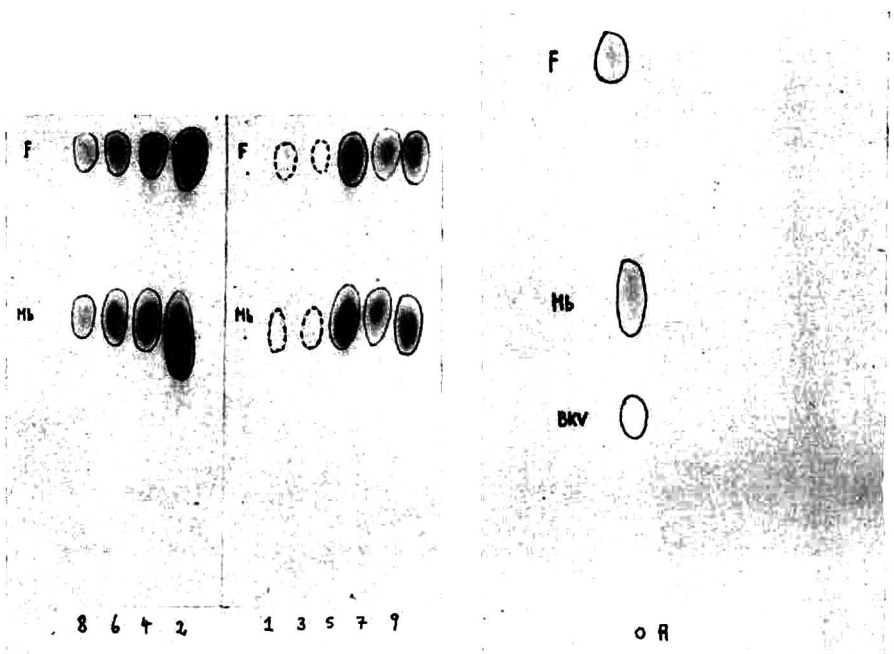

Fig. 2. Papierchromatogramme der einzelnen Aerosolfraktionen (1–9). Mb = Methylenblau; F = Fuchsin. Fliessmittel: *n*-Butanol–Essigsäure–Wasser (4:1:5).

Fig. 3. Papierchromatogramm eines niedergeschlagenen komplexen Aerosols, das aus den drei Komponenten Brenzkatechinviolett (BKV), Methylenblau (Mb) und Fuchsin (F) bestand. A = Aufschlagfläche des Misch-aerosols. Fliessmittel: *n*-Butanol–Essigsäure–Wasser (4:1:5).

Farbstoffaerosole wurden anschliessend in eine Mischkammer geleitet. Das Teilchenspektrum der Aerosole in der Mischkammer konnte durch eine spezielle Anordnung besonders eng im Bereich von 0.3 μ bis 1 μ gehalten werden.

Das Misch-aerosol wurde anschliessend für die Dauer von 5 Min. in den Pralldüsenabscheider nach DIRNAGL[9] eingeleitet. An 9 der 10 Austrittsdüsen erfolgte eine fraktionierte Abscheidung, je nach Grösse der Aerosolteilchen (0.3–1 μ), direkt auf Chromatographiepapierbögen (Schleicher & Schüll 2043), die an beiden Seiten des Teilchenanalysators eingespannt sind.

Fig. 1 zeigt deutlich die Aerosolabscheidungsverteilung auf den Papierbögen, die den verschiedenen Austrittsdüsen 1–9 entsprechen. Durch anschliessende aufsteigende Chromatographie in *n*-Butanol–Eisessig–Wasser (4:1:5) als Fliessmittel konnten die verschiedenen Abscheidungsfraktionen (1–9) in ihre Bestandteile Methylenblau und Fuchsin eindeutig aufgetrennt werden (siehe Fig. 2).

Die unterschiedliche Intensität und Grösse der Flecken zeigen die Konzentrationsverhältnisse in den einzelnen Niederschlagsfraktionen an. Die so lokalisierten Flecken von Methylenblau und Fuchsin können nun ausgeschnitten und eluiert werden. Durch Extinktionsmessung der Eluate am Spektralphotometer lassen sich die Konzentrationen der beiden Farbstoffe in den einzelnen Aerosolabscheidungsfraktionen bestimmen.

Ein aus den drei Komponenten Brenzkatechinviolett, Methylenblau und Fuchsin bestehendes Misch-aerosol konnte durch das gleiche Verfahren sehr gut aufgetrennt werden (siehe Fig. 3).

Die quantitative Bestimmung von ungefärbten Substanzen lässt sich auf die gleiche Art vielfach durch ihre Überführung auf dem Papier oder ihrer Eluate in gefärbte Verbindungen, oder in einigen Fällen direkt durch ihre Extinktionsmessung im U.V.-Bereich durchführen.

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Book Reviews

Steroid Chromatography, by R. NEHER, translated by R. H. BATHGATE, Elsevier Publishing Co., Amsterdam, 1964, xiii + 389 pages, price 60 s, Dfl. 30.

NEHER's review *Chromatographie von Sterinen, Steroiden und anderen Verbindungen*, which was first published in this Journal in 1958, was warmly received and was republished in English by *Chromatographic Reviews* in 1959 and then in French in E. LEDERER's *Chromatographie en chimie organique et biologique* in 1960. *Steroid Chromatography* is a completely revised and enlarged English edition of this review. It covers the literature up to the end of 1962 and lists additional references for the year 1963.

Since another book on the same subject (*The Chromatography of Steroids* by I. E. BUSH, Pergamon, 1961) has appeared only recently, the question seems justified whether NEHER's book has anything to offer that cannot be found in BUSH's book. NEHER's book is, of course, more up-to-date and includes the application of gas and thin-layer chromatography, which had just begun when BUSH wrote his book. But, moreover, it is more complete. While BUSH concentrates on steroid hormones, particularly the corticosteroids, to the exclusion of other classes of steroids, NEHER treats with equal thoroughness the sterols, bile acids and other steroid acids, cardiac glycosides and aglycones, sapogenins and saponins, aminosteroids and steroid alkaloids, as well as the steroid hormones, their metabolites and conjugates.

While BUSH emphasizes his own contributions to paper chromatography almost to the exclusion of other chromatographic techniques, NEHER deals with equal competence with adsorption and partition chromatography on both columns and sheets. He even includes some work on paper electrophoresis. While BUSH is steeped in theory and the hypothetical uses of paper chromatography, NEHER instructs the novice in the principles and techniques of chromatographic separations. Although NEHER also discusses the relation between constitution and chromatographic behavior, his approach is pragmatic and impersonal, rather than fanciful and enticing. Even when he deals with the subject of choice of chromatographic methods, NEHER manages to remain unbiassed most of the time, although his treatment betrays a large store of personal laboratory experience.

This book accurately draws on about 1000 references and contains a great number of tables and graphs in which data from diverse publications as well as the author's unpublished work are beautifully correlated. It is a pity that it is marred by unpardonable incompetence in translating and editing. In spite of its delayed appearance—the volume was originally advertised with a 1963 publication date—there is hardly a page free of errors that could have been eliminated by the editors. As an example of the execrable translation, I will only quote one sentence from p. 248 "Since there is a wide range of excellent PC and TLC methods for such compounds, and having regard to the high price and labile structure of glass fibre papers and the trouble taken in preparing them, there is in the author's view no justification in

using this method unless there is very good reason for doing so, especially since it is very sensitive to impurities in the extracts and to moisture, making it difficult to get reproducible results". This sentence occurs opposite to a very valuable unpublished chart of an "equieluotropic series". Truly, NEHER's work deserves better treatment than that.

E. HEFTMANN (Pasadena)

J. Chromatog., 18 (1965) 201–202

Ionenaustausch-Chromatographie, by KONRAD DORFNER, Akademie-Verlag, Berlin, 1963, viii + 236 pp., price DM 32.50.

This book is a descriptive account of ion exchange chromatography and is divided equally into sections on techniques and inorganic and organic applications. Though it is the third account of ion exchange that has appeared of late (the others are by O. SAMUELSON and by J. INCZEDY) it has neither the personal touch of the first nor the descriptions of complete analytical methods which make the latter so valuable.

The literature dealt with (946 references) contains few papers published in 1960 and none later than 1960 while SAMUELSON's book which appeared earlier in 1963 includes papers published in 1961. Most fields of application are mentioned in the various chapters, but the underlying principles are not well explained in all cases nor are numerical data given.

The book can be recommended as an introduction, perhaps also as a text book. It falls somewhat short as a work of reference and handbook. It is attractively produced and only minor printing errors were noted.

J. Chromatog., 18 (1965) 202

Analytische Anwendungen von Ionenaustauschern, by JÁNOS INCZÉDY, Verlag der Ungarischen Akademie der Wissenschaften, Budapest, 1964, 365 pp., price Dfl. 160.

The author has written what at first sight appears to be only another orthodox account of ion exchange. There are, however, a number of attractive features in this book. The most important of these for the analytical chemist is that numerous complete analytical methods are reported in full and that the author and his collaborators have checked most of them personally. Organic applications of ion exchangers are briefly but adequately discussed in about 40 pages, again with numerous complete methods. Two final chapters deal with miscellaneous topics such as the purification of water, of reagents, and the concentration of ions, both with ion exchange membranes and ion exchange papers.

The book is attractively produced and the reviewer found only unimportant errors.

J. Chromatog., 18 (1965) 202

Letter to the Editor

In international chromatographic literature "thin-layer chromatography" is called "Dünnschicht-Chromatographie" (German), "Chromatographie en couches minces" (French) and "Cromatografia su strato sottile" (Italian), etc. All of these are somewhat lengthy terms and I would like to propose that "Thin-layer chromatography" be called "Leptochromatography" (from the Greek $\lambda\varepsilon\pi\tau o\varsigma$ = thin).

If this suggestion were adopted "Leptochromatography" would then become "Leptochromatographie" in French and German and "Leptocromatografia" in Italian.

Institute for General Pathology and Experimental Medicine of the University of Parma (Italy) Prof. FRANCESCO REZZESI, M.D.

Announcement

"Industrial Organic Analysis" is the theme of a meeting to be held at the Drawbridge Inn, Sarnia, Ontario, October 4–6, 1965. Sponsored by the Analytical Chemistry Division, The Chemical Institute of Canada, sessions are being planned on such topics as:

1. Trends in industrial analysis.
2. Are classical analytical methods obsolete?
3. Advances in analytical spectroscopy.
4. Characterization of polymers.

There will be three additional general sessions, one of which will be devoted to *gas chromatography*. Papers are invited for these general sessions with particular interest centred on instrumental methods. Titles and short abstracts are requested by April 1, 1965, and should be sent to: R. M. Small, Research Department, Polymer Corp. Ltd., Sarnia, Ont., Canada.

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CRITICAL INLET PRESSURE FOR SEPARATION IN GAS CHROMATOGRAPHY

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The achievement of very difficult separations in gas chromatography requires the use of columns sufficiently long to generate the required number of theoretical plates. However, as length is increased the inlet pressure must also be increased in order to maintain flow. The question naturally arises as to whether manipulations in column length and flow velocity can be made in such a manner that the inlet pressure need not be increased indefinitely as the separation becomes more and more difficult. The answer to this question is important since there is a finite limit to the inlet pressure which can be used with any given system. Earlier theoretical results have indicated, however, that there is no simple escape from the pressure limitation imposed by difficult separations[1,2]. There appears to be a critical inlet pressure, p_c, for any specified separation. The separation cannot be achieved below this pressure. If, for example, one lowers the velocity of flow so as to reduce the flow resistance, the peak spreading is increased by molecular diffusion (due to the increase in residence time within the column). A longer column is thus needed to compensate for the added peak spreading and this, in turn, requires a further reduction in velocity. A theoretical limit is reached as column length approaches infinity and flow velocity approaches zero.

Two previous theoretical treatments have recognized the role of critical pressure in gas chromatography[1,2]. In the first case the concept arose as a rather incidental part of the study of minimum analysis time in gas chromatography. The derivation, directed toward other ends, was especially roundabout and difficult. In the second case the derivation was made on the basis of uniform columns, especially applicable to liquid chromatography. After the derivation was complete, averages were used to represent the highly non-uniform (due to compressibility) properties of the carrier gas. The results of this approximate treatment were close but not identical to those of the first treatment.

The object of this paper is to present a direct and exact treatment of the critical pressure in gas chromatography. This derivation, unlike the earlier ones, will demonstrate the role of gas compressibility and will relate the critical pressure directly to the partition coefficients of the component peaks.

LENGTH AND PRESSURE RELATIONSHIPS

Since high inlet pressures are necessary to maintain flow in long columns, the precise relationship between column length, pressure and flow velocity is needed.

We start out with a basic pressure-gradient equation[2]:

$$-\mathrm{d}p/\mathrm{d}z = 2\phi\eta v/d_p^2 \tag{1}$$

where $-\mathrm{d}p/\mathrm{d}z$ is the pressure gradient with the negative sign indicating the decrease of pressure toward the outlet. The term ϕ is a dimensionless structural parameter nearly constant for most packed columns at around 300 and equal to 16 for capillary columns. (For porous materials ϕ should be replaced by $\sim 300/\Phi$, where Φ is the fraction of gas in interparticle space.) The equation reflects the fact, observed in flow through granular materials, that the pressure gradient is proportional to the flow velocity, v, the viscosity, η, and inversely proportional to particle diameter, d_p, squared (the latter is in analogy to the Poiseuille equation).

Eqn. (1) can be easily integrated over the column length, L. First noting that the pressure–velocity product, pv, is constant in the column (as a result of Boyle's law), v may be written as $p_i v_i/p$, where p_i and v_i are inlet values. We then have:

$$\int_{p_i}^{p_0} p\,\mathrm{d}p = -(2\phi\eta p_i v_i/d_p^2)\int_0^L \mathrm{d}z \tag{2}$$

or:

$$p_i^2 - p_0^2 = 4\phi\eta p_i v_i L/d_p^2 \tag{3}$$

When P is used for the compression ratio, p_i/p_0, and Eqn. (3) rearranged, we get:

$$L = \frac{d_p^2 p_i}{4\phi\eta v_i}\left(\frac{P^2 - 1}{P}\right) \tag{4}$$

The very maximum use can be made of a given inlet pressure if the outlet is held near vacuum[3]. In this case P approaches infinity and the above equation reduces to:

$$L = d_p^2 p_i/4\phi\eta v_i \tag{5}$$

This is the desired relationship between length, inlet pressure and inlet velocity.

NUMBER OF THEORETICAL PLATES

A given separation, achieved at a fixed temperature, liquid load, etc., will require a certain minimum number of theoretical plates for its success. This can be shown to be:

$$N_{\min} = 16\,[Rs(V_m + KV_s)]^2/(V_s\Delta K)^2 \tag{6}$$

where Rs is the desired peak resolution, V_m and V_s the volume of mobile and stationary phases in the system, K the (mean) partition coefficient and ΔK the difference in K exhibited by the peaks in question. For difficult separations, where ΔK is small, the required number of plates, $N_{\min}$, is obviously large.

In order to see if a sufficient number of plates are available in a column, it is necessary to write $N = L/\hat{H}$, where $\hat{H}$ is the apparent (or measured) plate height. A simple but usually adequate equation for the latter is:

$$H = {}^9/_8\,(B'/p_i v_i + C_g' p_i v_i) + {}^3/_2\,C_l v_i \tag{7}$$

This equation is applicable only when $p_i \gg p_0$, as assumed here. The numerical factors, $^9/_8$ and $^3/_2$, originate as corrections for the pressure gradient[4]. The B' and C_g' terms represent longitudinal diffusion and non-equilibrium in the gas phase with the pressure dependence (originating in the diffusion coefficient) taken out. Thus these terms are equal to the usual B and C_g terms at unit pressure. Liquid non-equilibrium is represented by C_l.

With this expression for $\hat{H}$, and with the column length, L, as found in Eqn. (4), the number of plates, $L/\hat{H}$, becomes:

$$N = \frac{d_p^2 p_i}{4\phi\eta\left(^9/_8 \frac{B'}{p_i} + {}^9/_8\, C_g' p_i v_i^2 + {}^3/_2\, C_l v_i^2\right)} \tag{8}$$

The independent quantities of this equation are p_i and v_i; once these are chosen L is fixed by Eqn. (5). This equation shows that N decreases as the inlet velocity, v_i, becomes larger. With a given inlet pressure, particle size, etc., the very maximum in obtainable plates is approached as a limit when $v_i = 0$:

$$N_{\lim} = 2\, d_p^2 p_i^2 / 9\phi\eta B' \tag{9}$$

or, if B' is written in terms of the gaseous diffusion coefficient at unit pressure, D_g', *e.g.*, $B' = 2\,\gamma D_g'$, where γ is a structural parameter in the neighborhood of 0.6, this equation becomes:

$$N_{\lim} = d_p^2 p_i^2 / 9\phi\gamma\eta D_g' \tag{10}$$

This number of plates must be equal to or greater than the number, $N_{\min}$, required for the separation as shown in Eqn. (6). Thus for the separation of a given pair of components, the inlet pressure of the column must satisfy the following requirement:

$$p_i \geq (\phi\gamma\eta D_g')^{1/2}\, \frac{12\, Rs\, (V_m + KV_s)}{d_p V_s \Delta K} \tag{11}$$

THE CRITICAL PRESSURE

The right hand side of Eqn. (11) gives the lowest possible inlet pressure with which a resolution of Rs can be achieved for a pair of components having a difference ΔK in their individual partition coefficients. This pressure is termed the critical inlet pressure, *i.e.*:

$$p_c = 12(\phi\gamma\eta D_g')^{1/2}\, \frac{Rs\, (V_m + KV_s)}{d_p V_s \Delta K} \tag{12}$$

This can be conveniently expressed in terms of the R value:

$$R = V_m/(V_m + KV_s) \tag{13}$$

where R is the retention parameter indicating the velocity of the component peak divided by that of an inert (air) peak. After rearrangement:

$$p_c = 12(\phi\gamma\eta D_g')^{1/2}\, \frac{Rs}{d_p\, (\Delta K/K)\, (1 - R)} \tag{14}$$

where $\Delta K/K$ is the relative selectivity (this quantity also equals $\Delta t'/t'$, where t' is the retention time from the air peak).

An alternative to the above expressions for p_c can be obtained directly from Eqn. (10). Since N_{11m} is the largest number of plates obtainable with the inlet pressure p_i (assuming given values for d_p, η, etc.), we may solve for p_i and regard this as the critical (minimum) pressure for obtaining $N = N_{11m}$ plates, *viz.*:

$$p_c = (9\phi\gamma\eta D_g' N)^{1/2}\, d_p \tag{15}$$

This is equivalent to the rigorous equation derived earlier[1].

It is useful to calculate a numerical p_c value to illustrate the critical-pressure concept. All quantities must be expressed in consistent units, *e.g.*, cgs. We may assume a typical case in which $\phi = 300$, $\gamma = 0.6$, $\eta = 10^{-4}$ poise, $D_g' = 5 \cdot 10^5$ g·cm·sec^{-3} ($D_g = 0.5$ cm^2/sec at 1 atm), $d_p = 0.014$ cm ($\sim$ 100–120 mesh), $R = 0.5$ and $Rs = 1$. Upon substitution of these quantities into Eqn. (14) we get $p_c \cong 2 \cdot 10^5\, K/\Delta K$, in cgs units. Since 1 atm is approximately 10^6 dyne·cm^{-2}, this becomes:

$$p_c = 0.2\, K/\Delta K \text{ (in atmospheres)} \tag{16}$$

If, for example, it is necessary to separate two components with a relative selectivity, $\Delta K/K$, of $1/50$, the inlet pressure must exceed about 10 atm (this large value is not surprising when we consider that this separation would require 40,000 plates). This can be reduced to about 3 atm providing a coarse 30–40 mesh support is assumed.

While the treatment above is designed primarily for packed columns, it is equally valid for capillary columns. In the latter case the dimensionless constants are $\gamma = 1$ and $\phi = 16$. The column diameter, d_c, must replace the particle diameter, d_p, in the equations.

Strictly speaking, the equations for critical pressure are applicable only to columns existing as single segments. If several segments are linked together by pumps, these equations would apply only to the separation achieved in the individual segments.

The derivation given above is valid whether the column outlet is operated at reduced pressure or not. If the outlet is held at 1 atm, maximum use is not being made of the available inlet pressure. In any case the basic requirement for inlet pressure is $p_i \geq p_c$. If the outlet is held at 1 atm this simply increases the margin by which p_i must exceed p_c.

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SUMMARY

The concept of the critical inlet pressure, p_c, the lowest possible pressure for achieving a given separation, is discussed. The theoretical approach to this parameter starts with the basic flow equation for an ideal gas in a packed column. The integrated

flow equation and an expression for the plate height are then used to formulate the number of plates in the column. The maximum value of this must be greater than the minimum needed to separate a given pair. This criterion is used to formulate an expression for the critical pressure. Several alternate equations are given and a numerical example is presented.

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GAS CHROMATOGRAPHY ON SMALL DIAMETER COLUMNS*

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Packed columns of usually 4–6 mm inner diameter and capillary columns of diameter less than 1 mm find application in gas chromatography practice. Progress during the last two years in the sphere of increasing chromatographic resolution is related to the development of small diameter columns. Principal trends of work in this sphere were as follows:

(1) Formation of adsorbing layer on the capillary column inner wall by etching[1–4], the resulting large surface being coated with liquid film.

(2) Formation of an adsorbing layer by coating the wall with an active solid powder[5,6].

(3) Formation of an adsorbing layer by coating the wall with an active solid dispersed in a sol[7].

(4) Formation of a liquid film by coating the wall with powdered support impregnated with the stationary liquid[8].

(5) Filling a glass column with an active solid packing, then drawing out the column, decreasing its diameter, and pressing in active solid particles into the wall[9].

(6) Filling the column with an inert support followed by treating the latter and the column inner wall with a solution of the stationary liquid (frontal method)[10,11].

The aim of the above modifications is to increase the sorptive surface and to decrease column interstitial volume. This allows an increase in column selectivity without lowering efficiency in terms of H (height equivalent to a theoretical plate) and makes it possible in a number of cases to carry out an analysis on a short column. Maximum efficiency of H = 0.2–0.3 mm has been obtained by the authors using small diameter modified columns. But analysis using these columns presents certain difficulties and requires experience.

One more trend in the development of capillary columns consists in increasing the inner diameter up to 1.6 or even 2.5 mm[12–17]. The increase of sorptive capacity allows operation with samples large enough for the use of a thermal conductivity cell. In addition, there is the possibility of using very high velocities of carrier gas in long columns without substantial pressure gradients.

The advantages and disadvantages of capillary and packed columns are sufficiently discussed in the literature[18].

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In the choice of the optimum technique in the chromatographic process the following factors are particularly important: column selectivity, column efficiency, pressure drop, and the obtaining of reproducible data.

The selectivity coefficient of the column may be defined from the ratio:

$$K_c = \frac{\Delta\Gamma_0}{\Gamma_0} = \frac{\Delta\Gamma}{\Gamma + \frac{\varkappa}{\varkappa_1}} \tag{1}$$

where:

$\Delta\Gamma_0$ = difference of Γ_0 (overall Henry coefficients of adjacent components of sample);

$\Delta\Gamma$ = difference of Γ (true Henry coefficients);

$\varkappa$ = gas portion of the column volume, $\varkappa_1$ = portion of column volume occupied by stationary liquid.

The overall Henry coefficient is the ratio in a unit volume of the column of the total quantity of solute to the amount of solute in the gas phase. The true Henry coefficient is the ratio of the concentration of solute in the liquid phase to the concentration in gas phase.

It is quite evident that the K_c value will approach its asymptotic value $k_c = \Delta\Gamma/\Gamma$, when the $\varkappa/\varkappa_1$ value decreases. Thus, the most selectivity is provided by the packed columns.

To attain maximum efficiency it is necessary to have a large sorptive surface and a small thickness of liquid film, which can be obtained in different ways. In particular, a large surface is more readily obtained in packed columns.

The pressure drop per unit length is substantially lower for capillaries than for packed columns and this allows the use of capillary columns of greater length than packed columns.

Finally, it is evident that a packed column is much more readily reproducible than a capillary column. This is one of the chief reasons for using only packed columns in process chromatographs.

From the above it follows that to obtain high resolution the use of short packed columns is quite feasible. Decreasing the inner diameter enables the analysis time to be shortened and reduces the wall effect on peak spreading. An additional increase of efficiency is obtained by the reduction of the sorbent particles and by the use of small samples if a flame ionization detector is used (although the use of a thermal conductivity cell is possible). Further, due to the small sorptive capacity, the temperature may be lowered resulting in an additional increase in the selectivity coefficient.

In this connexion the examination of packed columns of less than 1 mm inner diameter was of interest. These columns, simple both in operation and design, possess valuable properties which enlarge the gas chromatographic possibilities. They have the advantages of satisfactory reproducibility, a proportionately small gas volume, and a reasonably uniform sorptive capacity throughout. All this favours the wide use of such columns in laboratory and process chromatographs. Also, low heat inertia due to the increased proportion of metal in the column section is an advantage in programmed temperature applications.

All experiments were done using either a Beckman flame ionization detector

and a recorder with a pen speed of 0.25 sec full scale, or a Gasofract 300 B (Dr Virus KG) with a flame ionization detector. Gaseous and liquid samples were injected by a microsyringe, a splitter being used if necessary. In the present work capillaries of 0.7–0.9 mm inner diameter were used. Column packings were obtained by coating the 0.10–0.14 and 0.14–0.20 mm fractions of Celite or Chromosorb W with various

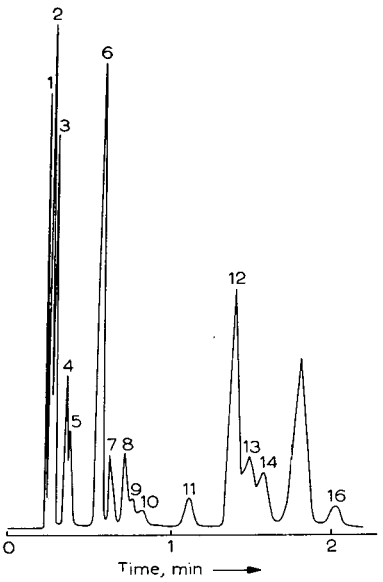

Fig. 1. Chromatogram of C_1–C_5 hydrocarbons mixture on the 1.25 m × 0.9 mm column. Quantity of column packing (20% w/w *n*-heptadecane on Celite) 0.390 g. Inlet pressure 2 kg/cm², temperature 38°. 1 = Methane; 2 = ethylene; 3 = ethane; 4 = propylene; 5 = propane; 6 = isobutane; 7 = isobutene + 1-butene; 8 = *n*-butane; 9 = *trans*-2-butene; 10 = *cis*-2-butene; 11 = 3-methyl-1-butene; 12 = isopentane; 13 = 1-pentene; 14 = 2-methyl-1-butene; 15 = *n*-pentane; 16 = 2-methyl-2-butene.

non-volatile liquids in the conventional manner. Columns were packed by two techniques. In the first a small flask containing column packing is joined to one end of the straightened column. The lower end of the column is closed by a small piece of metal gauze held in place by a very short length of capillary, which is attached to the main column by a metal collar. This end of the column is weighted by a hollow bolt and blind nut retained by the collar. Filling is done by tapping the lower end on a solid surface. Recently, column-filling has been done by another technique using a specially designed device involving a tripod and vibrator. Column filling is speedy taking only a matter of minutes.

The results of this research indicate that in the analysis of gaseous and liquid hydrocarbons efficiencies may be attained corresponding to H = 0.22–0.3 mm even with considerable inlet pressures (of the order of 2–3 kg/cm²); the reproducibility of the small diameter columns both in packing density and efficiency proved quite satisfactory.

This permitted the use of columns 0.14–1.25 m long for a rapid detailed analysis of pyrolysis gases, pentane–amylene fractions, and other mixtures involving com-

ponents up to C_{10}. As an example Fig. 1 shows a chromatogram of a C_1–C_5 hydrocarbons mixture, obtained with a 1.25 m column at 38°. In this case 16 components are separated in about 2 min; however, a further increase of carrier gas (nitrogen) flow-rate permits a reduction of the analysis time to 70 sec, the quality of resolution being only slightly lowered.

An analysis of paraffins and olefins with the same carbon number on a non-polar stationary phase is a complicated problem; nevertheless this problem can be solved for C_6–C_8 hydrocarbons using a length of only 14 cm of 0.9 mm diameter column packed with 15 % *n*-hexadecane or squalane, which carries out the separation in less than 8 min. On 1 m column the resolution is unnecessarily good (Fig. 2).

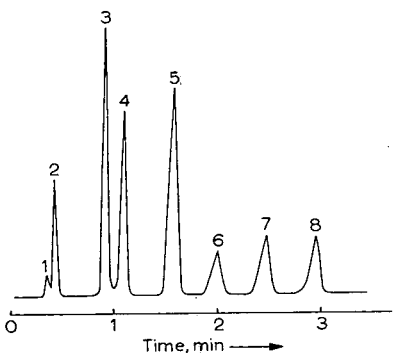

Fig. 2. Analysis of synthetic blend on the 1 m column. Inlet pressure 3 kg/cm², temperature 50°. 1 = 1-Pentene; 2 = *n*-pentane; 3 = 1-hexene; 4 = *n*-hexane; 5 = benzene; 6 = cyclohexane; 7 = 1-heptene; 8 = *n*-heptane.

When the partition coefficient of components is highly different, short columns naturally provide a rapid and complete resolution. Thus, for the separation of hexane, benzene, and toluene mixtures in 25 sec, a 38 cm column proved to be sufficient.

The application of small diameter packed columns opens a prospect for the development of techniques for the analysis of such complex mixtures as liquid petroleum products. Thus, a chromatogram of straight-run gasoline hexane fraction containing 20 peaks can be obtained in 5 min on a 52 cm column[19].

Fig. 3 shows a chromatogram of a mixture of oxygen-containing compounds and hydrocarbons obtained on the 30 cm column in about 3.5 min.

In papers recently published[10,11] packed capillary columns of 0.5 and 0.25 mm diameter with efficiencies up to 1500 theoretical plates per meter have been described. They were prepared by passing a solution of the stationary phase through a capillary filled with a solid support, both the packing and the wall being coated with liquid. In our opinion, confirmed experimentally, the preparation technique used in the present paper is quite simple and allows the packing of columns of the highest efficiency.

For a rapid analysis of substances with greatly different partition coefficients the use of short capillary columns is of considerable interest. Thus, Fig. 4 shows a chromatogram of a benzene, toluene, and *p*-xylene mixture, obtained on a 130 cm column of 0.9 mm inner diameter coated with *n*-heptadecane in 10 sec at room temper-

ature and at a carrier gas flow of the order of 200 ml/min. A complete resolution at very high gas velocities can be attained using a small film thickness. This results in a decrease of the liquid mass transfer term of the HETP equation and flattens the slope of the HETP *vs.* gas velocity curve after the minimum position. Thus a rise in flow

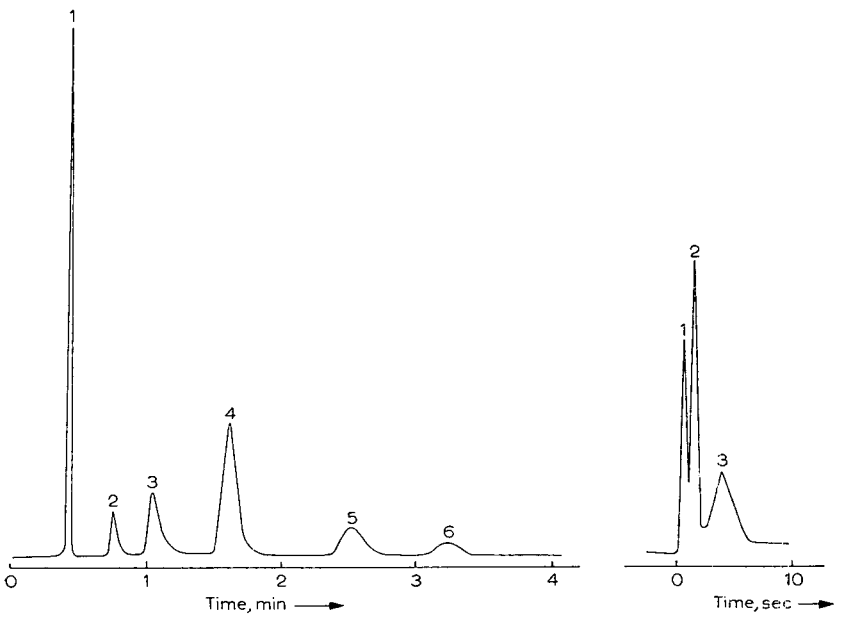

Fig. 3. Analysis of a mixture of oxygen-containing compounds and hydrocarbons on the 30 cm × 0.7 mm column with 15% w/w *n*-heptadecane on Celite (0.10–0.12 mm). Temperature 26°. 1 = Ethylene; 2 = acetone; 3 = allyl alcohol; 4 = methyl ethyl ketone; 5 = tetrahydrofuran; 6 = *n*-hexane.

Fig. 4. Chromatogram obtained on the 1.3 m × 0.9 mm capillary column coated with *n*-heptadecane. 1 = Benzene; 2 = toluene; 3 = *p*-xylene.

rate increases the HETP only to a very slight extent, but since the selectivity coefficient remains constant the high resolving power is retained.

Thus, small diameter short chromatographic columns are very powerful tools for the analysis of diverse organic mixtures and merit more extensive use.

SUMMARY

The principal trends of work in the sphere of increasing chromatographic resolution are considered. Short packed columns of inner diameter less than 1 mm are shown to possess high efficiency and resolving power. Examples are given indicating the possibility of using small diameter columns for the rapid analysis of various gaseous and liquid mixtures. Since small diameter packed columns can be readily and satisfactorily reproduced, their application in process chromatographs is feasible.

It is shown that with short capillary columns operating at low temperatures very high carrier-gas velocities can be used in some cases.

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THE USE OF BINARY STATIONARY PHASES IN GAS–LIQUID PARTITION CHROMATOGRAPHY*

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The separation efficiency in gas–liquid chromatography depends upon column performance (in terms of the number of theoretical plates) and upon the selectivity of the liquid phase. For analytical separations a stationary liquid is required to give a complete and selective separation. However, different liquid phases give different separations but in many cases an intermediate separation condition is required. Therefore, in more complicated analysis of mixtures with separation factors on one liquid phase close to unity, combined columns find increasing application. The following types of such columns have been used:

(1) Columns with various single liquid phases in each arranged in a sequence.
(2) Single columns composed of sections packed with various liquids.
(3) Single columns loaded with mixed binary liquid phases.

By changing the proportions of the lengths of the columns (or their sections) in the first and second case, or of the composition of the liquid phase in the third type, it is possible to effect a continuous change of properties between those of the pure liquids and thus to control within certain limits the relative retention times and the total duration of analysis.

Many examples of the use of combined columns have been reported in literature; some are distinguished by their quantitative approach to the problem of choice of optimal proportions of column (or section) lengths, or of the composition of the liquid phases, for practical analytical applications[1–7].

THEORETICAL CONSIDERATIONS

Determination of optimal composition of the stationary phase

Various methods of determination of the optimal weight or volume proportions of two stationary liquids have been suggested. ROHRSCHNEIDER[6] determined graphically the ratio of two section lengths containing different liquid phases. JUVET, CHIU AND SIMBORG[7] have derived mathematical relationships which permit the calculation of the weights of the two liquid phases necessary to obtain any intermediate retention time. From the weights of the two liquids, the necessary column length can be calculated.

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PRIMAVESI[3] has found a linear relationship between the relative retention volume (uncorrected for the pressure drop), or the retention time for a constant flow rate of the carrier gas, and the weight percentage of one of the liquid phases. For a small pressure drop along the column, and assuming an ideal mixing of the two stationary liquids, columns of the three types give similar separations[4].

If, however, the mixing deviates strongly from ideal behaviour, columns of the type 2 and 3 may give results differing from those obtained with columns of the type 1. In the limiting case of two practically immiscible liquids, columns of type 1 and 2 give very similar results which have been confirmed experimentally by PRIMAVESI[3]. For columns of the type 1, further mathematical relationships have been derived, permitting the calculation of the retention times for high pressure drops of the carrier gas[1].

In our investigations on the determination of optimal composition of the mixed liquid phase, we have utilized the KEMULA-BUCHOWSKI equation transferred from liquid–liquid partition equilibria to gas–liquid systems[8–10]. There are two forms of this equation, differing in their concentration units:

$$\log k' = u_1 \log k'_1 + u_2 \log k'_2 + \frac{V_{(m)}}{V^\circ_z} \cdot \frac{g^{E*}{}_{(m)}}{4{,}575\ T} \qquad (1)$$

$$\log {}^x k' = x_1 \log {}^x k'_1 + x_2 \log {}^x k'_2 + \frac{g^E{}_{(m)}}{4{,}575\ T} \qquad (2)$$

where:

$k' = Kr$ = partition number of the vapour in the system: mixed liquid phase–carrier gas;

K = partition coefficient;

r = ratio of cross-sectional areas of the fixed and mobile phase;

k'_1, k'_2 = partition numbers of the vapour in the system between a single component of the liquid phase and the carrier gas;

u_1, u_2 = volume fractions of the components of the mixed stationary phase;

${}^x k'$, ${}^x k'_1$, ${}^x k'_2$ = the respective "rational" partition numbers (concentrations in both phases expressed in mole fractions);

x_1, x_2 = mole fractions of the components of the mixed stationary phase;

V°_z = molar volume of the solute;

$V_{(m)} = \Sigma x_i V^\circ_i$ = molar volume of the mixed solvent;

V°_i = molar volume of a single pure solvent;

$g^E{}_{(m)}$ = excess free enthalpy of the mixed solvent;

$$g^{E*}{}_{(m)} = g^E{}_{(m)} - RT \sum_i x_i \ln \frac{l_i}{x_i}$$

where

$$l_i = \frac{V^\circ_i}{V^\circ_{(m)}}$$

$$-R \sum_i x_i \ln \frac{l_i}{x_i} = S^E{}_{(\mathrm{ath})} = \text{excess entropy of athermal solutions.}$$

For ideal solutions of two stationary liquids the relationship between $\log k'$ (log partition number of a vapour) and the molar per cent composition of the mixture is linear. With eqn. (2), however, linear relationships of $\log {}^x k' = f$ (composition)

are rarely obtained in practical investigations of binary liquid mixtures. Therefore, in our work[11–13] we have employed eqn. (1) for the determination of optimal composition of the mixed liquid phase for a given separation. Furthermore, linear relationships are often obtained when using eqn. (1), even for non-regular mixtures, which may be explained by a compensation of the deviations related to the excess free enthalpy of mixing $g^E{}_{(m)}$ by the excess entropy of mixing of athermal solutions $S^E{}_{(ath)}$ (*cf.* the commentary to eqns. (1) and (2)[9,10,13]). This further increases the possibilities of application of eqn. (2) for analytical purposes. Thus, from the relationship (1), the composition of the liquid phase can be determined at which the optimal separation of a given mixture is obtained.

The experimental determination of these relationships is carried out as follows:

(1) The determination of retention times for columns loaded with the pure stationary liquids.

The following parameters are to be identical in both cases: column length and diameter, volume of stationary phase at column temperature, carrier gas flow rate. The ratio p/p_i should be as similar as possible in both experiments. If the relationship $\log k' = f$ (composition) is to be determined more exactly, a few (2–4) measurements of the retention times must be carried out at intermediate compositions of the mixed liquid phase.

(2) Calculation of $\log k'$ (log partition number) from the retention times, using the formulae:

$$t = t_0 (1 + k') \tag{3a}$$

$$\log k' = \log \left(\frac{t}{t_0} - 1 \right) \tag{3b}$$

where:

t and t_0 = retention times of a given vapour and of an inert gas not retarded on the column, respectively.

(3) The plotting of the calculated values of $\log k'$ on 100 % composition axes (corresponding to pure liquids) and connecting the points with straight lines (Fig. 1).

(4) The determination of the composition of the liquid phase at which the distance between the $\log k' = f$ (composition) lines is the longest, taking into account the time axis t/t_0. This composition corresponds to optimal conditions of separation of the analysed mixture.

The above theoretical considerations have been discussed more fully and confirmed experimentally in our earlier work[11–13], in which the effect of the nature and composition of liquid mixed phases of various polarities on the separation efficiency has been investigated. It has been demonstrated that, by changing the composition of the mixed stationary phase, the separation efficiency can be controlled within certain limits, as well as the total duration of analysis. The following substances were analysed: cyclic and aromatic hydrocarbons, ethers, halogenated aliphatic hydrocarbons[11], aliphatic hydrocarbons C_5–C_7[12], methyl esters of *n*-, iso- and unsaturated fatty acids[13], the isomeric hexanes (petroleum ether)[14].

In Fig. 1 the relationship between $\log k'$ and volume composition of quinoline in diethyl malonate is illustrated for the following vapours: isopentane, pentane, 3-methylpentane, cyclopentane, hexane, furan, cyclohexane, heptane, cyclohexane, benzene and thiophene.

The fundamental differences between the relationship given by PRIMAVESI and eqns. (1) and (2) lie in the range of their application. The relationship between V_g and composition of the liquid phase holds well only for two immiscible, or ideally miscible stationary phases, when the experimental results coincide for all three types of column. Eqns. (1) and (2) do not apply at all to immiscible liquids, but only to two liquids miscible in all proportions, with the excess energy effect as small as possible (ideal systems).

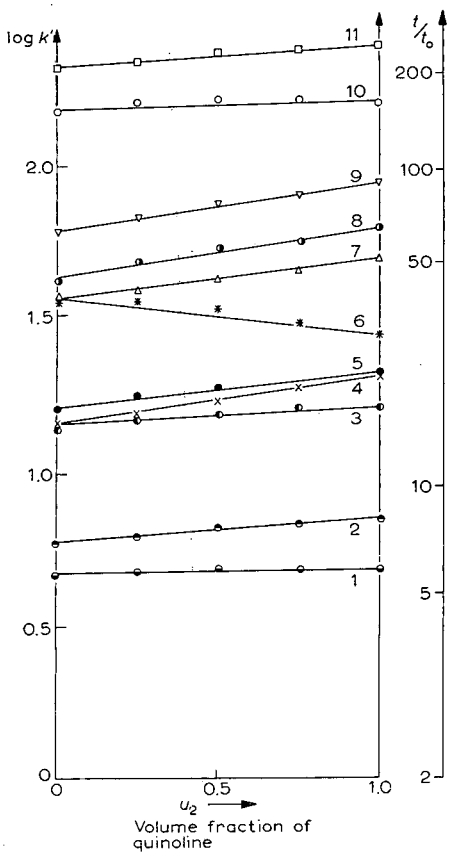

Fig. 1. Log of partition number k' of isopentane (1), *n*-pentane (2), 3-methylpentane (3), cyclopentane (4), *n*-hexane (5), furan (6), cyclohexane (7), *n*-heptane (8), cyclohexene (9), benzene (10) and thiophene (11). u_2 = volume fraction of quinoline in the system diethyl maleate–quinoline–nitrogen. Temperature of measurement: 35°; average inlet pressure 0.8 ± 0.01 atm; outlet pressure: atmospheric; carrier gas: N_2; flow rate at 20° (the permeability of capillary uncorrected for reference chamber): 60.1 ml/min; loading of stationary phase: 16.8% v/w; supporting material: Polish diatomite D (0.1–0.315 mm).

The determination of excess free enthalpy of mixing of binary solvents

As the value of K depends upon the nature of the solvent, it is possible to determine in this way the deviations from ideality of mixing of regular or approximately regular solutions employed as stationary phases. The value of K may easily be calculated from the retention times (eqns. (3a) and (3b)), thus, the method

described may be utilized not only for analytical purposes, but also for physico-chemical investigations, particularly for the determination of excess free enthalpy of mixing of binary solutions.

For the calculation of $g^E_{(m)}$, the relationship following from eqn. (2) was used[15]; as less simplifying assumptions were made in the derivation of eqn. (2), it reflects even small deviations of simple (regular) mixtures from ideality. In practice it leads to non-linearity of log ${}^xk'$ as a function of composition of the mixed solvent.

It should be pointed out that the partition coefficient K is related to V_g by the equation:

$$K = \frac{V_g T \rho}{273}$$

where ρ is the density of liquid phase at column temperature. It follows, therefore, that the excess free enthalpy of mixed solvents at column temperature can be found from deviations from linearity of the relationship $V_g = f$ (composition of stationary phase in mole fractions), provided that the flow rate is constant and the ratio p/p_i similar in all experiments. Of course, any variation of the density of the solution with its composition should be taken into account, as well as parallel changes of the ratio of mean molar volume of liquid to the molar volume of the gas (V°_i/V°_g). In the experimental part of this work, a method is given for the calculation of $g^E_{(m)}$ from the deviations of $\log {}^xk' = \log {}^xKr$, as a function of the composition of the mixed liquid phase (at a constant value of r). It is demonstrated that the partition coefficient K of the vapours at column temperatures can be calculated with good accuracy from values of k'; the variation of log K with the composition of the stationary phase may also be utilized for the determination of the excess free enthalpy of mixing.

EXPERIMENTAL

The measurements were carried out at 32° and 50° using a Model 116E Perkin-Elmer chromatograph with a thermoconductometric detector. Glass columns 105 cm long and of 5 mm internal diameter were employed. The stationary phases were triacetin, pelargonic acid (BDH, England), *n*-tetradecane (Light, England), quinoline (redistilled, Xenon, Poland); Polish diatomite D[16] was used as the supporting material; it was loaded with 16.8 % of the stationary liquids from their ether solutions. The flow rate of nitrogen was constant in all experiments and equal to 60 ml/min (measured with a bubble flowmeter at the column outlet). The flow rate was not corrected for the permeability of the capillary nor on the reference chamber of the detector. For a given binary system of stationary phases, care was taken to keep identical input pressures in all experiments, by a uniform packing of the columns. The chromatographed substances were: furan (Schuchardt, West Germany), *n*-hexane, cyclopentane (Light, England), cyclohexane, cyclohexene (Azoty Plant, Poland), and benzene (Chemical Reagents, Poland).

CALCULATION OF PARTITION COEFFICIENTS

It follows from the explanation to eqns. (1) and (2) that the partition number $k' = Kr$. Thus, for the calculation of the partition coefficients K, it is sufficient to

find the ratio of the volumes of the stationary (liquid) phase and the mobile (gas) phase and to divide the corresponding values of k' by r. Table I gives a comparison of partition coefficients calculated in this way (K_{I}) with those calculated from retention data (K_{II}). The volumes of the liquid and gas phase in the column, necessary for the calculation of r at column temperature were determined from retention data, according to the relationship:

$$V_{M(1)} = V_{A(1)}\, j\, \frac{T_1}{T_2}$$

$$V_{M(2)} = V_{A(2)}\, j\, \frac{T_1}{T_2}$$

$$V_{(L)} = V_{M(1)} - V_{M(2)}$$

$$r = \frac{V_{(L)}}{V_{(g)}} = \frac{V_{(L)}}{V_{M(2)}}$$

where:

$V_{M(1)}$ and $V_{M(2)}$ = the corrected retention volumes of an unadsorbed solute on a column containing the support alone (1) and with the stationary phase on it (2), respectively;

$V_{A(1)}$ and $V_{A(2)}$ = measured (uncorrected) retention volumes of unadsorbed solute on the two columns (as above);

V_L = volume of the stationary phase at column temperature;

T_1/T_2 = ratio of absolute column temperature (1) to temperature of measurement (2);

j = pressure correction factor.

The value of K_{II} calculated from the ratio of net retention volume and volume of liquid phase at temperature of analysis:

$$K = \frac{V_N}{V_L}$$

DETERMINATION OF $g^{E}{}_{(m)}$

As mentioned above, excess free enthalpies of mixing of binary solvents were determined graphically from the deviations of the relationship between logarithm of partition number (log ${}^{x}k'$) and the composition of the mixed phase, expressed in mole fractions (eqn. 2). From the retention times of analysed substances, the k' values were calculated from eqn. (3b) corresponding to partition numbers expressed in volume scale of concentrations. In order to calculate the value of log ${}^{x}k'$ (concentrations in molar fractions) the values of k' were multiplied by the ratio of the molar volumes of the liquid $V^{\circ}{}_L$ and $V^{\circ}{}_g$ under column conditions[9].

$${}^{x}k' = k' \frac{V^{\circ}{}_{(L)}}{V^{\circ}{}_{(g)}}$$

where:

$$V^{\circ}{}_{(L)} = \sum x_i V^{\circ}{}_i = x_1 V^{\circ}{}_1 + x_2 V^{\circ}{}_2$$

$$V^{\circ}{}_{(g)} = 22.4\, \frac{T_1}{273}\, j$$

TABLE I

COMPARISON OF THE PARTITION COEFFICIENTS CALCULATED FROM THE VALUES OF PARTITION NUMBER FOR FURAN, *n*-HEXANE, CYCLOPENTANE, CYCLOHEXANE, CYCLOHEXENE AND BENZENE WITH THOSE CALCULATED FROM RETENTION DATA

Liquid stationary phase / *Solute*	*n-Tetradecane* 50°		*Quinoline* 32°		*Tetralin* 32°		*Pelargonic acid* 50°		*Triacetin* 50°	
	K_I*	K_{II}**	K_I	K_{II}	K_I	K_{II}	K_I	K_{II}	K_I	K_{II}
Furan	57.680	57.134	223.565	225.565	153.111	150.780	94.466	94.192	177.979	178.05
n-Hexane	265.653	263.343	173.404	174.626	365.407	359.947	218.302	218.344	45.815	45.819
Cyclopentane	163.966	162.551	163.356	164.432	269.614	265.558	147.098	147.091	50.714	50.739
Cyclohexane	454.964	451.057	408.901	411.641	784.808	773.220	393.764	393.777	113.441	113.472
Cyclohexene	490.864	486.658	748.802	753.658	1033.397	1018.121	493.670	493.637	211.820	211.877
Benzene	367.760	364.580	1286.600	1294.956	1105.870	1089.551	479.613	479.604	556.244	556.294

* $K_I = k'/r$.
** $K_{II} = V_N/V_L$.

The calculated values of ${}^{x}k'$ were plotted on a diagram with a concentration scale expressed in mole fractions for the binary mixture of stationary phases. In Fig. 2, the relationship $\log {}^{x}k' = f$ (composition of stationary phase) is presented for furan (I), *n*-hexane (II), cyclohexane (III), cyclohexene (IV), and benzene (V) in the system triacetin–pelargonic acid. The values of deviations between the straight line

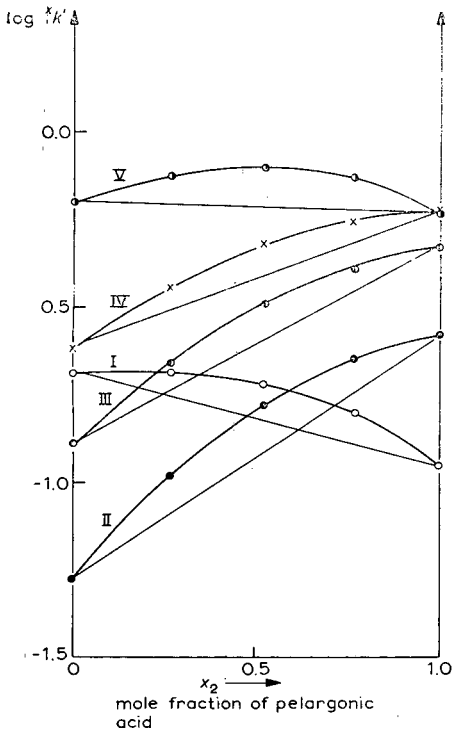

Fig. 2. Log of partition number ${}^{x}k'$ of *n*-hexane (●), furan (○), cyclohexane (◐), cyclohexene (×) and benzene (◑), x_2 = mole fraction of pelargonic acid in the system triacetin–pelargonic acid–nitrogen. Temperature of measurements: 50°; average inlet pressure: 0.97 ± 0.03 atm; the remaining conditions of experiments as in Fig. 1.

and the curve, measured vertically at a given composition of the mixed stationary phase are a quantitative measure of its deviation from ideality and are proportional to the value of $g^{E}{}_{(m)}$, as according to eqn. (2):

$$\Delta \log {}^{x}k'_{(ex)} = \frac{g^{E}{}_{(m)}}{4{,}575\,T_1} \tag{4}$$

From these deviations, determined graphically for the five substances given, mean values of $\Delta \log {}^{x}k'_{(ex)}$ were calculated for a series of compositions of the mixed stationary phase and also the corresponding values of $g^{E}{}_{(m)}$ in cal/mol. A plot of $g^{E}{}_{(m)}$ as a function of molar fraction of pelargonic acid in triacetin is presented in Fig. 3. The maximal calculated value of $g^{E}{}_{(m)}$ in this system is equal to *ca.* 160 cal/mol and corresponds to the concentration of *ca.* 0.52 molar fraction of pelargonic acid.

The method of calculation of partition coefficients from partition numbers (k') and the volume of the liquid phase in the column (V_L) is a simplification of calculation methods employing other retention data. It follows from the fact that, by mathematical transformations of the k' value, analogous relationships are obtained between partition coefficient and retention data.

$$k' = \frac{t - t_0}{t_0} = \frac{V_R - V_A}{V_A} = \frac{V'_R}{V_A}$$

As

$$k' = Kr = K\frac{V_L}{V_M} \quad \text{and} \quad \frac{V_M}{V_A} = j\frac{T_1}{T_2}$$

it follows that:

$$K = k'\frac{V_M}{V_L} = \frac{V'_R}{V_A} \times \frac{V_M}{V_L} = \frac{V'_R j}{V_L}\frac{T_1}{T_2} = \frac{V_N}{V_L}$$

It seems, however, that the use of relative values $(t - t_0)/t_0$ may eliminate partly the error due to the calculation of the correction factor for pressure drop along column, and the inaccurate measurement of actual carrier gas flow rate in some chromatographs. The agreement of partition coefficients calculated from k' values, and those calculated from other retention data, is quite good, as is seen from the comparison in Table I. Relatively large differences in K values observed in the case of tetralin as stationary phase may be explained by inaccurate measurements due to evaporation of the liquid from the column at the temperature employed. We were not able to ascertain which of these K_I or K_{II} values were more accurate.

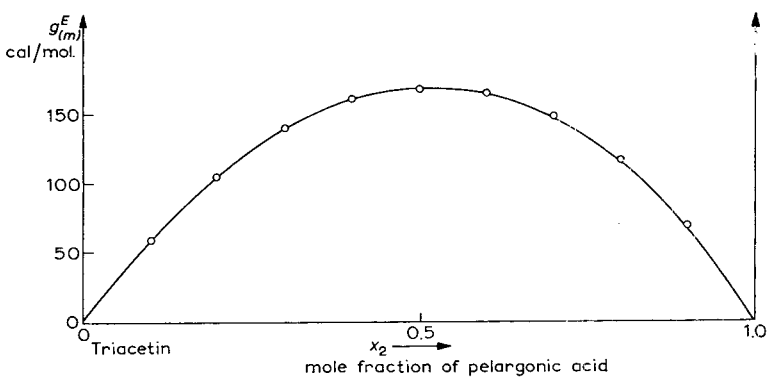

Fig. 3. Excess free enthalpies ($g^E_{(m)}$) of mixtures of triacetin and pelargonic acid, calculated from data of Fig. 2.

Excess free enthalpies of mixed solvents are a measure of deviations from ideality of the system studied and one of the measurable functions determining the thermodynamic state of the system. Therefore, they may be found useful for the estimation of the interaction forces between two solvents and between the solvent mixture and the solute.

The described methods by gas chromatography permit the determination of the function $g^E_{(m)}$ rapidly and quite accurately.

It is evident that the accuracy of determination will be proportional to the magnitude of deviations from linearity of the relationship log ${}^{x}k' = f$ (composition in mole fractions). According to eqns. (1) and (2), the value of $g^{E}{}_{(m)}$ found in practice decreases with the absolute temperature of measurement. It follows, therefore, that the determinations of $g^{E}{}_{(m)}$ by the chromatographic method should be carried out at as low temperatures as possible. It should also be pointed out that eqns. (1) and (2) were derived for single (regular) solutions (conformal system).

Assuming a limiting, very low concentration of the solute, the interactions between the solute and the mixed solvent may then be neglected. If, however, the mixture of solvents cannot be classed as regular (especially when stronger interactions of the hydrogen bond type are brought into play) then the nature of the solute may also influence the magnitude of deviations from linearity of the relationship log ${}^{x}k' = f$ (composition). It is evident that in these cases the deviations from linearity are not only a measure of $g^{E}{}_{(m)}$ but also reflect the magnitude and character of interactions between the solute and the mixture of solvents. In the system described pelargonic acid and triacetin were employed which belong to groups of compounds[1] capable of hydrogen bonding. It is probable that here lies the cause of slight differences in the magnitude of deviations observed for different solutes at a given composition of the mixed solvent. If that is the case, the deviations should be considered as due to a sum of excess free enthalpy of mixing and of excess thermodynamic potential, the latter related to interactions between the solute and the solvent.

Thus the differences between magnitudes of deviations for various solutes would be a measure of the differences of interactions between these solutes and the mixed solvent of a given composition. In our calculations of $g^{E}{}_{(m)}$ for the system pelargonic acid/triacetin/hydrocarbons we had too few experimental data to take these considerations into account, and thus the plot of $g^{E}{}_{(m)} = f$ (composition) in Fig. 3 represents arithmetic mean values. The curve representing the variation of mean values of $g^{E}{}_{(m)}$ is asymmetrical, and the maximum is shifted in the direction of higher concentrations of pelargonic acid.

Judging from its shape, the system studied is intermediate between regular (simple) and complex solutions.

SUMMARY

The theoretical basis of choice of optimal composition of the binary mixed stationary phase for a given separation problem is described; a number of practical applications is presented. The possibility of the application of binary solvent systems in gas chromatography is considered for the investigation of the energy effect due to deviations from ideality of binary liquid mixtures. In the experimental part a method of calculation of excess free enthalpies of mixing solvents $g^{E}{}_{(m)}$ is given, together with an experimental illustration. The values of $g^{E}{}_{(m)}$ have been calculated from the deviations from linearity of the relationship log ${}^{x}k' = f$ (composition of mixed phase). The values of partition numbers (k') determined from retention times have been used for the calculation of partition coefficients (K) of solutes at column temperature. The results obtained are compared with partition coefficients calculated from other retention data.

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THE THEORY OF GAS ELUTION CHROMATOGRAPHY WITH LARGE SAMPLES AND CALCULATION OF THE NECESSARY SENSITIVITY OF THE DETECTION SYSTEM*

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Within the scope of elution chromatography the usual requirements concerning the sensitivity of the detector are determined not only by the initial concentration, C_0, of the substance to be determined in the mixture analysed and the accuracy of determination required (permissible relative error σ) but also by the dilution of the injected sample in the chromatographic column, as a result of which the concentration, C, in the chromatographic peak is usually significantly less than the initial concentration in the sample. An increase in the amount of sample injected (q), although resulting in a corresponding increase in C is, however, limited by the decrease in the resolution power of the chromatographic unit.

It is generally thought necessary[1] that the limiting permissible sample q_m should be so small that the width of a chromatographic peak, τ, would be determined only by processes of dilution of the sample in the column and independent of sample size, where τ is the duration of a chromatographic peak, measured between the moments of appearance on the elution curve of the points, corresponding to half the value of the maximum signal, expressed in seconds. However, as will be shown later, in the great majority of cases such a requirement is excessive and results in an unjustified decrease of the actual sensitivity of the method.

In practice, it is a sufficiently good resolution of the peak of a component analysed from the neighbouring peaks on the chromatogram that is of interest, rather than the duration of the peak, τ, as such. As it is known[2], the separation is characterized by the resolution factor:

$$K_1 = \frac{\Delta t}{\tau_1 + \tau_2} \qquad (1)$$

depending on the difference Δt of the retention times of neighbouring components.

The required accuracy of chromatographic analysis determines the necessary resolution. To evaluate it in a quantitative manner, the relationship should be established between the resolution factor, K_1, and the error due to the overlapping of the peak of the component measured and the signal from the neighbouring in-

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completely resolved component. Such a calculation for the case of equal peak heights of the components to be separated on the elution curve has been made by one of the authors and N. M. TURKEL'TAUB[3]. In the general case the purity of a fraction, *i.e.* the proportion of the main component of the fraction, may be expressed in terms of two dimensionless parameters, one of which is the K_1 factor, and the other, the ratio of maximum heights of the peaks analysed, W.

The calculation, performed on the assumption that the shape of either of the two neighbouring peaks on the chromatogram may be described by a Gaussian curve equation with one value of the decrement index, enable us to relate the relative error, σ, introduced by an unresolved component, with the resolution factor, K_1, and the parameter W. In the case of analysis according to peak heights:

$$\sigma_h = We^{-16\,K_1^2} \tag{2}$$

When the analysis is made on the basis of peaks areas:

$$\sigma_s = \frac{W}{2}\left[1 - \phi\left(2\,K_1 + \frac{\ln W}{8\,K_1}\right)\right] \tag{3}$$

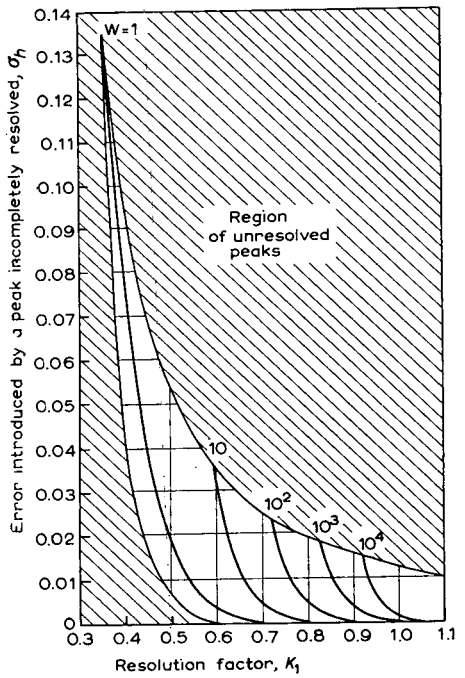
where:

$$\phi(x) = \frac{2}{\sqrt{\pi}}\int_0^x e^{-x^2}\,dx \text{ (the probability integral)}$$

These results, represented graphically in Figs. 1 and 2, make it possible to assess the necessary values of the resolution factor, K_1, from the precision of analysis required. The limiting values of K_1 and W, confining the region of unresolved peaks, have been found by determining the extremum of the function describing the result of superposition of two Gaussian curves, and by determining the regions where three extrema of this function are fused in one. An example of such a calculation may be found in the paper by GHENKIN[4].

The relation (3) permits the length of column necessary to secure the required purity of fraction to be calculated, depending on the relative content, W, of the interfering component. In Fig. 3 the results of such a computation are presented, showing precisely the dependence previously reported by one of the authors[5].

Let us now consider the dependence of the resolution factor K_1 and concentration C_m at peak maximum on sample size, q. If the initial peak width is comparable to the amount of diffusional dilution in the column, the shape of the peak cannot be described by the Gaussian curve equation. In this case, however, the elution curve may be considered as a result of superposition of a series of Gaussian curves, each of which corresponds to the elementary volume of sample, shifted in time with respect to each other. Therefore the shape of the peak may be found by multiplying the Gaussian curve by a function describing the distribution in time of concentration of the sample introduced and by integrating the product as a function of sampling time. Calculations of this type for different procedures of sample introduction are given in the literature[6]. To introduce the sample as a rectangular impulse with a concentration C_0 and duration B (the "plug" method), the relation describing the



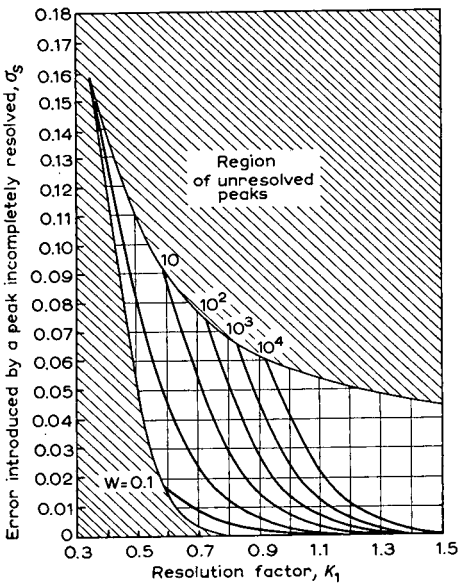


Fig. 1. Dependence of relative error (σ_h), introduced by the unresolved component, on the resolution factor (K_1), in quantitative analysis using peak heights. W = ratio of peak height of the interfering component, to that of the component to be determined.

Fig. 2. Dependence of relative error (σ_s), introduced by the unresolved component, on the resolution factor, (K_1), in quantitative analysis using peak areas. W = ratio of peak height of the interfering component, to that of the component to be determined.

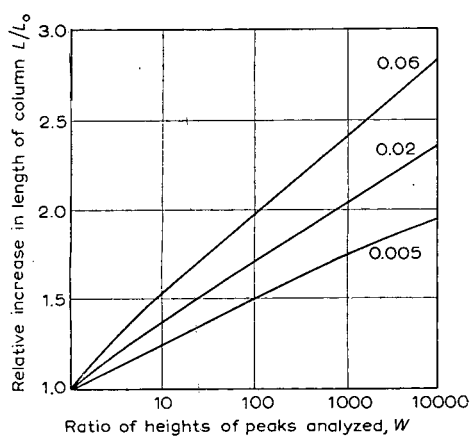


Fig. 3. Dependence of the relative increase in length of column (L/L_0) necessary for obtaining the required purity of fraction on the W parameter. Figures beside the curves indicate the selected value of the error σ_s due to incomplete resolution. L_0 = length of column securing the required purity of fraction (relative error σ_s) with W = 1.

shape of the peak as it emerges from the column may be represented as follows:

$$C(t) = \frac{C_0}{2}\left\{\phi\left[\sqrt{\frac{L\alpha}{4D}}\left(1 - \frac{t}{t_0} + \frac{B}{t_0}\right)\right] - \phi\left[\frac{L\alpha}{4D}\left(1 - \frac{t}{t_0}\right)\right]\right\} \tag{4}$$

where:

t_0 = retention time for an infinitely small sample size;
L = length of column;
α = linear velocity of carrier gas;
D = effective diffusion coefficient;
ϕ = probability integral.

The peak maximum emerges from the column at time $t_m = t_0 + B/2$. This is easily shown by differentiation of (4). Substitution in (4) gives the maximum concentration:

$$C_m = C_0\,\phi\left(\frac{B}{2\,t_0}\sqrt{\frac{L\alpha}{4D}}\right) \tag{5}$$

which is equivalent to:

$$C_0\,\phi\left(\frac{B}{4\,\Gamma}\sqrt{\frac{\alpha^3}{DL}}\right)$$

Considering that the duration of the peak, τ_0, observed at an infinitesimal sample size, q, is determined by the relation (3):

$$\tau_0 = 4\,\Gamma\sqrt{\frac{DL}{\alpha^3}}\ln 2 = 3.330\,\Gamma\sqrt{\frac{DL}{\alpha^3}} \tag{6}$$

where Γ is the Henry coefficient. Hence the expression (5) may be rewritten in the following form:

$$C_m = C_0\,\phi\left(0.8326\,\frac{B}{\tau_0}\right) \tag{7}$$

The peak width, τ, may be determined satisfying the equality $C(t) = 0.5\,C_m$. Using (4), (5) and (6) let us transform it to the following form:

$$\phi\left(Z + 1.6652\,\frac{B}{\tau_0}\right) - \phi\,(Z) = \phi\left(0.8326\,\frac{B}{\tau_0}\right) \tag{8}$$

where:

$$Z = \sqrt{\frac{L\alpha}{4D}}\left(1 - \frac{t}{t_0}\right) \tag{9}$$

Equation (8) has two roots, Z_1 and Z_2, to which correspond the moments, t_1 and t_2, of recording the points of half concentration. Obviously:

$$\tau = t_2 - t_1 \tag{10}$$

Since the probability integral $\phi(x)$ is an odd function, the roots Z_1 and Z_2 of eqn. (8) are related by the following relationship:

$$Z_2 = -Z_1 - 1.6652 \frac{B}{\tau_0} \tag{11}$$

Using the equalities (9), (10), and (11), the following expression may be obtained to determine the peak width, τ:

$$\frac{\tau}{\tau_0} = 1.201\, Z_1 + \frac{B}{\tau_0} \tag{12}$$

where Z_1 is the positive root of transcendent eqn. (8), which may be found by the common method for approximate solution.

From the known values of τ/τ_0 it is easy to calculate the relative impairment of resolution, K_1/K_0, due to the increase in sample size, by using the expression (1):

$$\frac{K_1}{K_0} = \frac{\tau_0}{\tau} \tag{13}$$

where K_0 is the maximum value of resolution factor for the particular conditions of the chromatographic run, corresponding to an infinitesimal sample.

The results of calculating C_m/C_0 (from (4)), τ/τ_0 (by an approximate solution (8)) and substitution of the value found (Z_1 into (12)), and K_1/K_0 (by using the relation-

TABLE I

DEPENDENCE OF THE RELATIVE CONCENTRATION (C_m/C_0) AT THE PEAK MAXIMUM, RELATIVE PEAK WIDTH (τ/τ_0) AND RELATIVE IMPAIRMENT OF RESOLUTION (K_1/K_0) ON THE RELATIVE SIZE OF THE SAMPLE INTRODUCED (B/τ_0)

B/τ_0	C_m/C_0	τ/τ_0	K_1/K_0
0.0	0.0000	1.0000	1.0000
0.1	0.0937	1.0042	0.9958
0.2	0.1862	1.0092	0.9909
0.3	0.2761	1.0209	0.9793
0.4	0.3624	1.0374	0.9639
0.5	0.4440	1.0590	0.9443
0.6	0.5201	1.0859	0.9209
0.7	0.5902	1.1179	0.8946
0.8	0.6538	1.1560	0.8651
0.9	0.7107	1.1997	0.8334
1.0	0.7602	1.2502	0.7999
1.2	0.8423	1.3662	0.7320
1.4	0.9007	1.5052	0.6644
1.6	0.9404	1.6633	0.6012
1.8	0.9660	1.8362	0.5446
2.0	0.9815	2.0196	0.4951
2.2	0.9904	2.2102	0.4525
2.4	0.9952	2.4050	0.4159

ship (13)) with different values of the parameter, B/τ_0, characterizing the size of the injected sample, q, are given in Table I and graphically represented in Fig. 4.

$$q = vC_0B \tag{14}$$

when v is the volume flow rate of carrier gas.

Obviously, an increase in sample size injected is an efficient method for increasing the sensitivity of chromatographic analysis, since with the consequent increase of concentration at the peak maximum there occurs only a relatively small increase in peak width and thus a relatively small impairment of the resolving power. Thus, at $B/\tau_0 = 0.4$ the concentration at peak maximum is about 36 % of the concentration in the sample, while the resolution factor decreases only to 96.4% of the value of K_0 corresponding to an infinitesimally small sample. For the sake of comparison it may be noted that KEULEMANS[1] in his monograph regards $B/\tau_0 = 0.02$

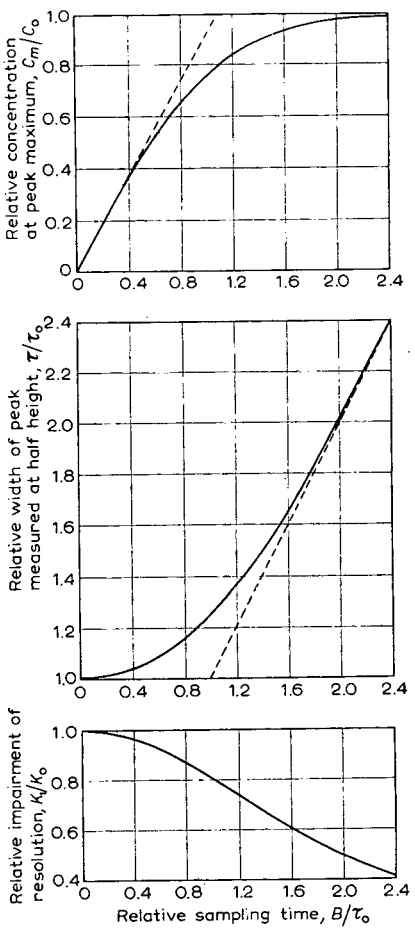


Fig. 4. Dependence of relative concentration (C_m/C_0) at peak maximum, relative peak width (τ/τ_0) and relative impairment of resolution (K_1/K_0), on the relative size (B/τ_0) of the sample introduced.

as the limiting permissible value for the sample injected, *i.e.* a value nearly 20 times less.

For an experimental verification of the relationships obtained, the height h and duration τ of chromatographic peaks were measured for each volume V of the liquid sample introduced into the sampling device. A study was made of the resolution mixture containing approximately equal amounts of benzene, octane, and nonane ($\sim$ 5% each), dissolved in diethyl ether. The analysis was made on a column 200 cm long, 5 mm in diameter, packed with Celite 545, 80–100 mesh, with 30 % of silicone oil DC 550 at a temperature of 130°. Hydrogen was used as carrier gas; its rate was maintained at 170 ml/min. The liquid sample was injected into the vaporizer by means of a syringe with a micrometric movement of the piston.

A comparison of the experimental with the calculated values (presented in Table I) was made by plotting the two relations on a double logarithmic scale and superimposing the calculated curve on the experimental one until the best coincidence

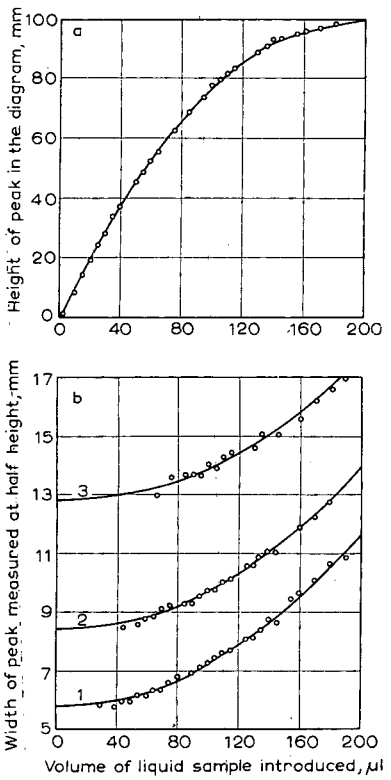


Fig. 5. Dependence of the height (h) and width (τ) of chromatographic peaks on the volume (V) of the liquid sample introduced. Solid lines show calculated curves plotted for the following values of the parameters: 1 = benzene, V_0 = 100.2 µl, h_0 = 102.3 mm, τ_0 = 5.74 mm; 2 = octane, V_0 = 125.9 µl, h_0 = 105.2 mm, τ_0 = 8.36 mm; 3 = nonane, V_0 = 162.2 µl, h_0 = 115.3 mm, τ_0 = 12.7 mm. The points show experimentally measured values. Column: 200 × 0.5 cm; inert support: Celite 545; grain size: 80–100 mesh; stationary phase: silicone DC 550, 30 % of the support by weight; t = 130°; carrier gas: hydrogen; hydrogen flow rate: 170 ml/min. Thermal conductivity detection. Because of an overlapping of curves in Fig. 5 (a), only the results for benzene are shown.

is reached, while the co-ordinate axes of the two curves are kept parallel; the position of the origin of co-ordinates of the calculated curve determined the values of the parameters h_0 and V_0, and τ_0 and V_0, respectively, for the dependence of the height h or duration τ of peaks on the sample volume V.

From the values of parameters thus found, calculated relationships were plotted, which were subsequently compared to the values experimentally measured.

The results of comparison are shown in Fig. 5, where the solid lines indicate the calculated values, and the points have been obtained by direct measurements. It is evident that the agreement of theoretical and experimental values is quite satisfactory. Therefore the use of the results of the theory described above for an assessment of the necessary values of sensitivity of detecting devices is fully justified.

To calculate the threshold sensitivity of a detecting system required for a particular chromatographic run the following quantities must be known:

(1) Concentration C_0 of the component to be determined in the system analysed.

(2) Limiting permissible relative error σ under the particular conditions of the analysis.

(3) The limiting value of the resolution factor K_0 obtained with a vanishingly small sample size. (The value of K_0 is determined by the physico-chemical characteristics of the components to be separated and by the choice of conditions for making the chromatographic run.)

(4) Relative concentration W of the neighbouring component on the elution curve.

The calculation is made as follows. From the given values of σ and W the value of the resolution factor, K_1, necessary for securing the required accuracy is determined from the curves presented in Figs. 1 or 2 (depending on the selected method of analysis from peak heights or areas). From the known value of the K_1/K_0 ratio, by using the curves in Fig. 4, the value of the maximum admissible sample and the corresponding value of C_m/C_0 are determined. Finally, the threshold sensitivity $C_{\min}$ is determined from the condition:

$$C_{\min} = \sigma C_m \tag{15}$$

Some typical examples are discussed below.

EXAMPLES OF APPLICATIONS OF THEORY

(a) *Analysis of comparatively large concentrations on ordinary packed columns*

Concentration of the component to be determined, 10%; with $M = 100$ g/mol this corresponds to $C_0 \approx 0.4$ mg/ml; permissible error $\sigma = 0.01$; $K_0 \approx 1$; $W \approx 10$; the calculation is made from peak areas.

From Fig. 2 it is shown that to secure the accuracy required a value of 0.95 is needed for the resolution factor, K_1, *i.e.*, the permissible impairment of resolution because of an increase in sample size, K_1/K_0, is 0.95. From the plot in Fig. 4 we determine that $B/\tau_0 = 0.46$ and $C_m = 0.41\ C_0$. Then from the equality (15) the necessary value of threshold sensitivity is calculated:

$$C_{\min} = 0.01 \times 0.41 \times 0.4 \frac{\text{mg}}{\text{ml}} \approx 1.6 \times 10^{-3} \frac{\text{mg}}{\text{ml}}$$

The value obtained is suitable for thermal conductivity detectors, which are actually used for such analyses.

(*b*) *Analysis of impurities on ordinary packed columns*

Concentration of the component to be determined, 10^{-3} %; *i.e.* $C_0 \approx 4 \times 10^{-5}$ mg/ml; $\sigma = 0.05$; $K_0 \approx 1$; $W \approx 10^4$.

In this particular case from the plot in Fig. 2, we find $K_1 = 0.97$ and then from Fig. 4 we determine: $B/\tau_0 = 0.36$; $C_m = 0.33\ C_0$.

The threshold sensitivity required:

$$C_{min} = 0.05 \times 0.33 \times 4 \times 10^{-5} \text{ mg/ml} \approx 7 \times 10^{-7} \text{ mg/ml}$$

It is evident that for this analysis the use of high sensitivity ionization detector is required.

(*c*) *Relatively precise quantitative analysis of components difficult to separate on capillary columns*

Concentration of the component to be determined, 1 %; *i.e.*:

$$C_0 \approx 4 \times 10^{-2} \text{ mg/ml}; \sigma = 0.02; K_0 = 0.74; S = 10^2$$

Due to a poor resolution, it is advisable to treat the result using peak heights. From the plot in Fig. 1 it is seen that the required value of K_1 is near to K_0, and it would be difficult to determine it with sufficient precision. Therefore we calculated K_1 by using formula (2) and find $K_1 = 0.737$. Hence, $K_1/K_0 = 0.997$. From Table I we determine by interpolation:

$$\frac{B}{\tau_0} = 0.07 \text{ and } C_m = 0.06\ C_0$$

Consequently:

$$C_{min} = 0.02 \times 0.06 \times 4 \times 10^{-2} \text{ mg/ml} \approx 5 \times 10^{-5} \text{ mg/ml}$$

Considering that in capillary chromatography ionization microdetectors of the flow type are used, and the rate of carrier gas flow is near 1 ml/min, let us determine the required value of the flow type detector sensitivity:

$$j_{min} = 5 \times 10^{-5} \text{ mg/ml} \times 1.7 \times 10^{-2} \text{ ml/sec} \approx 8 \times 10^{-7} \text{ mg/sec}$$

CONCLUSIONS

The proposed methods of calculation permit the assessment of the required values of the resolution factor and of the necessary length of column from the known conditions and also the calculation of the limiting sample size and of the necessary sensitivity of the detecting system.

The results obtained show that in practice it is advisable to work with samples of a substantially larger size than is usually recommended in the literature on chromatography.

SUMMARY

Formulae relating the error caused by incomplete resolution of peaks on the elution curve to the resolution factor and relative proportions of the components have been derived.

The dependence of peak height, peak width, and resolution factor on the size of the sample introduced has been theoretically established and experimentally confirmed. It is shown that increasing the sample size is an effective method for increasing the sensitivity of chromatographic analysis; the conditions for selecting the optimum sample size are formulated.

A method for an approximate calculation of the threshold sensitivity of a detector from the conditions of a given analytical problem is suggested and illustrated by typical examples.

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PREPARATIVE SCALE GAS CHROMATOGRAPHY. III.

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In two previous communications we investigated substance recovery[1] and choice[2] of carrier gas in preparative scale gas chromatography (PGC). The present paper is a further report on experimental work, using this technique.

THE CAPACITY OF PREPARATIVE SCALE GAS CHROMATOGRAPHY

In gas–liquid chromatography the plate number* should be derived from extrapolation to zero sample size and for a substance having a large partition factor. The number so obtained is then a measure of the analytical separation power of the column for that solute and for closely similar substances. With increasing sample size the relative band width increases and a graph of this, with relative band width expressed as "plate number", for a preparative scale gas-chromatographic column is shown in Fig. 1.

The smaller "plate number" for increased sample size reflects the separation power of the column for these large samples. This number could be named the "preparative scale plate number" and should be given with an indication of the sample size to which it refers.

In the case of mixtures which have to be separated, the sample size is determined by this preparative scale plate number and also by the relative retention of the substances. This relative retention, α, is equal to k_2/k_1 or K_2/K_1 or V'_{R_2}/V'_{R_1}; k, K and V'_R being the partition ratio, the partition coefficient and the adjusted retention volume[3], respectively. Large α values mean large samples of the mixtures and vice versa. The permissible sample size should obviously be as large as possible. This is the most important point in preparative scale gas–liquid chromatography.

In trying to increase the capacity of preparative scale gas–liquid chromatography, efforts have in the past been mainly directed to cancelling the adverse effect of increased sample size. This applies to the use of increased column diameters, parallel columns, automatic column operation repeating the same separation over and over again and also of continuous flow techniques.

The largest possible capacity could also be attained by finding the liquid phase which would give the largest relative retention, α, for the mixture in question. This,

* Plate numbers in this paper have been calculated with the equation $n = 16 \cdot (V_R \cdot V'_R)/Y^2$ in which V_R and V'_R are the retention volume (distance) and the adjusted retention volume (distance) and Y is the band width at the peak slope tangent intercepts with the base line. This equation is equivalent to $n = 16 \cdot (V_R/Y)^2/(1/k_2 + 1)$ and therefore partly takes into account the influence of the partition factor on plate numbers. For large partition factors it approximates to the equation usually employed, $n = 16 \cdot (V_R/Y)^2$.

however, leads to a large number of columns and to frequent column changes, which is an annoyingly time-consuming operation.

Still another approach, however, is to increase the separating power of the columns as much as possible and this is most simply obtained by increasing the column length of a relatively small bore column. Such a column will show an increased preparative scale plate number and will allow larger samples to be introduced.

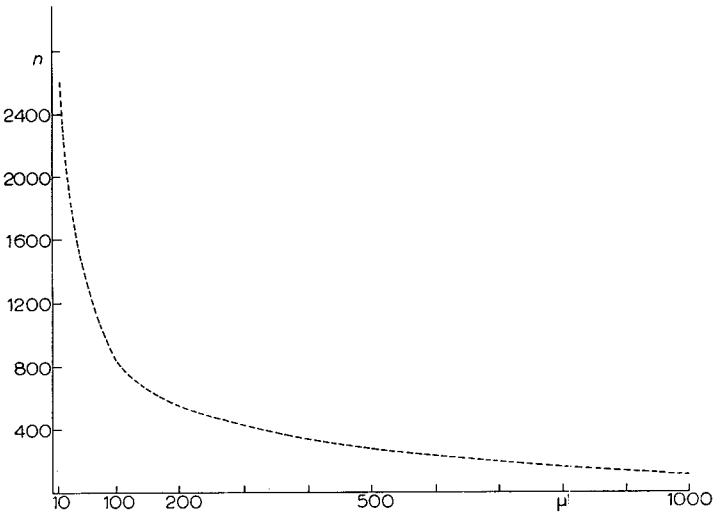


Fig. 1. Plate number, n, *vs.* sample size for a 6 m × 9 mm coiled glass Chromosorb P 30/60 mesh column coated with 35% SE. 30. 200 ml H_2/min and iso-octane as sample. The column was old and better results are obtainable.

It should also be possible to use only one or a very reduced number of such columns to solve most problems.

This paper discusses the experimental results of this approach, the parameters influencing the preparative scale plate number and the use of this number to find the permissible sample load.

The number of theoretical plates required for complete separation of two substances is given by the equation[3]:

$$N = 16\,[\alpha/(\alpha - 1)]^2\,[1/k_2 + 1]^2 \qquad (1)$$

This equation is, in principle, used to deduce the column length necessary for a given separation.

In preparative scale gas chromatography, eqn. (1) will not be used to find column length, but to find the maximum permissible sample size of a column. A sample calculation will serve to illustrate this. Consider the 6 m × 9 mm column of Fig. 1, a H_2 gas rate of 200 ml/min and a limiting duration of the separation < 2 h, which is equivalent to a partition factor in the range of 10 to 100. With such a partition factor the column will have its maximum separation power and the second term in eqn. (1) can be neglected. For an α value of 1.25 (this is the case for the *trans–cis*-decalin mixture at about 160°) eqn. (1) leads to 400 required plates. Fig. 1 shows that

the column gives that preparative scale plate number for sample sizes as large as 350 μl. In 500 μl decalin mixture there is about 350 μl of the *trans*-isomer and 500 μl is therefore the maximum possible sample size of this mixture which will still give complete separation.

This is in practice found to be the case, and a graph of this separation has already been published in ref. 2.

PERCENTAGE OF STATIONARY PHASE

The percentage of stationary liquid phase could have an influence in preparative scale gas chromatography. In order to verify that this parameter is critical, a 6 m × 9 mm glass column filled with Gas-Chrom P (Applied Science) as support was tested successively with 5, 10, 15, 20, 25, 30 and 35 % SE. 30 silicone gum as stationary phase in an Autoprep 700 instrument. The substances tested were iso-octane and cyclohexane. While there is a maximum in the plate number around 20–30 % coating with analytical size samples, the preparative scale plate number is still increasing slightly at 35 % coating with the larger samples (as shown for one case in Fig. 2).

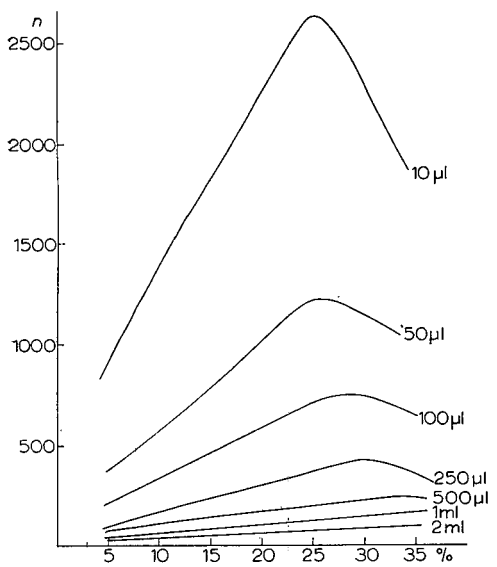


Fig. 2. Plate number, n, as a function of liquid coating percentage and sample size.

It could be concluded that liquid loadings should be further increased, but this is not so. Larger liquid loadings increase the separation time and especially increase the working temperature. This is often detrimental to the capacity of a column, since relative retention generally diminishes with higher temperatures. In fact, since Fig. 2 shows that the difference is quite small between 10 and 35 % coating, the lower percentage coatings can be used with advantage.

Fig. 2 was obtained with a low-boiling substance. Experimental results with the high-boiling decalin mixture confirm these results. Separation with 10 and 25 %

coating were remarkably similar and, even at 5 % coating, preparative scale work is possible (*cf.* Table I). The 500 μl decalin sample is nearly completely separated with the 5 % coating.

TABLE I

PLATE NUMBERS FOR A 20 m COLUMN FILLED WITH A LABORATORY-MADE COARSE GRAIN SUPPORT COATED WITH SE. 30 IN THE PERCENTAGES GIVEN

Decalin sample size	*Plate numbers*		
	Liquid coating (5 %)	*Liquid coating* (10 %)	*Liquid coating* (25 %)
10 μl *trans-*	—	—	1553
cis-	—	—	1908
100 μl *trans-*	954	1323	1351
cis-	2833	2919	1778
200 μl *trans-*	631	833	958
cis-	2303	2459	1631
500 μl *trans-*	292	427	618
cis-	1507	1745	1541
1 ml *trans*	211	307	354
cis-	1087	1404	1210

COLUMN DIAMETER AND PACKING METHOD

We have also tried to increase the capacity of gas chromatography by increasing the column diameter. The influence of this increase on the separation power (plate number) must be considered, and very variable claims for the plate number of increased diameter columns can be found in the literature. Some workers find a big drop in the plate number, others find no significant difference and this is often attributed to some special way of packing the column.

We have always found a large drop in the analytical plate number when the column diameter was increased. For 6 mm and 30 mm diameter columns of 2 m length, for example, and with a small sample load proportional to the section surface, the plate number showed a ten-fold difference under the most favourable circumstances. Preparative scale plate numbers per unit length are, however, more similar and with very large samples are of course even better on large bore columns.

The methods which we used for packing the columns varied from simply pouring in the material with or without lateral or lengthwise tapping or vibrating, to tamping the column with a glass rod as in the method used for packing liquid–liquid partition chromatography columns. Vacuum packing and adding the material in small portions or suspended in a liquid were also tested. The method which produces the largest density with celite (vertical tapping as hard as the metal column will stand) is not the most efficient for Chromosorb W (lateral vibrating).

This density is indeed a factor which markedly influences the column plate number for large bore columns. For "hard" supports like Chromosorb P and W and

Gas-Chrom P the packing density cannot be varied very much. Extreme values which we found with Chromosorb W 60/80 mesh and a 2 m × 30 mm column were between 0.37 to 0.415 g/ml with a corresponding plate number increase of 20 % (about 150 plates per metre for the highest density and for 150 μl samples).

With more sticky supports like the very fine mesh Chromosorb W or with Celite 545 (Johns Manville) screened by flotation to eliminate all but the coarsest 10 %, variations are much bigger. The extremes in density in this case are 0.24 to 0.46 g/ml for the celite coated with 20 % SE. 30, and the plate number is more than double for the denser packing. In fact there is an almost linear relationship, as shown in Fig. 3.

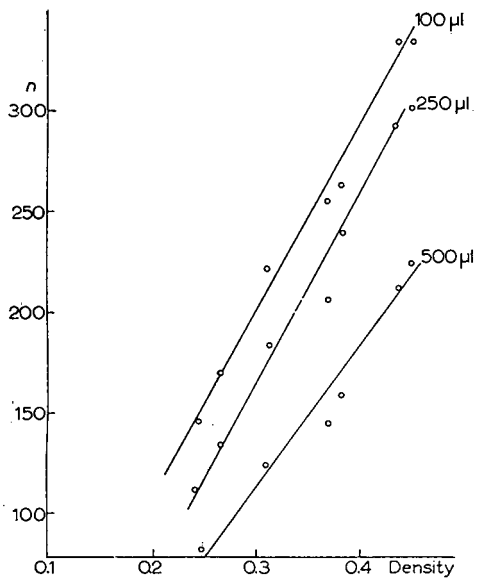


Fig. 3. Plate number, n, as a function of packing density and sample size.

It seems that, for these large bore columns, the denser the packing, the larger is the column plate number. In smaller bore columns the packing density effect seems relatively unimportant. For a 6 m × 9 mm glass column (Autoprep 700 Wilkens Instrument) filled with Chromosorb W 60/80 mesh and coated with 30 % SE. 30 we could vary the packing weight between 68 and 81 g, the column still being completely filled for the lower value. With these packing densities and for *trans–cis*-decalin, the preparative scale plate numbers at 160° and with 200 ml/min H_2 as gas rate were remarkably similar, and so were in fact the analytical plate number values. There was, however, a big difference in retention time because of the difference in inlet to outlet pressure ratio (compressibility factor). The loosely packed column is nearly twice as "fast" as the most densely packed column. It seems therefore that, for smaller bore preparative scale gas-chromatographic columns, the aim should not be maximum packing density but that on the contrary lightly packed columns should be used because of the reduction of analysis time.

Comparing a 2 m × 30 mm diameter column with a 6 m × 9 mm diameter column, filled with Chromosorb W 30/60 mesh and coated with 20 % SE. 30, the

preparative scale plate number per unit length is larger for the bigger column. For 500 μl samples the plate number is about the same for the two columns; thus the preparative scale HETP for this sample size using the wider column is about three times smaller than that obtained when using the longer column. Their separation power for these sample sizes is the same. The longer small-bore column contains, however, only about a quarter of the volume of stationary support of the wider bore column, and consequently requires much less carrier gas for the same separation. An important factor also is the higher concentrations in the effluent gas using the smaller column, with consequent improved recovery.

To quadruple the sample size, the long column should be made about four times longer. This is indeed experimentally confirmed, as will be discussed later. To quadruple the sample size by column diameter increase of the shorter and wider column would lead in principle to a 60 mm diameter column. It still remains to be seen if such a wide column would show the desired plate number. The volume ratio for the enlarged columns is again about four. The volume of support material for the wider-bore column becomes excessively large for a research laboratory. In conclusion, we think long small-bore columns are to be preferred to shorter wide-bore columns for preparative scale gas chromatography.

HIGH PRESSURE CHROMATOGRAPHY

Columns for preparative scale gas chromatography have normally a large percentage of liquid stationary phase (low β) and are therefore so-called liquid-controlled[3]. Although the influence of pressure in the column would therefore be expected to be negligible we have carried out experiments to confirm this, using a conventional Autoprep 700 unit. With the usual 6 m $\times$ 9 mm columns filled with some suitable support the inlet to outlet pressure ratio was *ca.* 3.

By attaching a "restrictor" (a small piece of very fine metal or glass capillary) to the instrument outlet, the inlet pressure had to be raised to 5–7 kg/cm^2 to obtain a gas rate of 200 ml/min at atmospheric pressure. The effect on the capacity of the instrument was indeed negligible.

The differences from comparable "normal" chromatograms were: increased analysis time, a diminished katharometer detector response and peak asymmetry reversal from tailing to leading. Where a "tailing" peak was obtained in normal circumstances, use of a restrictor resulted in a markedly "leading" asymmetric peak shape. This is for example the case for iso-octane at 110° and will be discussed in more detail further. These differences can all be explained easily. The increased analysis time is due to the lower linear gas velocity in the pressurised column, the lower detector sensitivity is caused by the lower concentration of the solute at high pressure, and the peak asymmetry reversal from tailing to leading (only for large PGC samples) is due to a fall in the gas rate while the vapor is forced through the restrictor.

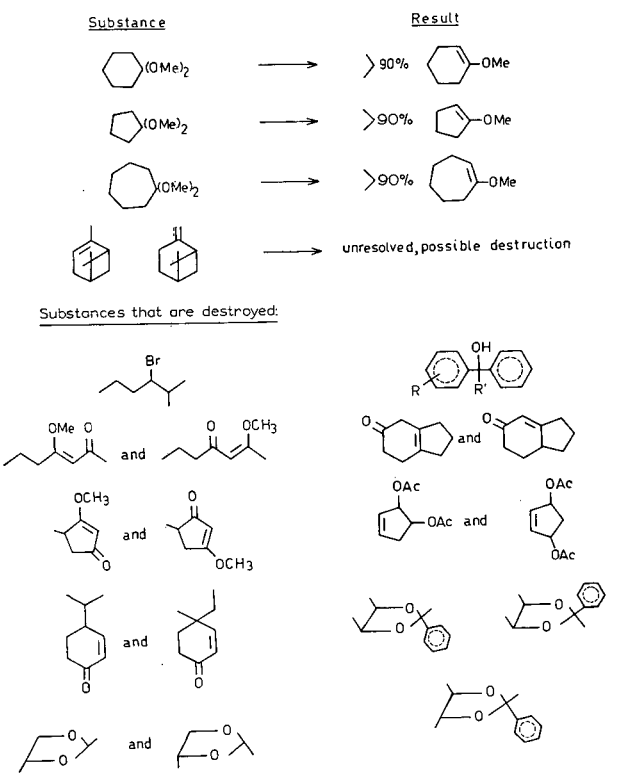
For 200 μl *trans*-decalin on the above column, for example, the measured gas rate fell gradually from 200 to 160 ml/min at peak maximum while returning to 200 ml/min when the base line was once again reached. These gas rate changes must result in a leading peak shape. With the restrictor between the column and the detector the sensitivity was restored. With a cold trap condensor placed ahead of

the restrictor no significant difference was found but we do not know if any condensation had occurred in the trap. Because of the negative result with regard to capacity, we did not press this point any further.

INFLUENCE OF THE STATIONARY SUPPORT

It is important that the stationary support material should be as inert as possible. For analytical gas chromatography of pesticides and steroids, for example, this has been generally recognised and has resulted in the introduction of glass columns and the exclusive use of deactivated white diatomaceous supports. For preparative scale gas chromatography this point is just as important. We have indeed encountered many separations which could not be carried out on metal columns with pink supports, but which were generally possible using glass columns and white supports. Because of this we are now working exclusively with the latter materials although the pink supports (Chromosorb P) give much more plates per unit length even for increased sample sizes. A number of examples of mixtures or pure substances which are destroyed or badly resolved through some support interaction on metal columns with pink supports are given in Table II.

TABLE II



Some supports are found to have a very high destructive power, *e.g.*, a laboratory-made support destroyed even SE. 30, which was depolymerised and blown off the column at temperatures as low as 150°. The support was made by backing Celite 545 with 3 % sodium carbonate at 1000°, crushing, screening, boiling with nitric acid and hydrogen chloride, washing, boiling with methanolic sodium hydroxide and thorough washing. The maximum allowable working temperatures (MAOT) for liquid phases must depend much more on the support than is generally believed, since SE. 30 can normally be used even at 350°.

Obviously the stationary supporting material can also affect the capacity of preparative scale gas chromatography. We have pointed out already in this paper that higher plate numbers, which go with better supports, mean greater capacity. But the support material giving the highest plate number for analytical scale work does not show necessarily the same superiority in preparative scale gas chromatography. This is the case for the seemingly very similar white diatomaceous supports Chromosorb W (Johns Manville) and Gas-Chrom P (Applied Science). Under identical circumstances, using the same 6 m × 9 mm coiled glass column filled with 30/60 mesh material, coated with 30 % SE. 30, at 160° and with 200 ml/min H_2 as carrier gas, the plate numbers shown in Table III were found for the *trans–cis*-decalin mixture.

TABLE III

PLATE NUMBERS FOR A 6 m × 9 mm COILED GLASS COLUMN

Chromosorb W/Gas-Chrom P mesh material coated with 30 % SE. 30. Isotherm at 160° with 200 ml H_2/min.

Decalin sample size (*μl*)	*Plate numbers*	
	Chromosorb W	*Gas-Chrom P*
10 *trans-*	2340	3240
cis-	2850	5080
50 *trans-*	1570	1130
cis-	3260	3770
100 *trans-*	720	540
cis-	2380	2150
200 *trans-*	420	300
cis-	1520	1470

These results show that while Gas-Chrom P is better for analytical work, Chromosorb W is superior for preparative scale gas chromatography. On truly analytical gas-chromatographic columns with openings of 4 and 2 mm we have also found consistently that Gas-Chrom P gives more plates per unit length than the similar Chromosorb W or Anakrom ABS (Analabs Inc.) in 10 mesh cuts.

RESULTS WITH COLUMNS OF INCREASED LENGTH

Plate numbers measured on the *trans*-decalin peak for increasing sample loads of *trans–cis*-decalin at 180° on 1.5 m, 6 m, 12 m and 20 m columns, filled with Chromosorb W 30/60 mesh with 25 % SE. 30 are shown in Fig. 4.

The results summarised in Fig. 4 show that increasing the column length indeed produces a linear increase of the plate number and improves the capacity of the column. The plate numbers are somewhat lower than can be obtained on these columns because of the high working temperature and the corresponding low partition factors.

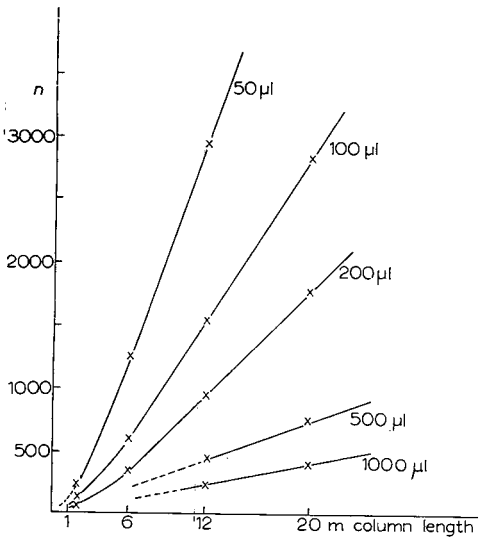
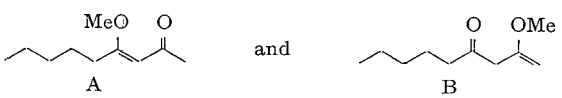


Fig. 4. Plate number, n, for increasing column lengths and sample sizes.

To fit the 12 m and 20 m columns to the Autoprep 700 unit, an insulated "hat" of sufficient size was adapted to the column oven. For the largest column the oven temperature was not very uniform although an additional fan was provided. Comparison with experiments in an oven in which the oven temperature *was* the same at all points showed, however, that this had no measurable effect. Fig. 4 can be used to find the permissible maximum sample size for a given problem and column. The sample sizes of Fig. 4 refer to the decalin mixture which has roughly about 75 % *trans*–25 % *cis*-composition. The plate numbers correspond therefore with only 75 % of the given sample size.

As already inferred, the approximate composition of the sample should be kept in mind; this is shown in the following example.

On methylating 2,4-nonanedione, a 50:50 mixture of the enol ethers A and B is produced:



This mixture has $\alpha = 1.17$ on 35 % butanediol–succinate polyester on Chromosorb W 60–80 mesh at 138°. About 760 plates are necessary according to eqn. (1) and Fig. 4 shows that this corresponds to a 100 µl sample on a 6 m column. The results of these experiments are found in Fig. 5.

Fig. 5 gives about 1400 plates for the two substances; this, as can be inferred from the chromatogram, shows that the sample size could have been doubled. The 100 μl of 50:50 sample contains indeed only 50 μl of each component. It is thus found that the preparative scale plate number is about the same for polyester and SE. 30. The α difference in this case between the two liquid phases just mentioned is, however,

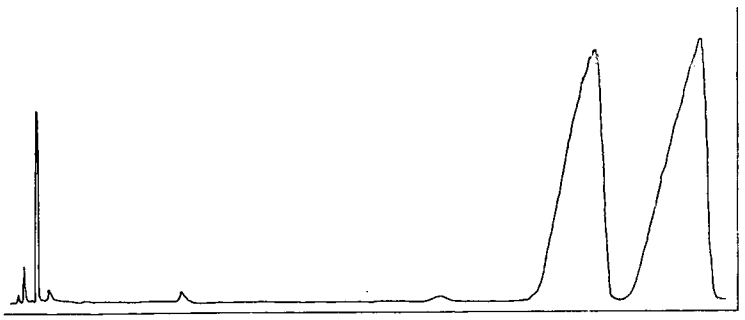


Fig. 5. Separation of isomeric enol ether ketones.

considerable. The separation of Fig. 5 is impossible on a metal column with a pink support (firebrick — see Table II).

DEVIATIONS FROM NORMAL CHROMATOGRAPHIC BEHAVIOUR DUE TO INCREASED SAMPLE SIZE

The approach described in the preceding paragraph works well as long as the sample size remains relatively small. With larger samples, deviations from normal chromatographic behaviour become more and more pronounced. There are typical changes in retention times and in peak asymmetry.

It is well known that the retention time increases with sample size and this aspect is, for example, briefly mentioned by DAL NOGARE AND JUVET[3]. This increase is attributed to peak width increase only in the direction of increasing carrier volume and the peak front is said to appear at the same retention volume regardless of sample size.

This is only exceptionally the case. In practice the first peak front of mixtures shows an increased or reduced retention time according to circumstances. For the second peak of mixtures, large increases in the peak front position are often found. This is exemplified in the results of Table IV obtained for decalin on a 12 m × 9 mm coiled glass column.

Decreasing values of peak front retention volume as a function of sample load are thus found when the gas rate is low, for substances with low partition factors, and/or with coarse supports. Increasing the gas rate considerably, without changing other conditions, again produces increasing peak front retention volumes.

An intermediate value of the gas rate can be found at which this peak front retention distance remains constant. This is shown in the results of Table V obtained on a 6 m × 9 mm coiled glass column filled with Chromosorb W 30/60 mesh, isooctane as sample, at 110° and with hydrogen as carrier gas (Autoprep 700 instrument).

Constant peak front retention times are found at a gas rate of 200 ml/min. Below this value there is a decrease, above this gas rate there is an increase.

TABLE IV

DECALIN ON 12 m × 9 mm COLUMN

Chromosorb W 30/60 mesh coated with 30% SE. 30. Isotherm at 180° with 200 ml H_2/min. Modified Autoprep 700 instrument.

Decalin sample size (μl)	*Peak front in mm on chart*	
	First peak, trans-decalin	*Second peak, cis-decalin*
10	92	111
50	95	115
100	96	117
200	97	120
500	98	127
1000	99	141

The decreasing values can be explained as the result of a displacement effect by the high maximum peak concentration. The tailing asymmetry of the peaks indicates a type I isotherm. With lower gas rates and with increased sample size the influence of exponential sample introduction is also stronger and can also lead to this decrease.

We suggest that the increase in the peak front distance for larger gas rates and on longer columns can be explained by the increase in the resistance to gas flow with larger samples. This is reflected, even when the flow rate change (because of the sample introduction) measured at outlet pressure is negligible, in a larger p_i/p_o ratio with resulting longer retention times. Large samples have, however, an influence on

TABLE V

DEPENDENCE OF PEAK FRONT RETENTION ON GAS RATE FOR A 6 m × 9 mm COLUMN FILLED WITH CHROMOSORB W 30/60 COATED WITH 30% SE. 30

Iso-octane sample size (μl)	*Peak front retention in mm for different H_2 rates*				
	50 ml/min	*100 ml/min*	*200 ml/min*	*400 ml/min*	*500 ml/min*
10	132	68	47	31	28
50	130	69	47	31	29
100	126	68	47	31	29
200	115	64	47	32	30
500	109	64	47	32	30
1000	105	63	46	33	31
2000	102	62	46	35	32

the gas rate measured at the outlet. For the isothermal experiment of Fig. 8, for example, the gas rate goes through a minimum which is 10% below the initial 200 ml/min and then increases again, even before elution of the mixture has started. For smaller samples the effect is less pronounced. This is probably the reason for the

increase in the peak front distance of the first peak of Table IV and also partly explains the displacement of the second peak. Additional confirmation is given by the fact that shorter columns show no increase of the peak front retention time with sample increase. The effect is much less pronounced with nitrogen as carrier gas. This could be expected, since the difference in resistance to gas flow between sample and carrier gas is smaller for nitrogen than for hydrogen.

The main factor determining the greater retention time increase of the front of the second peak of Table IV must, however, be due to something else. It is possible that with the larger sample sizes the substances no longer partition individually but that there is some other interaction than displacement. Temperature effects due to evaporation and condensation could also play a part here. At the front of the peak, condensation will cause a temperature increase while the rear boundary will have a lower temperature because of evaporation. This will retard second peaks and is related to the heat conductivity of the column material. In our glass columns the effect should be greater than in metal columns and the first experiments in this direction show that it is so. The same temperature effects could partly explain why our peaks show such a pronounced leading asymmetry. These explanations are rather unsatisfactory, but we can advance no other and the effect is important, since, as will be explained further, it increases the capacity of preparative scale gas–liquid chromatography columns. Even in conditions in which the first peak front is not markedly displaced (coarse supports, low liquid loading, relative low partition factors) 10 to 35 % increases for second peak front retention are found for larger samples. It occurs also with simple aromatic hydrocarbons on SE. 30 and with decalin on Carbowax 20M. We intend to investigate whether this effect is general.

The peak asymmetry changes mentioned above must be related to the peak front retention time changes. Peak asymmetry is generally discussed in terms of leading (—) or tailing (+). Tailing is the result of exponential sample introduction (large samples) with substances having a small partition factor. In preparative gas–liquid chromatography, leading is, however, more frequent and is normally encountered when dealing with higher concentrations and higher partition factors. The influence of the activity coefficient, γ, is predominant here and for dissimilar solute–solvent combinations, which is very often the case, γ is large, and leading skewness of the peaks results. This is adequately discussed by DAL NOGARE AND JUVET[3]. With preparative scale samples, peak asymmetry is very pronounced and leading and tailing for close homologues can be found on the same chromatogram. An example is shown in Fig. 6. On calculating the plate number for the different peaks a maximum value is found for the symmetrical peak.

Peak asymmetry can, however, be changed from this "normal" pattern by trivial factors. The effect of pressure has already been briefly mentioned. Indeed, iso-octane for example gives tailing peaks with all except the lowest sample sizes on a 6 m × 9 mm column filled with Chromosorb P 30/60 mesh, 30 % SE. 30 and at 110°. When a restrictor is placed on the column the peaks become leading at all except again the lowest sample sizes.

The gas rate, as could be expected, has also an influence on peak asymmetry. On a 6 m × 9 mm Chromosorb W 30/60 mesh column with 30 % SE. 30 at 110° and with iso-octane as sample, tailing is observed for all sample sizes from 10 μl onwards with a gas rate of 50 ml H_2/min. Under the same conditions, except that a gas rate

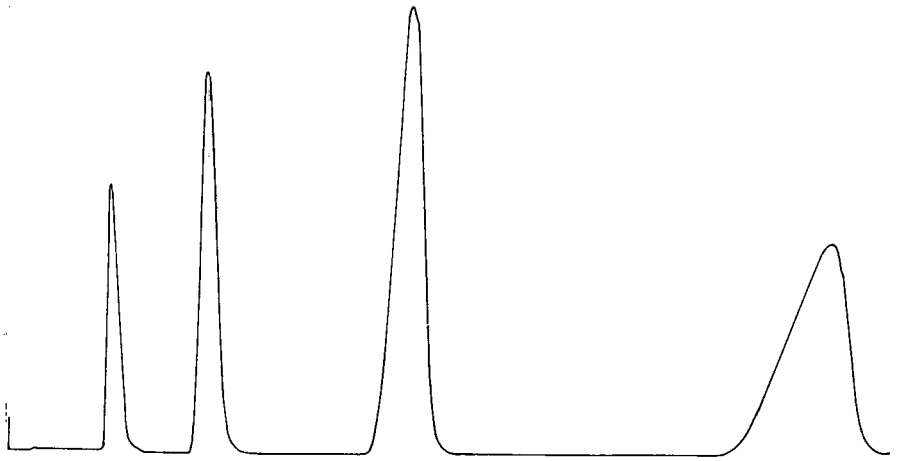


Fig. 6. 400 μl of the straight-chain hydrocarbons in C_6, C_7, C_8 and C_9 on a 12 m × 9 mm coiled glass column filled with sterchamol 0.5–1 mm grain size coated with 25% octylphthalate. 200 ml H_2/min at 120°. Note the transition of tailing to leading.

of 500 ml H_2/min is used, the peaks show leading, especially for large sample sizes, while symmetrical peaks are obtained with intermediate gas rates of 200–300 ml H_2/min. Using nitrogen as carrier gas, leading is observed even at 50 ml/min for the larger samples, while helium has an effect intermediate between those of hydrogen and nitrogen.

These results could be expected from exponential sample introduction and concentration effects (γ) but, as far as we know, have not been reported before.

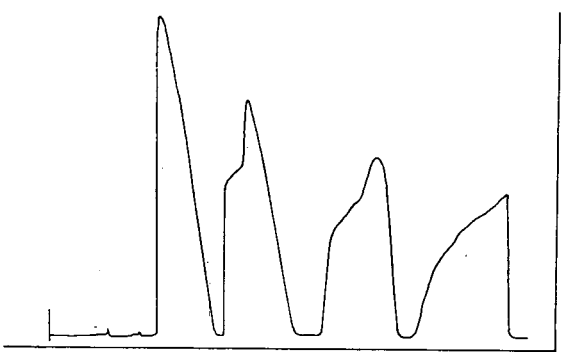


Fig. 7. 4 ml of benzene, toluene, ethylbenzene and cumene on 20 m × 9 mm coiled glass column filled with Chromosorb W 30/60 coated with 25% SE. 30. 200 ml H_2/min at 140°. Note high capacity which could still be increased. For peak shape irregularities, see text.

Yet another change in peak shape is encountered with large samples and at relatively low column temperatures. An indentation appears in the leading part of the curve. This is shown in the separation of Fig. 7 and is most evident here in the middle peaks. The same phenomenon is also seen to a lesser extent in the graph of Fig. 10. We do not know the reason for this.

TEMPERATURE GRADIENTS

The temperature gradients to be considered are of the chromathermography type where a temperature gradient is applied along the column, and of the so-called "temperature-programmed" type where the temperature is gradually and generally linearly increased in the oven as a whole.

Temperature programmation in preparative scale gas chromatography chiefly results in a narrowing of the peaks with corresponding higher concentration of solute in the outflowing carrier gas. This has a beneficial effect on the recovery of substances as we have pointed out before[1,4].

Chromathermography has also a peak-narrowing effect.

Application of the two gradients together is easily achieved experimentally. A 20 m × 9 mm coiled glass column is installed in a long narrow oven (1 m × 20 cm). The heater and a blower are placed at one end of the oven. Fresh air is introduced at the same end and is blown out through a small opening in the oven at the other end. Variable chromathermographic gradients are easily obtained in this way. Temperature programmation, but not uniform over the column length, is also easy. Isothermal operation is also possible with a relatively large fresh air flow. Results obtained with such an instrumental set up are shown in Fig. 8.

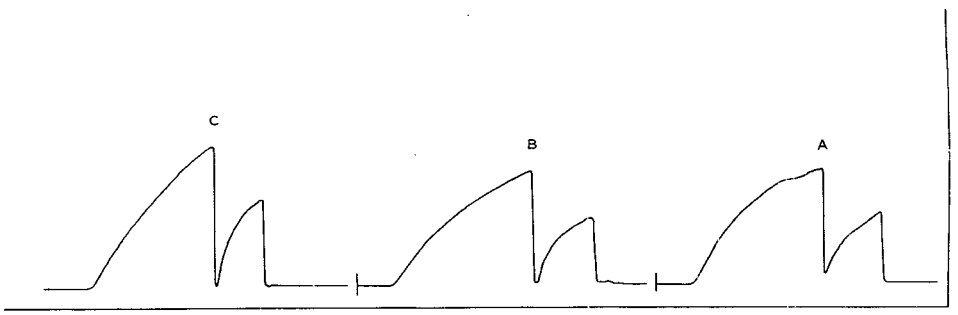


Fig. 8. Separation of 2 ml decalin samples on 20 m × 9 mm glass column (see text). Only part of each chromatogram is shown. The separation time was 80 min.

The separations of Fig. 8 were obtained on a 20 m × 9 mm coiled glass column filled with Chromosorb W 30/60 mesh coated with 25 % SE. 30. The sample size was 2 ml decalin and the carrier gas was hydrogen. The oven was as described above and was fitted with an injection port and a detector oven which was connected to an Autoprep 700 instrument.

Graph A is obtained with isothermal operation at 180° and with a carrier gas rate of 200 ml/min. The separation is not nearly complete. Graph B is obtained with a chromathermographic gradient from 185 to 145° and with a carrier gas rate of 200 ml/min. The separation is complete. The band width is only slightly larger, although the elution temperature is much lower than for graph A. Graph C then is obtained with programmation starting from 50°. At the end of the separation there is a chromathermographic gradient along the column from 205° to 158° (programmed chromathermography). The separation is complete and the peaks are obviously more narrow.

The three experiments were carried out in such a way as to obtain approximately equal retention times. The temperatures in the oven were measured with six permanently installed thermocouples.

For graph C the gas rate was 400 ml/min at the start and had fallen to 200 ml/min at the end of the experiment. Increased resistance to gas flow with a rise in temperature is a well known phenomenon. This is a desirable side effect, giving high speed for the initial separation and the necessary slow gas rate for recovery of the substances[2]. This change in gas rate does not adversely affect the separation. Whereas the plate number is fairly strongly dependent on the gas rate for small samples, this is not the case for large sample sizes. (*cf.* Fig. 9 for hydrogen. Similar results were obtained for helium and for nitrogen.)

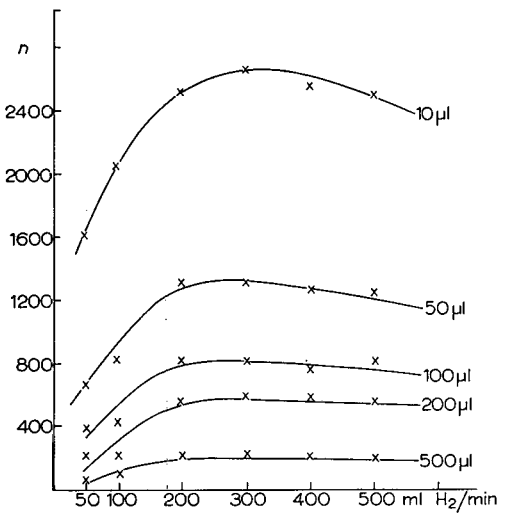


Fig. 9. Plate number, *n*, *vs.* gas rate and sample size showing that gas rate is less critical for preparative scale samples.

According to Fig. 4, the 20 m column should barely be capable of accepting a sample load of 1 ml decalin mixture. Fig. 8, however, obtained with the same column, shows that 2 ml can be separated on it. This is ascribed to the use of temperature gradients and to the special front peak retention increase of the second peak, already mentioned in this paper, which also occurs with the temperature gradients. In the specific case of the conditions of Fig. 8, B and C, the peak front retention of the first peak was independent of sample size but the peak front retention of the second peak showed a 10 to 20 % increase.

GRAIN SIZE OF THE SUPPORT

An objection to the use of longer columns is the increased resistance to gas flow and the larger pressure drop. We have therefore investigated the effect of a coarse support material. White diatomaceous supports of mesh size below 30/60 are commercially unavailable. We have, however, made our own supports as described

under Influence of the stationary support. Additional alkaline washings made it sufficiently inactive for use. Its mesh size was 15/20. Sterchamol of mesh sizes 10/15 and 15/30 was also tried. With analytical size samples the plate number is, as expected, very much higher for small grain size supports. The preparative scale plate number is, however, practically the same for all the supports and is therefore independent of grain size. The drop in plate number with increasing sample size is indeed not so pronounced for the coarse grain supports. This can be seen with the column given in Table I (25 % coating), which was obtained with the laboratory-made support of mesh size 15/20. The experimental results for separations on coarse support columns confirm the above statements. Decalin mixture samples (1 ml) are completely separated in 40 min at 165° on a 20 m × 9 mm column filled with a support of mesh size 15/20 and coated with either 10 or 25 % SE. 30. Samples (2 ml) in isothermal operations give only slightly inferior results to graph A of Fig. 8. We have not yet tried programmed chromathermography with a column of this type, but intend to do so. A major advantage of the coarse support column is the speed due to the negligible pressure drop and the possibility of reducing the working temperature. This can be deduced by comparing the figures just mentioned with those of Fig. 8. Support material of mesh sizes below 30 should also make it possible to use even longer small-bore columns. The pressure drop would be negligible and the separation power of the column for preparative scale samples would be high. The use of nitrogen as carrier gas should also be more easily possible because of the slight pressure drop. As we have shown before, the preparative scale plate number is independent of the nature of the carrier gas[2].

We are building a 75 m column preparative scale gas chromatograph.

SPEED AND LONGER COLUMNS

Instead of using longer columns in order to increase sample size, they can also be used with advantage to shorten the separation time and to increase recovery percentages. This may seem paradoxical but is exemplified by the following facts, illustrated by Fig. 10. A 0.5 ml decalin sample can just be separated on a 6 m × 9 mm

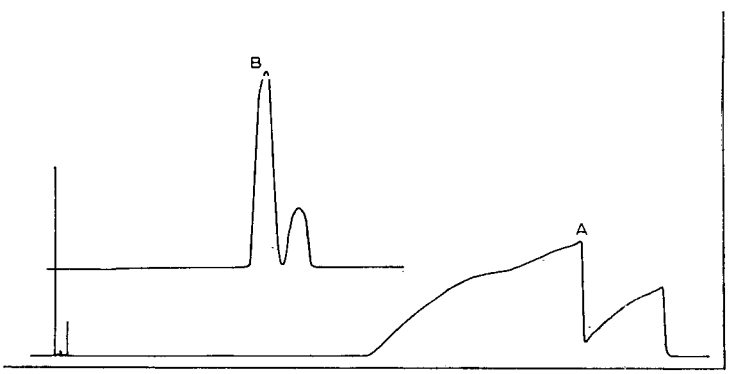


Fig. 10. Separation of 0.5 ml decalin mixture; same time scale and gas rate; (A) at 120° on a 6 m × 9 mm column; (B) at 220° on a 20 m × 9 mm column. The long column is more than twice as fast as the shorter column.

column filled with Chromosorb W 30/60 mesh coated with 30 % SE. 30. The maximum separation power of the column must be applied by using sufficiently low temperatures to ensure long developing times. In this case the separation is achieved at 120° and lasts over an hour. On the 20 m × 9 mm column used for the experiments of Fig. 8 a sample of 0.5 ml decalin is very easily separated and even when the temperature is increased to 220°, separation is still complete. The analysis time, using the same gas flow rate in the two comparative experiments, has, however, been reduced for the longer column to 25 min.

The concentration of the substances in the outflowing gas mixture is much greater for the longer column so that recovery problems should also be much simplified by working in this way. A point which was mentioned in our study of recovery in gas chromatography[2] but which was perhaps not sufficiently stressed is that the collection bottles must be filled as completely as possible with glass wool. The first drops of recovered substance form a liquid layer on the glass wool and this acts as a stripping gas-chromatographic column.

ACKNOWLEDGEMENT

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SUMMARY

Methods of improving preparative scale gas chromatography were investigated. The best results as regards separation power, sample size, recovery, economy of operation etc. should be obtainable on long narrow-bore columns filled with a loosely packed very coarse support, coated with 10 % liquid phase and with "programmed chromathermography".

Attention is drawn to the necessity of using glass columns and inert supports. It should be possible to replace a large number of columns with different liquid phases by a restricted number of columns of much increased length with general purpose liquid phases *e.g.* SE. 30, or Carbowax. The influence of preparative scale sample size on peak shape and retention is discussed. The most remarkable feature here is the big increase in peak front retention with increased sample size for the second peaks of decalin mixtures. There are indications that this could be a general effect. For not unduly large samples (shorter columns) and not unduly small α values, the maximum allowable sample load can be derived fairly accurately from the "preparative scale plate number". Where the conditions are different, the maximum sample load is best found experimentally and will be greater than could be expected from the preparative scale plate number. This is due to the retention effect on the second peaks.

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MULTI-DIMENSIONAL CHROMATOGRAPHY USING DIFFERENT DEVELOPING METHODS

III. THE IDENTIFICATION OF SUBSTANCES BY MEANS OF PROGRAMMED DISTRIBUTION OF FRACTIONS IN TWO-DIMENSIONAL CHROMATOGRAPHY

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In earlier papers[1,2] new possibilities were pointed out of preparation and identification by two-dimensional chromatography, where gas chromatography is used as a method for placing the fractions on the moving start line of a thin layer or paper. The possibility of identification by the programme-controlled shifting of the plate was also mentioned[2]. KAISER[3] tried the method suggested[1] and the start line of a thin layer was intermittently moved along as soon as some components left a gas-chromatographic column featuring programme-controlled temperature. The signal for movement was taken from the recording instrument. Each fraction from the gas chromatograph was separately plotted as a discrete point on the start line of the plate. This method, however, does not permit utilization of the characteristic distribution of spots on the thin-layer chromatogram for the purpose of identification.

In this communication the case formerly suggested[2] is dealt with, where the gas chromatogram remains isothermal, but the shifting of the plate is logarithmically programmed with time. Proof is furnished that by logarithmic shifting of the thin layer or chromatographic paper, the distance between the zones of the individual members of the homologous series is the same and the reading of the two-dimensional chromatogram greatly facilitated. Further, the possibility is pointed out of noting the position of the zones or of identifying them directly in KOVÁTS retention indices[4], and in a suitable group of substances of correlating them with their boiling points[5].

THEORY

The shift of the chromatographic zone of a substance 1 is proportional to the velocity of the carrier gas u so that $u_1 = R_F \cdot u$,

where: $R_F = 1/(1 + K_1)$

$K_1 = a \cdot e^{Q_1/RT}$

K_1 = the separation coefficient

a = a constant

Q = the heat of adsorption or solution

R = the gas constant

T = the absolute temperature.

For a pair of homologous substances 1 and 2

$$Q = RT \cdot \ln \frac{K_2}{K_1} = RT \cdot \ln \frac{t_2}{t_1} \tag{1}$$

where: Q = sorption energy increase for the homologous increment,
t_1, t_2 = elution times of maximum of zones of homologues 1 and 2.

K and likewise t, increase exponentially with an increasing number of increments. If we make a logarithmic recording t, the distance in the chromatogram corresponding to the increment of the $-CH_2-$ group will be the same, and additive, for substances having the same structure.

The position of the individual components in the chromatogram can be established for $y_1 = \log t_i$ and $y_{i-1} = \log t_{i-1}$ from the equations of the straight lines for

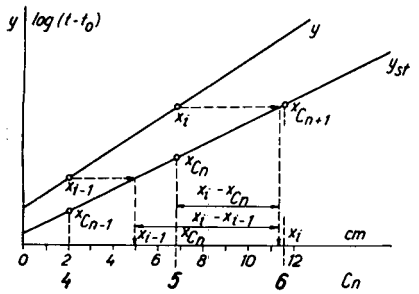

Fig. 1. Graphical illustration of position of substance i (in cm) measured from the edge of the thin layer, in relation to a chosen homologous series.

the standard series of n-paraffins and the homologous series where the component i occurs as follows from the definition of the KOVÁTS' indices[4]. The procedure is graphically illustrated in Fig. 1.

$$x_i - x_{i-1} = (x_{C_n} - x_{C_{n-1}}) \frac{\log t_i - \log t_{i-1}}{\log t_{C_n} - \log t_{C_{n-1}}} \tag{2}$$

$$x_{C_n} - x_i = (x_{C_n} - x_{C_{n-1}}) \frac{\log t_{C_n} - \log t_i}{\log t_{C_n} - \log t_{C_{n-1}}} \tag{3}$$

In the logarithmic shifting of the plate, $\log t$ is directly given by the linear section L on the start line of the plate. If we correlate some of the zones in the chromatogram to the elution indices of n-paraffins, we can read directly from the start of the plate the values for KOVÁTS' retention indices, corresponding to the component or increment of the series:

$$I_{\text{subst.}} = 100 \frac{L_i - L_{C_n}}{L_{C_n} - L_{C_{n-1}}} + 100 \cdot C_n \tag{4}$$

$$I_{CH_2} = 100 \frac{L_i - L_{i-1}}{L_{C_n} - L_{C_{n-1}}} \tag{5}$$

Similarly, proceeding from the assumption that the boiling points of non-polar substances are determined by the dispersion forces, we can, when using the non-polar stationary phases in a sufficiently wide range of boiling points, estimate the boiling point of the component i in the chromatogram:

$$(\text{B.P.})_i = (\text{B.P.})_x + \{(\text{B.P.})_y - (\text{B.P.})_x\} \frac{L_x - L_i}{L_x - L_y} \tag{6}$$

EXPERIMENTAL AND DISCUSSION

Gas chromatography

The experiments were performed on a chromatograph CHROM III (Laboratorní přístroje, N.E., Praha) equipped with exchangeable detectors (thermal conductivity, flame and argon ionization), columns for preparation and analysis in packed and capillary columns. The katharometer and the packed column with isothermal and programme-controlled heating system were chosen for use. The column measured 3 m in length and 0.6 cm in diameter. The packing was SE-30 silicon elastomer (General Electric Inc., U.S.A.), 20% weight on Chromosorb W (Johns-Manville, Ltd., London, Great Britain). The carrier gas was hydrogen. An adapter for the trapping device was used and under the outlet were placed the stage[2] and the carrier plate with the thin layer. The fractions ahead of the outlet to the thin layer were registered by means of a recording instrument.

Logarithmic shifting device

The apparatus illustrated in Fig. 2 was constructed for the logarithmic shifting of the plate. This apparatus consists of a Wheatstone bridge, the logarithmic potentiometer P_1 (Aripot, manufactured by ARITMA, N.E., Praha) with an electron-tube impedance transformer, the amplifier Z and the motor M moving the stage (for carrying the plate), coupled with the potentiometer slide P_6. The synchronous motor moves the slide of the logarithmic potentiometer P_1, thus producing on the cathodes of the EF-80 electron-tube a voltage which is deducted from the voltage of the bridge. The voltage difference is conveyed into the amplifier Z feeding the two-phase in-

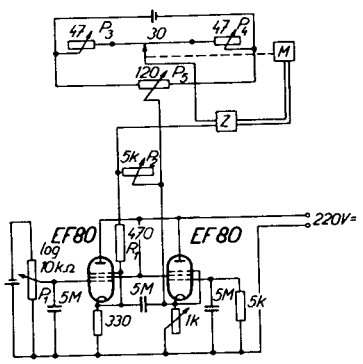

Fig. 2. Scheme of device for logarithmic shifting.

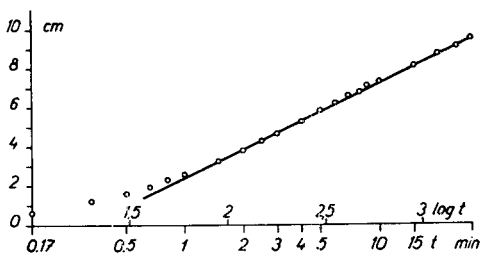

Fig. 3. Time behaviour of shifting of plate with thin layer.

duction motor (type RD 09, manufactured by VEB Magdeburger Armaturen Werke, D.D.R.). The slide of the Wheatstone bridge moves so that the voltage difference on the input side of the amplifier approaches zero. In the position of the stage corresponding to the displacement of the logarithmic potentiometer P_1, the amplifier input shows zero voltage and the stage stops. With the aid of the potentiometers P_2, P_3 and P_4 the modulus of the logarithmic shifting can be changed. The normal position of the stage can be adjusted by means of the potentiometer P_5. The actual moving of the stage depending on time is graphically illustrated in Fig. 3. The shift corresponds, with the exception of the first minute, satisfactorily to the logarithmic course.

Thin-layer chromatography

The technique of the fraction trapping on a thin layer was described in a previous paper[2]. The chromatograms were developed in a chamber corresponding to the S-chambers[6] for dried slurry plates. The type used was adapted for work with layers made with dry powder. This is shown in Fig. 4. The quantity of solvent used

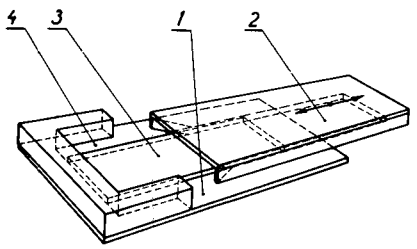

Fig. 4. Glass chamber for developing chromatograms on a thin powder layer. (1) Bottom part of chamber; (2) lid of chamber; (3) plate with thin layer; (4) space for solvent.

was approximately 25 ml. Examination of Merck thin-layer silica gels showed that only used silica gel PHH (see ref. 2) gave no colouring after treatment with tetracyanoethylene.

Chemicals

n-Propylbenzene and *n*-butylbenzene were synthetized by Friedel-Crafts' reaction and purified by means of rectification. The other substances used were commercial preparations: benzene, toluene (Lachema, Brno), ethylbenzene (Fluka, Buchs, Switzerland). Silica gel, tetracyanoethylene, aromatic hydrocarbons, phenols, indoles, and quinoline were the same as those used in our previous experiments[2].

The reading of the zone position in Kováts' indices and the correlation to the boiling points of aromatic hydrocarbons

In order to verify the theoretical assumptions, the homologous series of aromatic hydrocarbons benzene to *n*-butylbenzene was chosen. This mixture was complemented by C_6, C_7, C_8 and C_{10} *n*-paraffins. The individual components of the mixture were adsorbed on a logarithmically moving thin layer of silica gel, placed under the outlet of the column. The logarithmic shifting was begun always at the time interval $t - t_0 = 1$ minute. The position of the aromatics was detected with tetracyanoethylene, the silica gel having been moistened with a solution of tetracyanoethylene and

dried prior to the experiment. The positions of the aromatic hydrocarbons quoted are recognized by the yellow to orange-yellow spots of the complexes formed. Only the detection of benzene, which evaporates quickly and thus reduces the possibility of exact localization of the spot, presents certain difficulties. The position of *n*-paraffins, which do not yield a colour complex with tetracyanoethylene, was registered by reference to the linear recording on the gas chromatograph, taking place at the same time. Fig. 5 presents the chromatogram of the mixture with linear movement of the chromatographic chart paper.

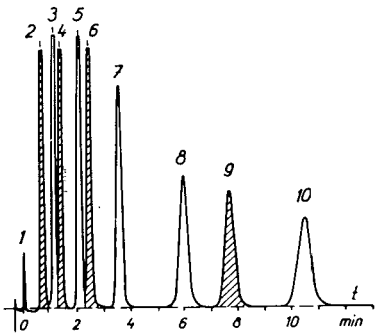

Fig. 5. Isothermal chromatogram of aromatic hydrocarbons and *n*-paraffins (t = 110°, F = 100 ml H_2/min). (1) Air; (2) *n*-hexane; (3) benzene; (4) *n*-heptane; (5) toluene; (6) *n*-octane; (7) ethylbenzene; (8) *n*-propylbenzene; (9) *n*-decane; (10) *n*-butylbenzene.

Table I gives the values $L_{max} - L_0$ for linear shifting (measured on the chart paper of the chromatograph) and L_{max}' (measured from the plate edge) for the bulk of spots for logarithmic displacement. KOVÁTS' retention indices were calculated for linear shifting from the known relations[4] and for logarithmic shifting from the values read on the start line of the thin layer according to eqn. (4).

The comparison between the positions of the individual aromatics on the plate

TABLE I

COMPARISON OF KOVÁTS' INDICES, CALCULATED FROM A CHROMATOGRAM WITH LINEAR MOVEMENT OF THE CHART (GAS CHROMATOGRAPH RECORDING) AND WITH LOGARITHMIC SHIFTING (THIN LAYER)

Substance	*Linear shifting*		*Logarithmic shifting*	
	$L_{max} - L_0$ (*mm*)	$I^{SE-30}_{170°}$	L_{max}' (*mm*)	$I^{SE-30}_{170°}$
n-Hexane	6.5	600	—	—
Benzene	11.5	676	5.5	675*
n-Heptane	13.0	700	14.0	700
Toluene	20.5	774	39.0	772
n-Octane	24.0	800	48.0	800
Ethylbenzene	35.5	867	69.0	865
n-Propylbenzene	59.5	956	97.0	951
n-Decane	77.0	1000	113.0	1000
n-Butylbenzene	105.0	1052**	128.0	1046**

* Computed by extrapolation from C_8 and C_7.
** Computed by extrapolation from C_8 and C_{10}.

(measured from the plate edge) calculated from eqn. (2) and the actual ones is given in Table II. Toluene and ethylbenzene were taken as standards.

Similarly, the position of the zones, referring to any optimal standard, *e.g.*, *n*-paraffin, can be calculated from eqn. (3). Finally, the boiling points could be calculated from eqn. (6) (Table II, right-hand side). As a starting point for the calculations, the boiling points of toluene and ethylbenzene were used.

TABLE II

COMPARISON OF CALCULATED AND MEASURED POSITIONS OF MODEL SUBSTANCES ON A THIN LAYER AND OF THE CALCULATED AND MEASURED BOILING POINTS

Substance	*Position (mm)*		*Boiling points (°C)*	
	Calculated	*Measured*	*Calculated*	*Measured*
Benzene	5.5	8.5	83	80
n-Propylbenzene	97.0	100.0	159	158
n-Butylbenzene	130.5	128.0	184	183

Applications in coal tar chemistry

The applicability of this method to the examination of the composition of wash oils from coal tar was tested on a model mixture consisting of the following fifteen substances: 2-methylnaphthalene, 2,7-dimethylnaphthalene, 2,3-dimethylnaphthalene, 2,3,6-trimethylnaphthalene, acenaphthene, fluorene, indole, 3-methylindole, 2-methylindole, 4-hydroxyhydrindene, 5-hydroxyhydrindene, 2-hydroxydiphenyl, 1-naphthol, 2-naphthol and quinoline. The individual fractions were

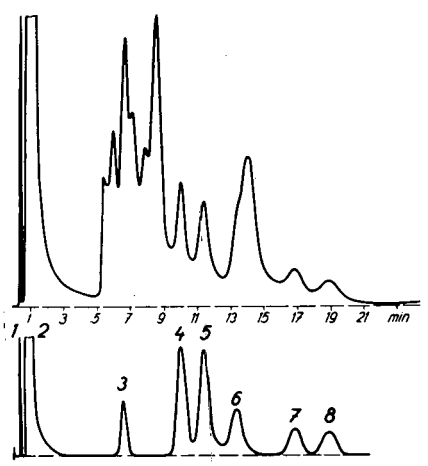

Fig. 6. Isothermal chromatograms. (a) Model mixture (t = 170°, F = 100 ml H_2/min), containing: 2-methylnaphthalene, 2,7-dimethylnaphthalene, 2,3-dimethylnaphthalene, acenaphthene, 2,3,6-trimethylnaphthalene, fluorene, indole, 3-methylindole, 2-methylindole, 4-hydroxyhydrindene, 5-hydroxyhydrindene, 2-hydroxydiphenyl, 1-naphthol, 2-naphthol, quinoline. (b) Fraction of aromatic hydrocarbons (t = 170°, F = 100 ml H_2/min). (1) Air; (2) solvent; (3) 2-methylnaphthalene; (4) 2,7-dimethylnaphthalene; (5) 2,3-dimethylnaphthalene; (6) acenaphthene; (7) 2,3,6-trimethylnaphthalene; (8) fluorene.

deposited by the aforementioned technique on a logarithmically moving thin layer of silica gel. The thin layer was developed with benzene and the individual substances were detected with a solution of tetracyanoethylene. Fig. 6a presents the chromatographic recording of the complete mixture.

In Fig. 7, the completed two-dimensional chromatogram on the plate after elution with benzene is presented.

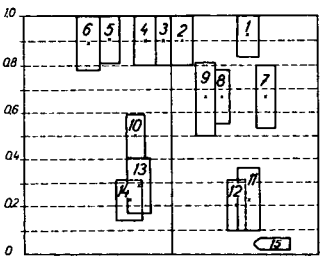

Fig. 7. Two-dimensional chromatogram on plate. Gas chromatogram: $t = 170°$, $F = 100$ ml H_2/min; chromatogram on thin layer: $t = 20°$; adsorbent: silica gel; solvent: benzene. (1) 2-Methylnaphthalene, violet; (2) 2,7-dimethylnaphthalene, black-violet; (3) 2,3-dimethylnaphthalene, blue-violet; (4) acenaphthene, green; (5) 2,3,6-trimethylnaphthalene, blue-violet; (6) fluorene, red-violet; (7) indole, brown; (8) 3-methylindole, violet, later brown; (9) 2-methylindole, black-violet, later brown; (10) 2-hydroxydiphenyl, dark ochre; (11) 4-hydroxyhydrindene, brown; (12) 5-hydroxyhydrindene, violet; (13) 1-naphthol, orange; (14) 2-naphthol, grey-violet; (15) quinoline.

Fig. 6b shows the chromatogram of the hydrocarbon portion. Both chromatograms in Fig. 6 were made under identical conditions.

The individual components were identified on the basis of the various colours of their complexes with tetracyanoethylene, the R_F values[2,6] and the quantities quoted above.

In the case of elution with benzene on silica gel, we can assume that the substances at the front of the plate, $R_F = 0.9$, are hydrocarbons (*cf.* Fig. 6b). If we use two known substances, in our case 2-methylnaphthalene and fluorene, we can establish the boiling points of the other fractions with the aid of eqn. (6). From Table III, it can be seen that the calculated and the actual boiling points of substances, whose presence is assumed, agree to a sufficient extent.

By comparing the chromatograms in Figs. 6a and 7 (6b respectively), we see that the zone of acenaphthene lies in the forward part of the common zone with the higher phenols, the zone of 2-methylnaphthalene forms the third peak in the chromatogram, etc.

TABLE III

CALCULATED AND ACTUAL BOILING POINTS OF HYDROCARBONS WITH R_F 0.90

Substance	*Boiling point* (°C)	
	Calculated	*Measured*
2,7-Dimethylnaphthalene	265	262
2,3-Dimethylnaphthalene	271	269
Acenaphthene	277	277
2,3,6-Trimethylnaphthalene	290	288

The importance of eqn. (5) can be verified in the case of the indoles, whose R_F values are around 0.75. The dimethylindoles in the tar fractions would be found on the plate at the same distance from the methylindoles as that formed by the interval between the indole and the methylindoles of a certain structure. The procedure is similar with phenols, whose R_F values generally vary over a range from 0.2–0.55 depending on the steric hindrance of the hydroxyl functional group. Finally, if we know the values of the KOVÁTS' retention indices for substances that are likely we can, with their help, very well determine directly the position of the individual substances on the plate.

SUMMARY

It was shown that by logarithmic shifting of the thin layer (or chromatographic paper), an equivalent distance for each of the individual members of an homologous series can be obtained along the start line, when developing a gas chromatogram. The identification of the substances in the two-dimensional chromatogram is greatly facilitated. The type of the substance can be found through measuring the distance directly in accordance with the values of KOVÁTS' retention indices, or with suitable groups of substances according to the boiling points of appropriate standards. In this manner, the position of a spot in the two-dimensional chromatogram, can be anticipated according to its structure and molecular size.

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A GAS-CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF PROGESTERONE IN HUMAN PLASMA

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INTRODUCTION

Until recently, the endogenous production of progesterone by the adrenal cortex, the ovary and the placenta has been evaluated indirectly through the determination of its major metabolite, pregnanediol.

In the last few years, biological[1] and chemical[2–5] methods for the determination of progesterone in peripheral venous blood have been developed. The chemical methods are suitable for routine assays, especially for the study of ovarian and placental function both in physiological and pathological conditions. Recently WOOLEVER AND GOLDFIEN[6] have described a double isotope derivative technique with a high degree of specificity, sensitivity and accuracy. This method has been used to investigate plasma progesterone levels in non-pregnant women[7], but it cannot be easily employed in every laboratory.

In this paper a thin-layer and gas–liquid chromatographic technique for the determination of plasma progesterone is described.

Gas–liquid chromatography has already been applied to the separation and determination of several hormonal steroids in biological materials. In particular, we have applied this technique to the quantitation of urinary oestrogens in normal[8] and pregnant women[9] and young boys[10].

Gas-chromatographic methods have also been described for progesterone evaluation in biological materials. However, the amounts of hormone which are required could be easily detected by colorimetry or spectrophotometry[11].

Methods employing progesterone-7-^{3}H have been used for the determination of plasma progesterone in pregnant women[12].

Our method for the determination of plasma progesterone consists of extraction with ether, solvent partition, thin-layer chromatography to purify the extract and separate the progesterone fraction, and finally gas–liquid chromatography for the identification and quantitation of progesterone.

APPARATUS AND MATERIALS

A gas-chromatographic apparatus, Fractovap, model C (Carlo Erba, Milan), was used. It was equipped with a hydrogen flame ionization detector, and with U-shaped

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micro-glass columns 80 to 90 cm long whose inside diameter was 2 mm. The columns were packed with silanized Chromosorb W, and coated to 3 % with SE-30, or packed with NPG, and coated to 1 % with QF-1.

The columns were preconditioned by heating at 250° in a nitrogen atmosphere for 12 h before use. All analyses were carried out at a column temperature of 200° and a nitrogen carrier pressure of 0.45 atm. The vaporization chamber was kept at 285°.

The samples, dissolved in acetone, were introduced by means of a 1 or 10 μl Hamilton syringe. Samples were usually run at a detector voltage of 1,500 V, and at recorder attenuation of 10.

For the quantitative determination, the area of the progesterone peak was calculated by means of the planimetric and triangulation methods, and then compared with the peak areas of known amounts of authentic standard of progesterone run on the same day.

For the purification of the extract, horizontal thin-layer chromatography was carried out using the "B.N.-Kammer" (Desaga, Heidelberg). The horizontal thin-layer technique permits the flow of solvents for a very long time, so that a greater purification of the extracts can be achieved[13].

All the solvents were of A.R. grade, and were redistilled immediately before use. The diethyl ether was peroxide free. For thin-layer chromatography silica gel G (Merck, Darmstadt) was used. Progesterone, obtained from Mann Research (New York) was prepared as standard solution in absolute ethanol.

PROCEDURE

The plasma, obtained by centrifugation of heparinized blood immediately after collection, was stored at —18° for at least 12 h.

Two extractions with 3 volumes of diethyl ether were performed by shaking gently for 10 min. The ether extracts were collected in a centrifuge tube with a conical base and then dried on a water-bath under nitrogen. The dry residue was dissolved in 10 ml of warm 70 % methanol and then kept overnight at —18° in order to precipitate lipids. The tube was spun in a refrigerated centrifuge for 25 min at 3,000 r.p.m. The upper liquid phase was carefully transferred to another tube, and the precipitate washed with 3 ml of cold 70 % methanol, which was decanted to the same tube. The pooled methanol extracts were then shaken with an equal volume of heptane. The heptane phase was separated and re-extracted with $^1/_2$ volume of 70 % methanol, which was added to the pooled methanol extracts. Twenty ml of distilled water was added to the combined methanol extracts, which were then extracted 3 times with 15 ml of petroleum ether, b.p. 35–40°. The combined petroleum ether extracts were then dried as previously described.

The dry residue is quantitatively spotted on silica gel G plates, using three times 15 μl of acetone, according to a previously described technique[14]. A standard solution of progesterone is also spotted on the plate. Horizontal development was carried out, at room temperature, by employing the "B.N.-Kammer", with 70 % methanol (v/v) saturated heptane. In this system progesterone does not move from the origin, while the interfering substances are displaced; therefore good purification of the plasma fraction containing progesterone can be obtained. Other solvent systems, *e.g.* methanol–benzene (1:9, v/v), which causes progesterone displacement

TABLE I

R_F AND R_{Oe_1}* VALUES OF AUTHENTIC AND PLASMA PROGESTERONE

Horizontal thin-layer chromatography in a cyclohexane–ethyl acetate (1:1, v/v) system. S.D. = standard deviation.

Compound	$R_F \pm$ S.D.	$R_{Oe_1} \pm$ S.D.
Authentic progesterone	0.550 ± 0.01	0.851 ± 0.01
Plasma progesterone	0.548 ± 0.03	0.846 ± 0.04
Oestrone	0.640 ± 0.01	— —

* Oe_1 = oestrone.

(R_F 0.60), do not reach the degree of purification of our proposed solvent system. On completion of the first development, the plate was left in the tank and the solvents were allowed to evaporate. A second development was then performed, using the cyclohexane–ethyl acetate (1:1, v/v) system. In this system, progesterone migrates and can be characterized by its mobility. The R_F values and the R_{Oe_1} values (R_F values of progesterone relative to oestrone) are reported in Table I.

The progesterone was visualized by spotting the plates with a hexane-saturated solution of iodine (yellowish brown spots), or an ethanolic solution of H_2SO_4 (spots fluorescent under U.V. light), or a Zimmermann reagent (violet spots).

The progesterone separated and purified is eluted from the silica gel by shaking in a centrifuge tube with absolute ethanol. After centrifugation, the ethanol extract was separated and dried. The residue was dissolved in acetone and quantitatively introduced into the gas-chromatographic apparatus as previously described.

RESULTS

Determinations of progesterone were carried out, by means of the method described above, on plasma obtained from 10 normally menstruating women at various stages of the cycle and from a pregnant woman. The results are reported in Table II.

TABLE II

PLASMA LEVELS OF PROGESTERONE IN NORMALLY MENSTRUATING AND PREGNANT WOMEN

Plasma volume extracted: 10 or 20 ml.

Subject	*Duration of cycle (days)*	*Day of cycle*	*Plasma progesterone (µg/100 ml)*
1	28	5th	0.25
2	27	6th	0.45
3	28	8th	0.50
4	30	15th	1.9
5	28	16th	2.5
6	28	22nd	3.5
7	28	26th	2.6
8	33	26th	2.4
9	30	26th	3.7
10	33	29th	1.1
11	Twenty-eighth week of pregnancy		15.5

Figs. 1 and 2 show, respectively, typical gas chromatograms of authentic progesterone and of plasma from a normal woman, which had been subjected to the thin-layer purification. The retention time of progesterone in both chromatograms was calculated by the time in minutes elapsing from the introduction of the sample into the vaporizing block to the highest point of the peak.

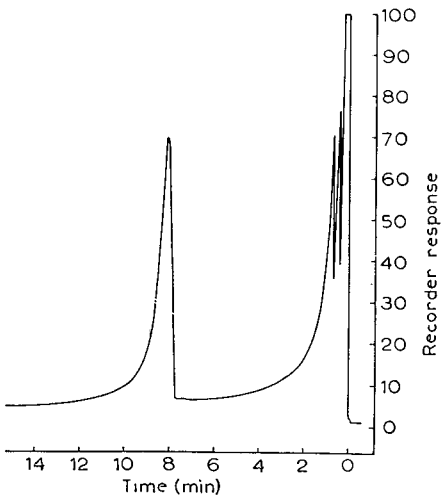

Fig. 1. Typical gas chromatogram of authentic progesterone. The retention time is 8.42 min (Attenuation 10)

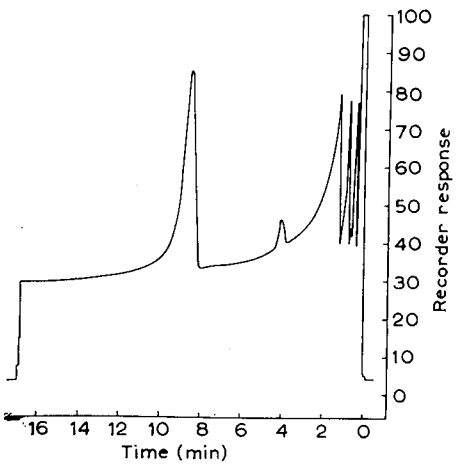

Fig. 2. Gas chromatogram obtained by the analysis of 10 ml of plasma from a subject in the follicular phase of the menstrual cycle. The retention time of progesterone is 8.42 min. (Attenuation 1)

The retention time of authentic and plasma progesterone was, under our conditions, 8.42 ± 0.07 (S.D) and 8.42 ± 0.15 (S.D.) min, respectively. As already mentioned, for the quantitative determination of progesterone the area of the authentic compound was compared with the area of the peak of the unknown sample showing the same retention time.

The amounts of progesterone determined by our method in plasma from normal women in the luteal phase of the menstrual cycle were comparable to those reported by other authors[5,7]. Very recently[15], similar results have been obtained by the application of two-dimensional thin-layer and gas–liquid radiochromatography.

DISCUSSION

Our gas-chromatographic method appears to be useful for determining amounts of progesterone in plasma ranging from 0.23 to 3.1 μg. Since the sensitivity of our gas-chromatographic apparatus can be greatly increased, it might be anticipated that smaller quantities (about 0.025 μg) of progesterone could be assayed with adequate reproducibility. This has, in fact, been confirmed by estimating very small amounts of authentic progesterone (≥ 0.025 μg). For the gas-chromatographic analysis of such small quantities of steroids it is necessary to use short columns in order to obtain sharp and easily measurable peaks. The efficiency of short columns, on the other hand,

is somewhat limited and only highly purified extracts can be applied to them. This can be achieved with a series of preliminary steps, namely cold methanol precipitation, solvent partition and thin-layer chromatography. The first two steps are used to remove the major part of the lipidic contaminants; horizontal thin-layer chromatography with 70 % methanol-saturated heptane permits a further purification of the progesterone fraction from interfering material, and the second development with cyclohexane–ethyl acetate (1 : 1, v/v) separates it from other steroids of similar mobility. The usefulness and efficiency of this procedure is apparent from Figs. 3 and 4, where

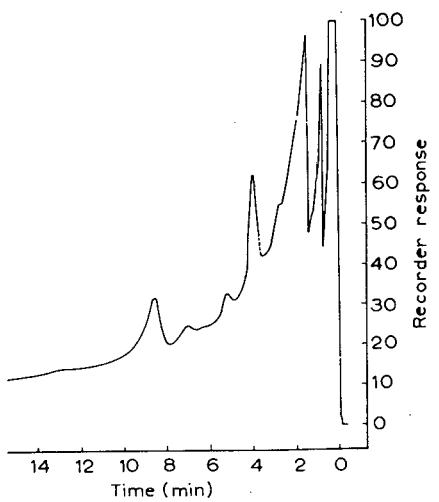

Fig. 3. Gas chromatogram obtained from 20 ml of female plasma without thin-layer purification. The retention time of progesterone is 8.42 min. (Attenuation 10)

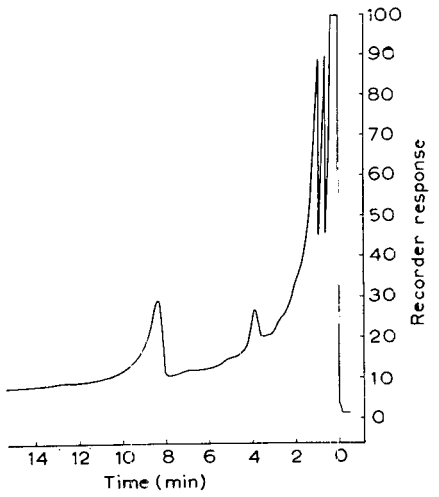

Fig. 4. Gas chromatogram obtained from 20 ml of plasma from the same subject as in Fig. 3, after thin-layer chromatography. The retention time of progesterone is 8.42 min. (Attenuation 10)

gas chromatograms of plasma, with and without purification by thin-layer chromatography, are shown. The peak with a retention time characteristic for progesterone emerges from a very low baseline, which does not interfere with the quantitative analysis. Horizontal thin-layer chromatography is particularly suitable for our purpose, since it permits two successive runs on the same plate with different solvent systems, simply by evaporating the first solvent system, and without moving the plate from the chromatographic tank.

TABLE III

PERCENTAGE RECOVERY OF VARYING AMOUNTS OF PROGESTERONE ADDED TO MALE PLASMA

No. of samples	*Amounts of progesterone added (μg)*	*Recovery % from male plasma (10 ml)*
10	2.5	94.3 ± 3.6*
6	5.0	88.5 ± 4.8*

* Standard deviation.

TABLE IV

ANALYSIS OF DUPLICATE SAMPLES OF PLASMA PROGESTERONE FROM FOUR SUBJECTS

Plasma volume extracted: 20 ml.

Subject	*Concentration of progesterone (μg/100 ml of plasma)*		*Deviation from the mean (%)*
	Sample I	*Sample II*	
1	3.0	3.5	7.7
2	1.6	2.1	13.5
3	2.0	1.7	8.5
4	2.5	2.7	3.8
X ± s.e.m.*	2.3 ± 0.3	2.5 ± 0.4	8.4 ± 2.0
	P = N.S.		

* S.e.m. = standard error of means.

The relationship between different quantities of injected authentic progesterone and the areas of the peaks so obtained is linear and follows the postulate of BEER. This relationship is shown in Fig. 5.

The sensitivity, accuracy, precision and reproducibility of the method have been checked by means of recovery experiments and duplicate analyses.

Table III shows the results obtained for recovery experiments of 2.5 and 5.0 μg of authentic progesterone added to 10 ml of male plasma.

Further evidence of the accuracy of this method has been obtained by recovering progesterone from silica gel after thin-layer chromatography. During these trials a negligible amount (about 2 %) is lost.

The results of the analyses in duplicate are shown in Table IV. It will be seen that there is satisfactory agreement, and that the difference is not statistically significant (P = N.S.). This confirms the reproducibility of the method.

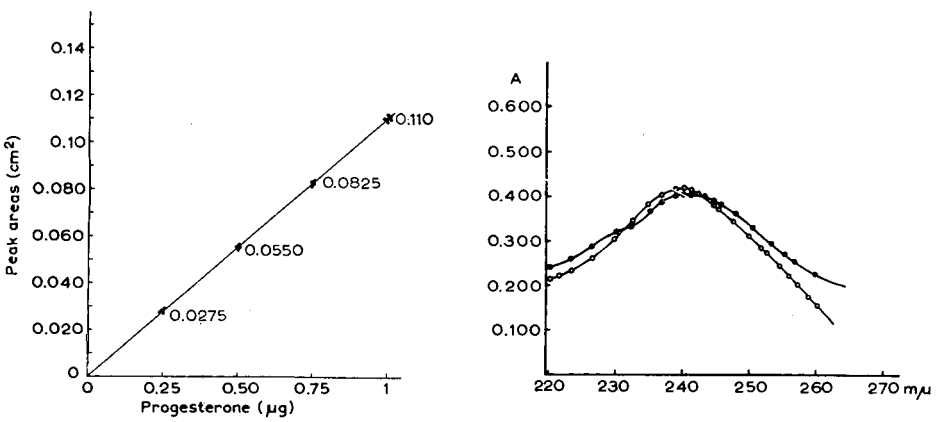

Fig. 5. Linear relationship between peak area and quantity of progesterone injected. The experimental conditions are described in the text.

Fig. 6. U.V. absorption spectra of an ethanolic solution of crystalline progesterone (open circles) and of the progesterone fraction of a pool of plasma from women (filled circles) after purification by horizontal thin-layer chromatography.

The specificity of the method depends on a preliminary purification and on the separation of progesterone from other Δ^4-3-ketosteroids. In fact, no other peaks, apart from that with the same retention time as progesterone, have been identified when extracts of a large pool of plasma were chromatographed on a 90 cm micro-glass column packed with 1 % QF-1. Further evidence of specificity of the method has been obtained by comparing the U.V. spectra of plasma fractions containing progesterone (after thin-layer chromatography) with the spectrum of the authentic compound. The spectra are recorded in Fig. 6.

Concluding, the results reported indicate that gas–liquid chromatography can be applied to the qualitative and quantitative determination of plasma progesterone in non-pregnant women. The importance of a preliminary purification of the extracts by means of horizontal thin-layer chromatography is shown, in order to obtain well defined and measurable peaks.

With some changes in the preliminary extraction and chromatographic separation, it is possible to apply this general technique to the quantitative determination of various steroid hormones in both tissue and biological fluids.

ACKNOWLEDGEMENTS

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SUMMARY

A method for the quantitative determination of progesterone in human plasma is described. This method consists of extraction with ether, solvent partition, thin-layer chromatography and finally gas–liquid chromatography for the quantitation of progesterone. The method is suitable for application to peripheral blood levels at all stages of the menstrual cycle and pregnancy. Data are presented on the sensitivity, accuracy, precision and reproducibility of the method.

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ÜBER DIE QUANTITATIVE ZUCKERBESTIMMUNG IN GLYKOSIDEN UND OLIGOSACCHARIDEN MIT HILFE DER GASCHROMATOGRAPHIE

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EINLEITUNG

Bei Untersuchungen über den Aufbau von Saponinen[1] trat häufig das Problem auf, die Zuckerbausteine quantitativ genau zu bestimmen. Ganz allgemein führt man solche Bestimmungen an Glykosiden, Oligo- und Polysacchariden derart durch, dass ein durch saure Hydrolyse erhaltenes Monosaccharidgemisch zunächst papierchromatographisch aufgetrennt wird. Nach Lokalisieren der Zucker durch Anfärben von Randstreifen auf dem Chromatogramm werden diese dann eluiert und titrimetrisch oder colorimetrisch bestimmt. Man kann die Zucker auch direkt auf dem Chromatogramm anfärben und die Konzentration aus der Fleckengrösse densitometrisch oder nach Elution ermitteln[2].

Der Fehler dieser Methoden liegt im allgemeinen über 5%; für Zuckerbestimmungen in Glykosiden ist er oft beträchtlich grösser. Bei jeder Bestimmung müssen Vergleichszucker mithydrolysiert und mitchromatographiert werden, da die Trennung, die Elution und die Farbstoffausbeute nicht streng reproduzierbar sind. Ferner müssen die Blindwerte des Papiers sorgfältig berücksichtigt werden.

In Anbetracht der Häufigkeit und Wichtigkeit solcher Bestimmungen erschien es angebracht, eine weniger umständliche und genauere Methode auszuarbeiten. Hierfür bot sich die Gaschromatographie der silylierten Zucker an. Während in früheren Versuchen in begrenztem Umfange die Gaschromatographie der acetylierten und methylierten Zucker untersucht wurde[3], konnte durch die Verwendung der leicht quantitativ herstellbaren und gut flüchtigen Persilylverbindungen die Anwendungsbreite der Gaschromatographie bei Zuckern wesentlich erweitert werden.

SWEELEY, BENTLEY, MAKITA UND WELLS[4] haben in einer grundlegenden, umfangreichen Untersuchung an über 100 Kohlenhydraten die Leistungsfähigkeit dieser Methode zur analytischen Identifizierung von Zuckern demonstriert. Sie trennten persilylierte Mono-, Di- und Trisaccharide, die entsprechenden Methylglykoside und andere Derivate an SE 52- und Polyäthylenglykolsuccinat-Säulen. Sie studierten die Silylierungsreaktion genauer und fanden, dass Kohlenhydrate in Pyridin mit Hexamethyldisilazan und Trimethylchlorsilan in wenigen Minuten quantitativ reagierten.

Bei eigenen Untersuchungen stellte sich heraus, dass für die quantitative Auswertung dieses Verfahrens eine Reihe von Bedingungen variiert werden mussten.

Ferner hatte man den Einfluss der Säure bei der Hydrolyse zuvor sorgfältig zu studieren. Die quantitative Bestimmung wurde zunächst auf Glucose, Galaktose, Mannose, Rhamnose, Xylose und Arabinose beschränkt, die in erster Linie als Zucker in Glykosiden*, Oligo- und Polysacchariden vorkommen. Die Hydrolyse wurde nach zwei verschiedenen, standardisierten Verfahren vorgenommen. Nach der Silylierung und gaschromatographischen Trennung der Silylzucker an Silikonfett DC konnten mit Hilfe allgemein verwendbarer Korrekturfaktoren die molaren Verhältnisse der Zucker aus den Peakflächen auf 3–5 % genau ermittelt werden.

Während dieser Untersuchungen erschien eine Arbeit von SWEELEY UND WALKER[5], die sich mit der quantitativen Bestimmung von Glucose, Galaktose, Galaktosamin und Neuraminsäure in Glykolipiden und Gangliosiden beschäftigt. Die Verfasser bestimmten diese Substanzen nach Methanolyse und Silylierung quantitativ gaschromatographisch nach Trennung an einer SE 30-Säule.

EXPERIMENTELLER TEIL

Substanzen

Die für diese Untersuchung benutzten Zucker D-Glucose (Gluc), D-Galaktose (Gal), D-Xylose (Xyl), D-Mannose (Mann), L-Arabinose (Arab), L-Rhamnose (Rham) und Lactose stammten von der Fa. E. Merck, Darmstadt.

Von den Glykosiden wurde Tomatin[6] von der Fa. C. Roth, Karlsruhe bezogen. Die übrigen Glykoside waren isolierte Reinsubstanzen aus *Digitalis purpurea* Samen (Digitonin)[7], aus Knollen von *Cyclamen europäum* (Cyclamin)[8], aus *Radix sarsaparillae* (Parillin)[9] sowie aus Blättern von *Hedera helix* (α-Hederin und Hederasaponin C)[10].

Alle Substanzen wurden vor der Einwaage sorgfältig im Hochvakuum getrocknet. Die Wägungen wurden auf einer Bunge-Halbmikrowaage durchgeführt (± 0.03 mg).

Silylierung

Das nach verschiedenen Verfahren erhaltene Zuckergemisch wurde durch dreimaliges Abdampfen im Rotationsverdampfer mit trockenem Benzol vollkommen wasserfrei erhalten. Man löste es in 0.8 ml Pyridin und setzte 0.4 ml Hexamethyldisilazan und 0.4 ml Trimethylchlorsilan zu. SWEELEY und Mitarb.[4] benutzten diese Lösung nach 5 Min. direkt zur Gaschromatographie. Für die quantitative Bestimmung der Zucker erwies es sich als günstiger, den Silylierungsansatz noch 1 St. auf 95° zu erhitzen, da das meist sirupöse Zuckergemisch sich im Pyridin dann besser umsetzte und der Niederschlag von NH_4Cl bei der weiteren Aufarbeitung sich besser filtrieren liess. Die Lösung wurde darauf bei 60° im Rotationsverdampfer zur Trockne gebracht, zweimal mit wasserfreiem Benzol abgedampft und der Rückstand in 2 ml wasserfreiem Benzol aufgenommen. Nach Filtration und Waschen mit weiteren 2 ml Benzol wurde wieder bei 60° eingeengt, so dass nur noch Spuren von Benzol und wenig Pyridin (10 % des Rückstandes) vorhanden waren. Dieses sirupöse Gemisch wurde je nach eingesetzter Menge mit wenig Benzol (10–100 µl) verdünnt, und es wurden 0.5 µl dieser Lösung mit einer 10 µl Hamilton-Pipette eingespritzt. Pro Monosaccharid etwa 100–200 γ.

* Ausgenommen sind hier vor allem die Herzgift- und C-21-Glykoside, die nicht ubiquitäre Zucker enthalten.

Äquilibrierung der Zucker

Etwa je 60 mg Glucose, Galaktose, Mannose, Rhamnose, Xylose und Arabinose wurden in je 3 ml H_2O gelöst und 20 Std. stehengelassen. Danach wurden die Zucker bei 60° im Rotationsverdampfer zur Trockne gebracht und silyliert.

Hydrolyse (Methode 1)

Etwa je 60 mg Glucose, Galaktose, Mannose, Rhamnose, Xylose und Arabinose, sowie genau eingewogene Mischungen zweier Zucker (im molaren Verhältnis), nämlich: Gluc–Arab, Gluc–Xyl, Gluc–Rham, Gluc–Mann, Gluc–Gal, Gal–Xyl, Gal–Rham, Mann–Arab, Rham–Arab, Rham–Xyl (zumeist in doppelter Ausführung) wurden in 3 ml 3 *N* HCl 2 Std. auf 95° erhitzt. Anschliessend wurde die wässrige Lösung mit Dow 3 neutralisiert und die Lösung zur Äquilibrierung 20 Std. stehengelassen. Darauf wurde eingeengt und wie beschrieben silyliert.

Je 5–50 mg der Glykoside Cyclamin, Digitonin, Parillin und Tomatin wurden in 3 ml 3 *N* HCl, 1 ml Dioxan und 3 ml Benzol 4 Std. auf dem Wasserbad auf 95° erhitzt. Nach dem Abkühlen trennte man die Benzolschicht ab, schüttelte die wässrige Phase noch zweimal mit Chloroform aus und wusch die organischen Phasen mit wenig Wasser einmal aus. Die wässrige Phase neutralisierte man mit Dow 3. Im Falle des Tomatins wurde nach der Neutralisierung noch einmal mit Chloroform ausgeschüttelt. Die wässrigen Phasen wurden wie beschrieben eingeengt und silyliert.

Falls die Glykoside in Wasser nicht genügend löslich sind, kann auch ein grösserer Anteil Dioxan (unter Verzicht auf das Überschichten mit Benzol) zugesetzt werden. Es darf möglichst kein Methanol oder Äthanol hinzugefügt werden, da unter diesen Bedingungen ein beträchtlicher Teil der Zucker (bis 60 %) in Methyl bzw. Äthylglykoside übergeht. In einem solchen Fall müssen dann hinterher in rein wässriger HCl die Zucker nachhydrolysiert werden. In dieser Weise wurde in einem Fall beim Cyclamin verfahren, wobei eine entsprechende Mischung von Vergleichszuckern analog behandelt wurde. Auch dann sind genaue quantitative Ergebnisse möglich (siehe Tabelle V).

Hydrolyse (Methode 2)

Etwa je 60 mg Glucose, Galaktose, Mannose, Rhamnose, Xylose, Arabinose, Lactose und β-Methylgalaktosid, sowie genau eingewogene Mischungen zweier Zucker (im molaren Verhältnis, zusammen *ca.* 60 mg) nämlich: Gluc–Arab, Gluc–Xyl, Gluc–Rham, Gluc–Mann, Gal–Xyl, Gal–Rham, Gal–Mann, Mann–Rham, Rham–Arab wurden je in 5 ml 5 % wasserfreier methanolischer HCl unter Feuchtigkeitsausschluss 4 Std. zum Sieden erhitzt. Nach 2 Std. war in jedem Falle der gesamte Zucker in Lösung gegangen. Nach dem Abkühlen verdünnte man mit dem doppelten Volumen H_2O und neutralisierte mit Dow 3. Nach dem Eindampfen wurde durch Abdampfen mit wasserfreiem Benzol getrocknet und anschliessend silyiert.

Die einfache in der Literatur beschriebene Methode des direkten Eindampfens der methanolischen Reaktionsmischung im Stickstoffstrom ergab Veränderungen im Verhältnis der Peakflächen eines Zuckers. Ausserdem traten Nebenpeaks auf, die auf eine teilweise Hydrolyse der Methylglykoside hindeuteten.

Je etwa 5–50 mg Tomatin, Cyclamin, α-Hederin und Hederasaponin C wurden in 5 % methanolischer HCl 5 Std. unter Rückfluss wie vorher beschrieben erhitzt und nach dem Abkühlen mit dem doppelten Volumen Wasser versetzt. Das ausge-

fallene Genin wurde durch dreimaliges Extrahieren mit Chloroform entfernt. Darauf wurde wie üblich mit Dow 3 neutralisiert, die Lösung anschliessend eingedampft, der Rückstand getrocknet und silyliert.

Gaschromatographie

Die gaschromatographischen Analysen wurden auf dem Gerät von Perkin-Elmer F 6/4 H F mit dem elektronischen Integrator D 2 und dem Kienzle-Digitaldrucker durchgeführt. Als Säulen dienten Stahlsäulen von 3 mm Innendurchmesser und 2 m Länge, die mit 15 % Silikonfett DC auf Celite 545 (60–100 mesh) gefüllt waren. Die Arbeitstemperatur betrug im allgemeinen 190°. Bei einem Eingangsdruck von 1.8 kp/cm^2 ergab sich eine Durchflussgeschwindigkeit für Helium von 34 ml/Min. Als Detektor diente eine Wärmeleitfähigkeitszelle mit einem Brückenstrom von 124 mA. Detektor und Einspritzblock waren auf 250° geheizt. Der Papiervorschub des Schreibers betrug 0.5 cm/Min. Die von der Wärmeleitfähigkeitszelle kommende Gleichspannung wurde auf den Perkin-Elmer Integrator D2 gegeben, der die Spannung in eine ihr proportionale Impulsfrequenz umwandelte. Diese wurde gespeichert und konnte bei Bedarf z.B. am Anfang und am Ende eines Peaks abgefragt werden. Die Summe wurde dann auf dem Kienzle-Digitaldrucker ausgedruckt. Die Anzahl der Impulse ist den Peakflächen und damit der Substanzmenge proportional. Unter den angegebenen Bedingungen betrug sie für 1 cm^2 Peakfläche etwa 6400 Impulse.

ERGEBNISSE UND DISKUSSION

In Vorversuchen sollten zunächst die günstigsten Bedingungen für eine quantitativ auswertbare Gaschromatographie von Silylzuckern ermittelt werden. Hierfür wurden alle verfügbaren Säulenfüllungen, vor allem die in der Literatur bereits für die Gaschromatographie von Silylzuckern beschriebenen, wie Apiezon M[11], Apiezon L[12], SE 30[5], SE 52[4] und Polyäthylenglykolsuccinat[4] auf verschiedenen Trägern, in ihrer Brauchbarkeit überprüft. Silikonfett DC auf Kieselgur erbrachte dabei die günstigsten Ergebnisse. Die Trennungen waren denen vergleichbar, die SWEELEY *et al.* auf SE 52 und SE 30 erhielten. Die Säule zeigte jedoch eine bessere Konstanz der Nullinie, und nach dem Lösungsmittelpeak wurde die Nullinie rasch und ohne Nachstellen wieder erreicht. Möglicherweise war dieses Verhalten teilweise darauf zurückzuführen, dass die Trennung bei einer vergleichsweise höheren Temperatur durchgeführt werden musste (190° statt 140° bei SWEELEY).

Nach Modifizierung der Aufarbeitung des Silylierungsansatzes wurde insgesamt eine störungsfreie und konstante Nullinie bei der Gaschromatographie erhalten, so dass die quantitative Auswertung mit einem elektronischen Integrator erfolgen konnte. Auf diese Weise liessen sich die auftretenden Peakflächen besonders leicht und genau erfassen. Selbstverständlich kann auch jede andere genau arbeitende Methode der quantitativen Auswertung benutzt werden (z.B. Berechnung aus Chromatogrammdaten, graphische Auswertung oder einfach durch Zerschneiden des Chromatogramms und Auswägen der Peakflächen).

Eine Besonderheit bei der Gaschromatographie von Zuckerderivaten tritt dadurch auf, dass von einem einheitlichen Zucker nach Aufarbeitung aus Lösung mehrere Peaks gegeben werden. Die auftretenden Peaks entsprechen den in der Lösung im Gleichgewicht vorhandenen Formen des Zuckers. Solche Peaks können

den α- und β-Glykosiden der pyranoiden, der furanoiden und septanoiden Form der Zucker entsprechen[4,12,13]. Ausserdem kann auch noch die Ketoform vorhanden sein[13]. Im allgemeinen hat man allerdings nach wässriger Äquilibrierung (Mutarotationsgleichgewicht) nur mit 2 bis 3 Hauptpeaks zu rechnen, während die anderen Verbindungen unter 1 % liegen. Diese Trennmöglichkeit ist zwar zur Untersuchung der Gleichgewichte der Zucker in Lösung von grossem Wert, sie erschwert aber die qualitative Identifizierung und noch mehr die quantitative Bestimmung der Zucker. SWEELEY *et al.*[4] haben daher versucht, durch Überführung in geeignete Derivate nur noch einen Peak zu erhalten. Durch Reduktion oder Oximierung der Aldehydgruppe konnten sie zwar dieses Ziel erreichen, es liessen sich dann aber die Peaks der verschiedenen Zucker nicht mehr gut trennen.

In der Tabelle I, Spalte a sind die relativen Retentionszeiten (bezogen auf persilylierte α-Glucose) der nach wässriger Äquilibrierung auftretenden Peaks angegeben. In der Spalte b findet man das prozentuale Verhältnis der zugehörigen Peakflächen. Dieses Verhältnis wies infolge der leichten Umwandelbarkeit während der Aufarbeitung Schwankungen auf.

Zur quantitativen Bestimmung der Zucker in Glykosiden, Oligo- und Polysacchariden muss vor der eigentlichen Bestimmung eine saure Hydrolyse erfolgen. Es ist bekannt, dass während dieser Hydrolyse die Zucker sich teilweise zersetzen,

TABELLE I

RELATIVE RETENTIONSZEITEN DER PERSILYLIERTEN VERBINDUNGEN AUS WÄSSRIGEN ZUCKERGLEICHGEWICHTSMISCHUNGEN UND DIE PROZENTUALE ZUSAMMENSETZUNG DER MISCHUNGEN

Glucose	a	0.73		0.81	1.00		1.50	
	b	—		1.0 %	43.0 %		56.0 %	
	c	2.0 %		1.5 %	41.5 %		55.0 %	
Galaktose	a	0.74		0.88	0.97		1.07	
	b	12.0 %		34.0 %	—		54.0 %	
	c	11.5 %		33.5 %	2.0 %		53.0 %	
Mannose	a		0.74			1.07		
	b		80.0 %			20.0 %		
	c		80.0 %			20.0 %		
Rhamnose	a	0.29		0.32	0.38		0.44	0.52
	b	—		72.0 %	—		27.0 %	1.0 %
	c	4.0 %		69.0 %	1.0 %		25.0 %	1.0 %
Xylose	a	0.25–0.41		0.31 0.32	0.45		0.55	
	b	—		3.0 %	46.0 %		51.0 %	
	c	20 %		—	37.0 %		43.0 %	
Arabinose	a	0.31		0.35	0.39			
	b	48.0 %		41.0 %	11.0 %			
	c	48.0 %		41.0 %	11.0 %			

(a) Relative Retentionszeiten bezogen auf persilylierte α-Glucose = 1.00; absolute Retentionszeit für Persilyl-α-glucose = 55 Min; Trennung bei 190°, 34 ml He/Min, an Silikonfett DC.
(b) Prozentualer Anteil der auftretenden Peakflächen nach wässriger Äquilibrierung der Zucker (siehe Experimenteller Teil).
(c) Prozentualer Anteil der nach Erhitzen mit wässriger HCl und nachfolgender Äquilibrierung erhaltenen Peakflächen (siehe Experimenteller Teil).

und es muss bei jeder quantitativen Bestimmung diese Tatsache berücksichtigt werden. Dabei interessiert vor allem, wieviel des Zuckers unter bestimmten Hydrolysebedingungen nicht wiedergefunden wird. In der Gaschromatographie ist auch damit zu rechnen, dass gewisse Zersetzungsprodukte im Gaschromatogramm neue Peaks ergeben, die möglicherweise die Trennung von anderen Zuckern erschweren. Zur Untersuchung dieser letzten Frage wurden zunächst Monosaccharide den Hydrolysebedingungen ausgesetzt, die für die Hydrolyse der Glykoside und Oligosaccharide sich als gut geeignet erwiesen hatten. Dabei kamen zwei standardisierte Hydrolysemethoden zur Anwendung. Bei der ersten Methode benutzte man 3 *N* wässrige HCl (gegebenenfalls mit Dioxanzusatz) und bei der zweiten 5 % wasserfreie methanolische HCl zur Hydrolyse bzw. Methanolyse. Über den prozentualen Anteil an neu auftretenden Peaks bei der wässrigen Hydrolyse und ihre relativen Retentionszeiten unterrichtet Tabelle I, Spalte c. Dieser prozentuale Anteil lag bei den meisten Zuckern zwischen 1 und 5% und war bei gleichen Hydrolysebedingungen weitgehend konstant. Auffallend war der hohe Prozentsatz von Nebenpeaks bei der Xylose. Es muss allerdings darauf hingewiesen werden, dass bei weiterer Verschärfung der Hydrolysebedingungen auch bei den anderen Zuckern z.T. sehr beträchtliche Nebenpeaks entstehen, so aus Glucose (bei 0.73), aus Galaktose (bei 0.97) und aus Arabinose (bei 0.36).

Bei der Methanolyse zeigte sich ein anderes Bild (Tabelle II). Da direkt die stabileren Methylglykoside gebildet wurden, war der Anteil an Zersetzungsprodukten geringer. Die persilylierten Methylglykoside zeigten gegenüber den persilylierten Zuckern erheblich verschiedene relative Retentionszeiten, wobei besonders der

TABELLE II

RELATIVE RETENTIONSZEITEN DER PERSILYLIERTEN METHYLGLYKOSIDE AUS DER SAUREN METHANOLYSE VON MONOSACCHARIDEN UND DIE PROZENTUALE ZUSAMMENSETZUNG DER REAKTIONSMISCHUNG

Glucose	a	0.63 0.72	0.93	1.02	
	b	2.0 %	71.0 %	27.0 %	
Galaktose	a	0.63	0.73	0.83	0.91
	b	18.0 %	59.0 %	21.0 %	2.0 %
Mannose	a	0.65	0.73	0.84	
	b	90.5 %	9.0 %	0.5 %	
Rhamnose	a	0.23	0.27	0.29	0.34
	b	1.5 %	87.0 %	9.5 %	2.0 %
Xylose	a	0.20–0.27	0.36	0.39	
	b	10.0 %	60.5 %	29.5 %	
Arabinose	a	0.23 0.24	0.28	0.36	
	b	90.5 %	8.5 %	1.0 %	

(a) Relative Retentionszeiten bezogen auf persilylierte α-Glucose = 1.00; Trennung bei 190°, 34 ml He/Min, an Silikonfett DC.
(b) Prozentualer Anteil der durch Methanolyse von Monosacchariden erhaltenen Peakflächen (siehe Experimenteller Teil, Methode 2).

geringere Unterschied zwischen α- und β-Anomeren auffiel. Auch das Verhältnis der Peakflächen zueinander hatte sich beträchtlich verschoben. Im Gegensatz zur wässrigen Hydrolyse war aber der prozentuale Anteil der Peakflächen gut reproduzierbar (auf ~ 1 %). Offenbar bildete sich in der methanolischen HCl ein konstantes Gleichgewicht der Anomeren aus; denn auch Maltose und Cellobiose ergaben das übliche Glucoseverhältnis, und selbst eingesetztes β-Methylgalaktosid zeigte nach der Methanolyse keinen Unterschied gegenüber eingesetzter Galaktose.

Betrachtete man jetzt die Trennmöglichkeiten zwischen den Zuckern, so liessen sich folgende Paare nach wässriger Hydrolyse als persilylierte Verbindungen gut trennen: Glucose von Rhamnose; Galaktose von Rhamnose; Mannose von Rhamnose; Glucose von Xylose; Galaktose von Xylose; Mannose von Xylose; Glucose von Arabinose; Galaktose von Arabinose; Mannose von Arabinose.

Bei der Trennung Glucose von Mannose und Glucose von Galaktose trat eine gewisse Überlappung der Peaks auf, bei der aber eine quantitative Bestimmung noch möglich war. Berücksichtigte man bei den Trennungen Xylose von Rhamnose und Xylose von Arabinose rechnerisch die 20 % Zersetzungsprodukte zwischen 0.25–0.41 der Xylose, so liessen sich auch diese Paare nebeneinander bestimmen. Trennungen der Paare Arabinose von Rhamnose sowie Mannose von Galaktose waren nicht möglich.

Günstiger noch waren die Trennmöglichkeiten nach der Methanolyse. Es liessen sich alle sechs Zucker gut nebeneinander bestimmen. Ohne weiteres trennbar waren: Glucose von Galaktose; Glucose von Mannose; Glucose von Rhamnose; Glucose von Xylose; Glucose von Arabinose; Galaktose von Rhamnose; Galaktose von Xylose; Galaktose von Arabinose; Mannose von Rhamnose; Mannose von Xylose; Mannose von Arabinose.

Bei rechnerischer Berücksichtigung kleinerer Nebenpeaks (die in der Menge weitgehend konstant anfielen), konnten auch Rhamnose–Xylose, Rhamnose–Arabinose und Xylose–Arabinose gut nebeneinander bestimmt werden. Lagen Galaktose und Mannose nebeneinander vor, fielen die einzelnen Peaks der beiden Zucker fast direkt aufeinander. Da aber das Verhältnis der Peakflächen zueinander bei einem Zucker weitgehend konstant war und sich dieses Verhältnis bei Galaktose und Mannose stark unterschied, konnte aus dem Verhältnis der resultierenden Peakflächen rechnerisch mit genügender Genauigkeit auf die zur Galaktose und Mannose gehörenden Peakflächen geschlossen werden. Man konnte auch eingewogene Mischungen von Galaktose und Mannose chromatographieren und daraus das Verhältnis ermitteln.

Zur Bestimmung der vorhandenen Mengen Zucker in einem Hydrolysat mussten die ermittelten Peakflächen noch mit zwei Korrekturfaktoren multipliziert werden, die einmal den molaren Respons des betreffenden Silylzuckers für die Wärmeleitfähigkeitszelle enthielten, zum anderen die unterschiedliche Zersetzung der Zucker bei der Säureeinwirkung berücksichtigten. Wenn man dabei auf die Ermittlung von Absolutwerten der Zuckerkonzentration verzichtete und das meist nur interessierende molare Verhältnis bestimmte, so konnte man beide Korrekturfaktoren zu einem einzigen zusammenziehen und diesen Faktor auf Glucose beziehen, für die er unter standardisierten Hydrolysebedingungen gleich 1 gesetzt wurde.

Für die übrigen Zucker wurden dann die Faktoren bestimmt, indem man Mischungen zweier Zucker, von denen einer meist Glucose war, im molaren Verhältnis

den standardisierten Hydrolysebedingungen aussetzte, aufarbeitete, silylierte und nach Gaschromatographie die Peakflächen für beide Zucker bestimmte. Wenn einer der Zucker Glucose war, erhielt man bei molarem Verhältnis den Faktor aus der Division der beiden Peakflächensummen. In der Tabelle III sind diese Faktoren für die wässrige Hydrolyse und in der Tabelle IV für die Methanolyse aufgeführt.

TABELLE III

AUF GLUCOSE BEZOGENE KORREKTURFAKTOREN BEI DER GASCHROMATOGRAPHIE DER SILYLZUCKER NACH SAURER WÄSSRIGER HYDROLYSE

Glucose	1.00	Rhamnose	1.16
Galaktose	1.06	Xylose	1.27
Mannose	1.15	Arabinose	1.24

TABELLE IV

AUF GLUCOSE BEZOGENE KORREKTURFAKTOREN BEI DER GASCHROMATOGRAPHIE DER SILYLIERTEN METHYLGLYKOSIDE NACH SAURER METHANOLYSE

Glucose	1.00	Rhamnose	1.16
Galaktose	1.04	Xylose	1.17
Mannose	1.08	Arabinose	1.20

TABELLE V

QUANTITATIVE ZUCKERBESTIMMUNG NACH HYDROLYSE DER METHODE 1

Substanz	*Molares Verhältnis*					
	Vorhandene Zucker					*Gefundene Zucker*
	Gluc	*Gal*	*Xyl*	*Arab*	*Rham*	
Tomatin	2	1	1			2.00:1.03:1.02
Digitonin	2	2	1			2.00:1.93:0.98
Cyclamin	3		1	1		3.00:0.95:0.95
Cyclamin*	3		1	1		3.00:1.00:1.05
Parillin	3				1	3.00:1.04

* Nicht unter Standardbedingungen (siehe Experimenteller Teil).

Um die so gewonnenen Korrekturfaktoren zu prüfen, wurden jetzt Glykoside und Oligosaccharide unter Standardbedingungen (Methode 1 und 2) hydrolysiert und die Zucker silyliert und gaschromatographiert. Zur Ermittlung der Korrekturfaktoren waren die freien Zucker etwas kürzer hydrolysiert worden als jetzt die Glykoside und Oligosaccharide, da sie der Säure von Anfang an ausgesetzt waren, während die Zucker der Glykoside erst allmählich in Freiheit gesetzt wurden. Es entstand auch hierbei eine Fehlermöglichkeit, da einzelne Zucker sehr viel schneller als andere abhydrolysiert wurden und daher auch länger der Säure ausgesetzt waren.

Das Ergebnis einiger quantitativer Zuckerbestimmungen zeigen die Tabellen V und VI sowie die Figuren 1–4. In Fig. 2 ist ein Beispiel für eine Berechnung bei teilweisem Überlappen der Peaks gegeben.

TABELLE VI

QUANTITATIVE ZUCKERBESTIMMUNG NACH HYDROLYSE DER METHODE 2

Substanz	*Molares Verhältnis*					
	Vorhandene Zucker					*Gefundene Zucker*
	Gluc	*Gal*	*Xyl*	*Arab*	*Rham*	
Tomatin	2	1	1			2.00:0.98:1.03
Cyclamin	3		1	1		3.00:1.02:1.03
Eingewogene Zuckermischung	3			2	3	3.00:2.06:2.96
α-Hederin				1	1	1.00:0.98
Hederasaponin C	2			1	2	2.00:1.02:1.98
						2.00:0.99:1.94*
Lactose	1	1				1.00:1.02
						1.00:1.03*

* Doppelbestimmung.

Aus den Tabellen V und VI sowie den Figuren 1–4 ist ersichtlich, dass die angegebene Methode sehr gut geeignet ist, die Zucker in Glykosiden und Oligosacchariden quantitativ zu bestimmen. Der Fehler liegt bei der Bestimmung nach Methode 1 bei 5 % während er bei der zweiten Methode 3 % nicht überschreitet. Damit ist eine Genauigkeit erreicht, die mit anderen Methoden nicht zu erzielen ist. Ausserdem ist dieses Verfahren der Zuckerbestimmung, sofern man einen Gaschromatographen besitzt, einfacher und weniger zeitraubend als die früheren Bestimmungen.

Der grössere Teil des Fehlers geht hierbei auf die Hydrolyse zurück, da entsprechende Zuckerbestimmungen ohne vorhergehende Säurebehandlung auf 1–2 % genau zu machen sind. Die Ermittlung von Absolutwerten ist grundsätzlich auch möglich. Sie ist jedoch recht umständlich und in den meisten Fällen auch nicht erförderlich.

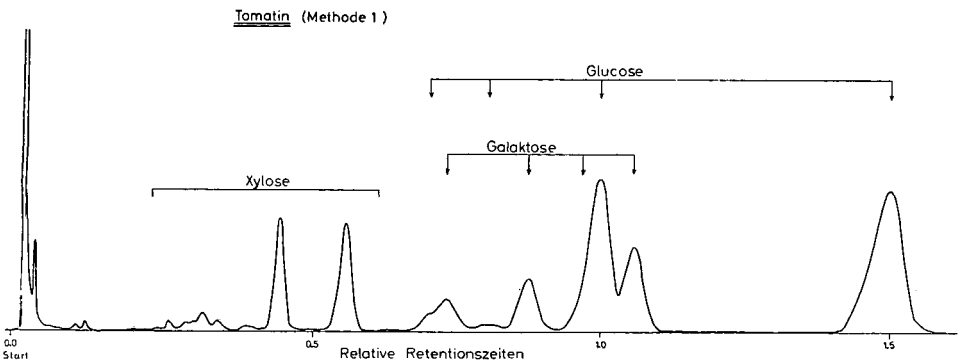

Fig. 1. Gaschromatogramm der silylierten Zucker aus Tomatin (Hydrolyse nach Methode 1). Trennung bei 190° an Silikonfett DC auf Kieselgur, Strömungsgeschwindigkeit 34 ml He/Min; Retentionszeiten bezogen auf Persilyl-α-glucose ≡ 1; absolute Retentionszeit für Persilyl-α-glucose = 55 Min.

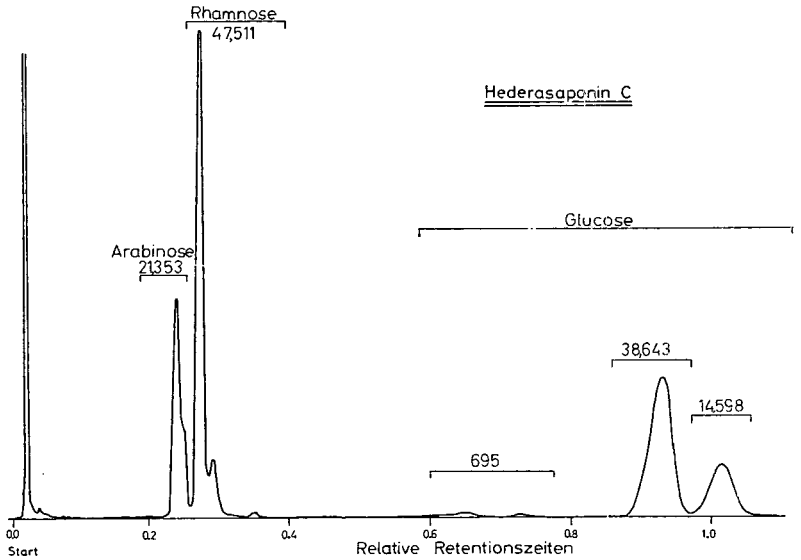

Fig. 2. Gaschromatogramm der silylierten Methylglykoside aus Hederasaponin C (Hydrolyse nach Methode 2). Trennungsbedingungen wie in Fig. 1. Quantitative Auswertung dieses Chromatogramms: Durch elektronische Integration wurden für die Peakflächen folgende Impulse erhalten: Arabinose 21,353 Imp., Rhamnose 47,511 Imp. und Glucose 53,936 Imp.; 9.5% der Fläche für die Arabinose (2,240) liegt unter den Rhamnose-Peaks (siehe Tabelle II) und muss daher der Arabinose zugezählt (23,593) und von der Rhamnose abgezogen (45,271) werden. Entsprechend müssen die 1.5% (690) des Rhamnose-Vorpeak berücksichtigt werden (22,903 bzw. 45,961). Nach Multiplikation mit den Korrekturfaktoren (1.20 bzw. 1.16) lässt sich das molare Verhältnis berechnen: 27,484 (1.02):53,315 (1.98):53,936 (2.00).

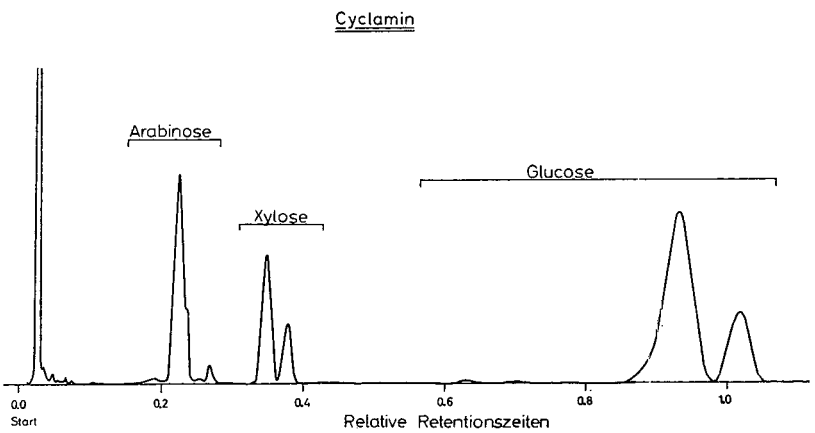

Fig. 3. Gaschromatogramm der silylierten Methylglykoside aus Cyclamin (Hydrolyse nach Methode 2). Trennungsbedingungen wie in Fig. 1.

Für eine Bestimmung benötigt man mindestens 5 mg, es werden am besten 5–50 mg für die Hydrolyse eingesetzt. Pro Zucker sollen optimal 100–200 γ eingespritzt werden. Bei Verwendung eines FID könnte diese Menge wesentlich verringert werden, doch müssten dann andere Korrekturfaktoren eingesetzt werden. Im allgemeinen werden jedoch 5 mg für eine quantitative Bestimmung zur Verfügung stehen.

Anzuwenden ist die Methode grundsätzlich für alle Glykoside, Oligo- und Polysaccharide, die mehr als eine Zuckerart und nicht andere Zucker als die angegebenen enthalten. Für Uronsäuren enthaltende Glykoside empfiehlt sich eine Doppel-

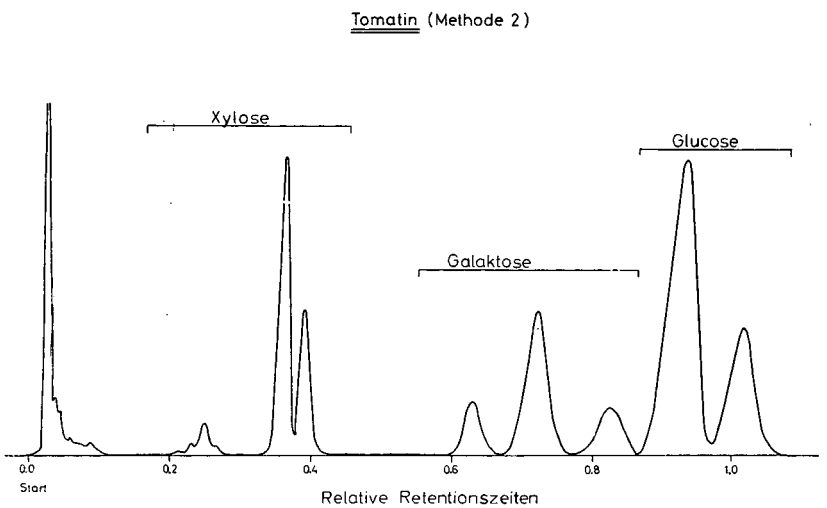

Fig. 4. Gaschromatogramm der silylierten Methylglykoside aus Tomatin (Hydrolyse nach Methode 2). Trennungsbedingungen wie in Fig. 1.

bestimmung, wobei in einem Falle nach der Hydrolyse die Uronsäure mit Dow 1 entfernt wird, im anderen Fall vor der Hydrolyse der mit Diazomethan hergestellte Methylester mit $NaBH_4$ zur Aldose reduziert wird. Aus der Differenz der beiden Bestimmungen lässt sich dann neben den Aldosen auch die Uronsäure bestimmen.

Müssen wegen der Natur des vorhandenen Glykosids andere Hydrolysebedingungen gewählt werden, so müssen auch die entsprechenden Korrekturfaktoren neu bestimmt werden. Am besten benutzt man dazu eine gleich oder ähnlich zusammengesetzte Mischung von Zuckern, wie man sie aus dem Glykosid erwartet. In den meisten Fällen wird man jedoch eine der beiden angegebenen Methoden zur Hydrolyse benutzen können, wobei häufig die Methanolyse vorzuziehen sein dürfte.

DANK

Herrn Prof. R. TSCHESCHE danke ich für die grosszügige Förderung meiner Arbeit, Herrn Prof. F. KORTE und Herrn Dr. U. CLAUSSEN für die Erlaubnis zur Benutzung ihres Gaschromatographen und Frl. L. WINTERFELD für die fleissige und geschickte Mitarbeit bei der Durchführung dieser Arbeit.

ZUSAMMENFASSUNG

Es wurde eine neue Methode zur quantitativen Bestimmung des molaren Verhältnisses von Glucose, Galaktose, Mannose, Rhamnose, Xylose und Arabinose in Glykosiden und Oligosacchariden ausgearbeitet. Dazu hydrolysierte man die Substanz unter standardisierten Bedingungen entweder mit wässriger oder methanolischer HCl. Die entstandenen Zucker bzw. deren Methylglykoside wurden silyliert und gaschromatographisch an einer Silikonfett DC-Säule getrennt. Mit Hilfe allgemein verwendbarer Korrekturfaktoren konnte aus den Peakflächen das molare Verhältnis der vorhandenen Zucker auf 3–5 % genau bestimmt werden.

SUMMARY

A new method was developed for the quantitative determination of the molar ratios of glucose, galactose, mannose, rhamnose, xylose and arabinose in glycosides and oligosaccharides. The substances were hydrolyzed under standard conditions, either with aqueous or with methanolic HCl. The sugars or methylglycosides formed were converted to trimethylsilyl derivatives and were separated by gas chromatography on a silicon grease DC column. By use of generally applicable correction factors the molar ratio of the existing sugars could be determined with an error of 3–5 %.

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THE GAS-CHROMATOGRAPHIC SEPARATION OF MIXTURES OF HYDRAZINE, METHYLHYDRAZINE AND 1,1-DIMETHYLHYDRAZINE

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Mixtures of hydrazine and some of its alkyl derivatives, which have recently become important in many fields, were studied with the aid of gas chromatography, a technique used by the present authors in earlier investigations on aliphatic amines[1].

Apart from the resolution of the mixtures, it was possible to carry out a rapid quantitative determination of the individual components under discussion and to avoid the numerous causes of interference which are usually encountered in other methods[2–13].

MATERIALS

1,1-Dimethylhydrazine (Eastman Kodak Co.) was used after a preliminary gas-chromatographic check had shown the presence of negligible quantities of impurities. The methylhydrazine was supplied by K. and K. Laboratories, Plainview, New York.

The following adsorbent systems were examined in order to determine the best experimental conditions:

(a) Carbowax 400 on Celite C 22;

(b) Carbowax 400 on Celite C 22, subsequently treated with KOH (7 % by weight with respect to the solid support);

(c) Carbowax 400 on Celite C 22, previously rendered alkaline with KOH (7 % by weight with respect to the solid support);

(d) Carbowax 1500 on Celite C 22;

(e) Carbowax 1500 on Celite C 22, subsequently rendered alkaline as under (b);

(f) Carbowax 1500 on Chromosorb W.

The gas-chromatographic determinations were carried out with the aid of a Fractovap type B instrument (C. Erba, Milan). The samples were introduced into the vaporisation chamber by means of an Agla microsyringe (Burroughs Wellcome Co., London) with a capacity of 0.5 ml. The flow-rate of the carrier gas was measured with a soap-bubble flow meter.

EXPERIMENTAL

Polyethylene glycols of various degrees of polymerisation were chosen as the stationary phase, owing to their high polarity. Alkalisation of the entire adsorbent system after permeation with the stationary phase had been tried with good results

in the work mentioned above[1]. This alkalisation differs from that used by HARDY AND POLLARD[14] on the support alone before permeation with the stationary phase.

Adsorbent system (b) clearly proved to be best for the present work. Carbowax 1500 on Chromosorb W was extremely sensitive and gave peaks even for extremely low concentrations. However, although the peak for hydrazine, whether hydrated or anhydrous, was very similar to that obtained with system (b), the peaks corresponding to the two methyl derivatives were less symmetrical and did not show a satisfactory separation. Lastly, Carbowax 1500 on Celite C 22, whilst having the same shortcomings as Carbowax 1500 on Chromosorb W as regards the methyl derivatives, did not even exhibit the same sensitivity for hydrazine as system (f).

Concluding that Carbowax 400 on Celite C 22, after alkalisation, was the best system for the present work, a series of tests were carried out at different temperatures to calculate the retention volumes and to determine the optimum temperature for the gas-chromatographic determination of the components of the mixtures in question. The tests were carried out using a column 2 m long, at a helium flow rate of 109 ml/min, and at the following temperatures: 85°, 90°, 100°, 105° and 110°.

The retention volumes of hydrazine, methylhydrazine and 1,1-dimethylhydrazine, calculated for different temperatures, are listed in Table I. A plot of the

TABLE I

RETENTION VOLUMES (V_r) AS A FUNCTION OF $1/T$

$1/T^*$	$(CH_3)_2N\text{-}NH_2$	$CH_3\text{-}NH\text{-}NH_2$	$N_2H_4 \cdot H_2O$
$2.790 \cdot 10^{-3}$	1035	2905	—
$2.755 \cdot 10^{-3}$	891	2450	—
$2.717 \cdot 10^{-3}$	747	2043.4	5346
$2.681 \cdot 10^{-3}$	650.5	1730	4288
$2.645 \cdot 10^{-3}$	540	1423.4	3440
$2.610 \cdot 10^{-3}$	440.5	1230	2864

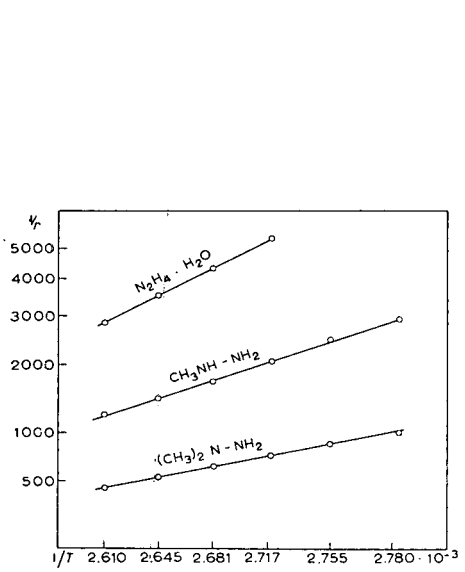
* Temperature in degrees absolute.

experimental values of the retention volumes on a semi-logarithmic scale, against the reciprocal of the absolute temperature, gives linear graphs, as shown in Fig. 1.

Some authors[15] have found, by thermodynamic methods, that the graphs for gas–liquid systems are linear when the partial molar heat of vaporisation of the compound in question is constant within the temperature range investigated.

It can be seen from Fig. 1 that the considerable differences in the retention volumes of the three hydrazines makes their qualitative separation possible in the temperature range under consideration. The V_r values of hydrazine fall off more steeply with increasing temperature than do those of methylhydrazine and especially those of 1,1-dimethylhydrazine. This may be explained by assuming that hydrazine and the polyethylene glycol of the stationary phase interact to form hydrogen bonds, and that this interaction decreases with increasing temperature.

On the other hand, the formation of hydrogen bonds between the alkyl hydrazines (especially 1,1-dimethylhydrazine) and the stationary phase is less likely or, at any rate, less frequent. It must also be borne in mind that hydrazine has a greater dipole moment than the methyl derivatives in question (see Table II), so that a



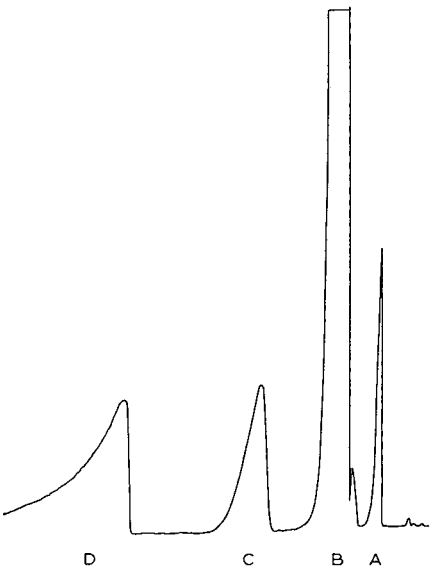


Fig. 1. The variation of the retention volumes with the reciprocal of the absolute temperature.

Fig. 2. Gas-chromatographic separation. A = $(CH_3)_2N\text{-}NH_2$; B = $CH_3\text{-}CH_2\text{-}OH$; C = $CH_3\text{-}NH\text{-}NH_2$; D = $N_2H_4 \cdot H_2O$. Experimental conditions: Support: Celite C 22 (30–60 mesh); stationary phase: Carbowax 400 (25%) + 7% of KOH; carrier gas: helium; column temperature: 130° ± 0.2°; inlet pressure: 0.7 kg/cm²; flow rate: 204 ml/min; current of bridge circuit: 24.5 mA; chart speed: 1.25 cm/min; length of column: 2 m; internal diameter of column: 0.6 cm. Retention time: dimethylhydrazine: 1 min 59 sec; methylhydrazine: 6 min 36 sec; hydrazine: 11 min 42 sec.

stronger association takes place between the dipoles of hydrazine and the strongly polar polyethylene glycol. This association decreases with increasing temperature. This effect is more noticeable with hydrazine because it is initially more highly associated than the alkyl hydrazines. This behaviour results in a steeper gradient of the top graph in Fig. 1 in comparison with the other two, or more precisely, in a decrease in the gradient with decreasing polarity of the compound.

The analytical separation of mixtures of hydrazine hydrate, methylhydrazine and dimethylhydrazine has been achieved under the conditions described, in strict agreement with the retention volume graphs in Fig. 1. Fig. 2 shows a chromatogram

TABLE II

Compound	*Dipole moment*	*B.p.* (°C)	V_r *(at 100°)*
$(CH_3)_2N\text{-}NH_2$	1.35[a]	62.2	650.5
$CH_3NH\text{-}NH_2$	1.68 ± 0.14[b]	87.5	1730
$N_2H_4 \cdot H_2O$	1.83 – 1.85[c]	118.5	4288

[a] Measured in *n*-heptane at 25°[16].
[b] Measured in benzene at 15°[17].
[c] Measured in benzene at 18°[18].

of the three components in question. The excellent resolution of the peaks achieved under the given experimental conditions can clearly be seen.

The calibration curve of each individual component of the mixtures was plotted and used to find experimental conditions which would permit a good separation, together with symmetrical peaks, and the maximum possible sensitivity at low concentrations. The first calibration curve studied was that of 1,1-dimethylhydrazine, when it was found necessary to dilute the compounds as far as possible with a solvent in order to record the curve at very low concentrations. A solvent was chosen which was completely miscible with the samples, and whose retention time did not interfere with the analysis.

Hydrazines are completely soluble in alcohols but less soluble in other solvents examined, such as *n*-heptane, ether, chloroform and dioxane. *n*-Propanol was therefore chosen as the solvent. A mixture of three hydrazines in this alcohol was chromatographed and the following sequence of peaks was obtained: 1,1-dimethylhydrazine (b.p. 62.2°), *n*-propanol (b.p. 97°), methylhydrazine (b.p. 87.5°) and hydrazine hydrate (b.p. 118.5°). The analysis was carried out at a temperature of 90° and a flow rate of 109 ml/min, the other experimental conditions being as reported for Fig. 2. The fact that an impurity in the propanol gave a peak which was partly superimposed on that of the 1,1-dimethylhydrazine made planimetry difficult. The peaks were therefore suitably resolved and the areas of the triangles obtained were measured geometrically.

1,1-Dimethylhydrazine was used in 2% and 0.4% solutions. The absolute detection limit of the analytical method for dimethylhydrazine was $1.95 \cdot 10^{-7}$ moles with the detector used. At higher dilutions, the method is of purely qualitative value, since the areas of the peaks are no longer reproducible and are also difficult to measure.

The same experimental conditions were used in the study of methylhydrazine. This compound gave an excessively high retention time, and above all, gave an asymmetric and flattened peak which would make planimetry difficult. By suitable variation of temperature and flow rate, it was found that at 115° and 204 ml/min a more regular and easily measurable methylhydrazine peak was obtained. A 2% (by volume) solution in propanol was used, since the use of more dilute samples resulted in the superposition of the solvent peak on that of the methylhydrazine. An irreversible adsorption of methylhydrazine on the stationary phase was observed, and in the first determinations this gave rise to a poor reproducibility of the areas of the peaks. Constant, and hence reliable, values were obtained only when saturation had been achieved, *i.e.* after the third or fourth run. The density of methylhydrazine, determined at 20°, was found to be 0.884.

The calibration curve of methylhydrazine was recorded under conditions specified in Fig. 2, except that the temperature and the flow rate were 115° and 204 ml/min, respectively. A temperature of 130° and a flow rate of 500 ml/min were used in the determination of the calibration curve of hydrazine hydrate. The other experimental conditions remained unaltered. A more concentrated (10%) alcoholic solution was used, since it was found that hydrazine was also subject to strong irreversible adsorption on the stationary phase, which interfered with its determination at very high dilutions.

The limiting concentrations for quantitative determinations were as follows: $2.5 \cdot 10^{-6}$ moles for hydrazine, $1.95 \cdot 10^{-7}$ moles for 1,1-dimethylhydrazine, and $0.46 \cdot 10^{-7}$ moles for methylhydrazine.

CONCLUSIONS

Examination of the results reported shows that 1,1-dimethylhydrazine has the highest thermal conductivity, which is followed by that of methylhydrazine, whilst the thermal conductivity of hydrazine is much lower. Consequently, in order to obtain peaks of the same surface area, *e.g.* 2 cm^2, the following quantities have to be used: $1.95 \cdot 10^{-7}$ moles of 1,1-dimethylhydrazine, $2.2 \cdot 10^{-7}$ moles of methylhydrazine, and $1.03 \cdot 10^{-5}$ moles of hydrazine, under the conditions specified above.

The gas-chromatographic determination of water in mixtures of hydrazines was not undertaken, since this problem, which has already been subjected to gas-chromatographic investigation by other authors[16,20], may be solved indirectly by the same technique as was described above. In fact, the water peak is well resolved in relation to those of the hydrazine peaks, although it is unsuitable for direct planimetric or geometric measurement, owing to its extremely flat and asymmetric shape. However, the water can be calculated by difference. It is quite probable that the concentrations of all the components in the mixtures discussed can be determined by one single measurement using a programmed gas-chromatographic instrument.

SUMMARY

The experimental conditions used for the separation of mixtures of hydrazine, methylhydrazine and 1,1-dimethylhydrazine are described. The limits of sensitivity found with the apparatus used in the quantitative determination of these products are given.

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GAS CHROMATOGRAPHY WITH STATIONARY PHASES CONTAINING SILVER NITRATE

VI. THE METHYLCYCLOHEPTENES AND METHYLENECYCLOHEPTANE*

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(Received September 9th, 1964)

It has been shown previously[1–5] that the complex-forming silver nitrate–glycol phase can be conveniently employed for the gas chromatographic separation of different types of isomeric olefins.

In order to gain information on the behavior of olefins possessing a seven-membered ring, the study of the relationship between structure and complex stability has been extended in the present work to the series of the isomeric methylcycloheptenes and methylenecycloheptane (compounds I–V, Table I). Furthermore, a rapid and precise method was needed for the determination of these isomers at equilibrium[6].

TABLE I

RETENTION VOLUMES OF THE ISOMERIC METHYLCYCLOHEPTENES AND METHYLENECYCLOHEPTANE
Stationary phase 30 g $AgNO_3$/100 ml triethylene glycol; column dimensions 3 m × 0.6 cm; weight ratio of solid support[1] to liquid phase 3:1; helium flow rate 120 ml/min; temperature 40°.

No.	*Compound*	*B.p. at 760 mm(°C)*	r^*	r^{**}	R^{***}
I	Methylenecycloheptane	138.2	1.81	0.97	3.20
II	1-Methylcycloheptene	137.2	1.33	0.72	3.14
III	3-Methylcycloheptene	129.8	3.97	2.14	2.64
IV	4-Methylcycloheptene	130.6	3.13	1.68	2.69
V	5-Methylcycloheptene	131.1	3.52	1.89	2.72
VI	Cycloheptene	116.4	2.98	1.61	—

* Relative to benzene.
** Relative to toluene.
*** Relative to methylcyclohexane on a 150 ft. capillary column coated with squalane (temperature 25°; nitrogen pressure 8.5 p.s.i.).

The difficulties encountered in the separation of compounds I–V are illustrated by the fact that a mixture of the compounds gives only two or at most three poorly resolved peaks when examined in columns packed with non-complex forming phases, *e.g.* silicone gum rubber, β,β′-oxydipropionitrile and di-2-ethylhexyl tetrachlorophthalate. For instance, a mixture of I–V gives only two peaks on a 2.5 m × 0.6 cm

* For Part V of this series, see ref. 5.
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column filled with 10 % silicone gum rubber on Chromosorb (temperature 70°; helium flow rate 30 ml/min), the first peak corresponding to the lower boiling isomers III–V (Table I) and the second to a mixture of I and II. The situation is even less satisfactory with more polar phases as differences in the volatility and polarity of the isomers examined seem to be counteractive. For example, a 2 m × 0.6 cm column packed with 20 % β,β'-oxydipropionitrile on Chromosorb (temperature 25–40°; helium flow rate 85 ml/min) gives only three overlapping peaks, while a 3 m × 0.6 cm column containing 10 % di-2-ethylhexyl tetrachlorophthalate on Chromosorb (temperature 75°; helium flow rate 85 ml/min) proves to be completely ineffective in the present case.

Experiments with a 150 ft. capillary column coated with polypropylene glycol (temperature 55°; nitrogen pressure 12 p.s.i.) showed some resolution of the closely boiling isomers I and II and of III, IV and V, respectively. Better separation is obtained (Fig. 1) with a 150 ft. capillary column coated with the less polar squalane (temperature 25°; nitrogen pressure 8.5 p.s.i.). Although the resolution observed is still not satisfactory for quantitative analysis, a complete separation of the isomers with a longer column of this type is feasible.

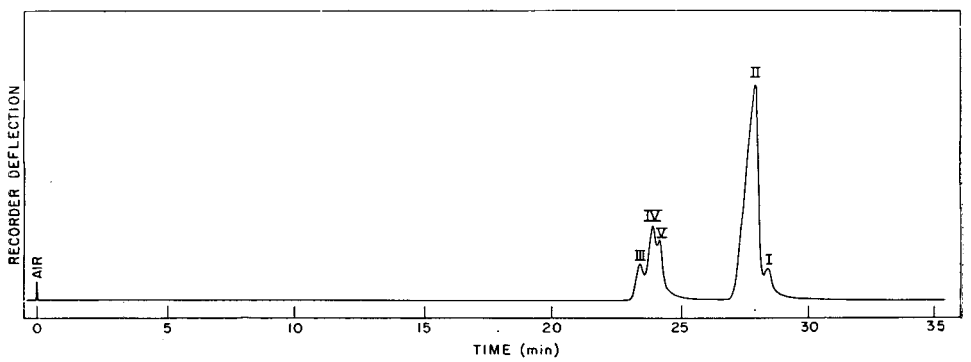

Fig. 1. Chromatography of a mixture of the four isomeric methylcycloheptenes(II–V) and methylenecycloheptane(I) on a 150 ft. capillary column coated with squalane (column C).

Examination of compounds I–V on columns containing silver nitrate–glycol showed that smooth separation can be obtained by proper choice of the salt concentration, type of glycol and working temperature. Solutions of silver nitrate in concentrations varying from 10 to 30 % in ethylene glycol, triethylene glycol and tetraethylene glycol were examined at temperatures from 20° to 70°. The best separations were achieved (Fig. 2) on a 3 m × 0.6 cm column filled with silver nitrate–triethylene

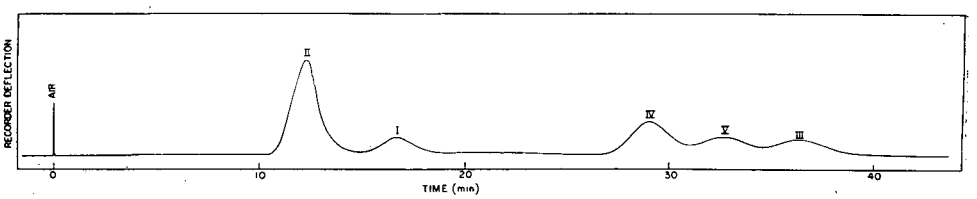

Fig. 2. Chromatography of a mixture of the four isomeric methylcycloheptenes and methylenecycloheptane on a silver nitrate–triethylene glycol column (column A).

glycol on 30–60 mesh firebrick (solution concentration 3 g/10 ml; weight ratio of support[1] to liquid phase 3:1; helium flow rate 120 ml/min; temperature 40°). The retention volumes of the isomers, relative to benzene and toluene, are summarized in Table I.

Quantitative analysis of synthetic blends of I–V, containing more than 5 % of each component, showed that the isomers can be determined with an accuracy of at least ± 1 % of the total. In addition to its application to equilibrium studies[6], the method was employed on a preparative scale (see Experimental) for the isolation of the previously unknown 4-methylcycloheptene and of an enriched sample of 5-methylcycloheptene.

DISCUSSION OF RESULTS

It has been shown[7,8] that the relative rentention volumes of closely boiling isomers parallel the stabilities of the silver ion–olefin complexes formed during the gas chromatographic process. On this basis, an interpretation of the data given in Table I can be made, as follows:

(a) 1-Methylcycloheptene (II) shows the lowest retention volume among the isomers examined. This is in accord with the behavior of 1-alkylcyclenes possessing smaller ring systems[1–4] and can be attributed to the decrease in complex stability caused by steric hindrance of the alkyl substituent at the double bond.

(b) 3-Methylcycloheptene (III) shows the highest retention volume among the isomers. Examination of models of this compound, based on the chair, boat and skewed boat conformations of the cycloheptene ring shows there is practically no steric interference by the methyl group in the approach to the double bond. A second factor which probably enhances the relative stability of the complex formed by III is the inductive effect of the methyl substituent. It has been already observed, both in cyclic[1–3] and in open-chain olefins[5,8], that the inductive effect of a methyl group in the 3-position is stronger than that exerted by the same substituent at a more remote position.

(c) Among the three closely boiling isomers III–V, which have the same type of endocyclic bond (disubstituted) and are therefore easily distinguished from II (trisubstituted double bond), the 4-methylcycloheptene (IV) forms the least stable complex ($r_{IV} < r_V < r_{III}$). An examination of models of IV shows that this is probably due mainly to steric factors, because there is a certain hindrance of the double bond by the 4-methyl group in the presumably predominant chair conformer (Fig. 3a). A clear steric effect is also evident in the twist-boat conformation (Fig. 3b).

(d) Models of 5-methylcycloheptene (V) show that, although steric hindrance of the double bond by the methyl substituent may exist in the boat conformation, there is no interference in the more stable chair conformation (Fig. 3c) or in the twist-boat conformation. This would explain the higher retention volume of V as compared to IV.

(e) The retention volume of methylenecycloheptane relative to that of 1-methylcycloheptene ($r_I : r_{II} = 1.36$) is considerably lower than that observed for corresponding pairs of methylenecyclane–1-methylcyclene analogues possessing smaller ring systems[1–4] (for instance, a ratio of 7.1:1 was found for the retention volumes of the methylenecyclohexane–1-methylcyclohexene pair of isomers).

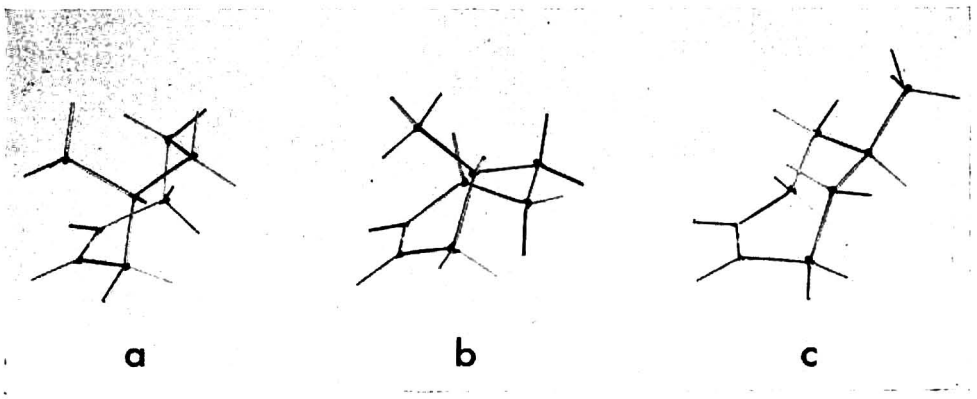

Fig. 3. Models of 4-methylcycloheptene (a and b) and 5-methylcycloheptene (c).

This observation is probably due in part to the stronger complexation ability of the endocyclic double bond in a 7-membered ring as compared to a 6- or 5-membered ring[8,9]. On the other hand, it may be dependent to some extent on the existence of certain steric hindrance of the double bond in methylenecycloheptane due to the flexibility of the cycloheptane ring. By analogy with the conformational analysis of cycloheptane[10] and cycloheptanone[11], one can expect that in methylenecycloheptane the ring will predominantly possess the twist-chair and twist-boat conformations. Examination of models shows that in the twist-chair form of I there appears to be a somewhat stronger hindrance of the double bond by the ring α-hydrogens compared to that in methylenecyclohexane, especially if opening (widening) of the bond angles in the cycloheptane ring is considered[10]. On the other hand, considerable steric hindrance of the double bond by one of the C_4 hydrogens is observed in the twist-boat conformation.

EXPERIMENTAL

Apparatus and procedure

Two Perkin-Elmer Model 154 Fractometers, one provided with a thermistor detector cell and the other with a flame ionization detector, were used in the study.

The following columns were mainly employed:

Column A. An analytical silver nitrate–triethylene glycol column (see text and Table I).

Column B. A preparative column, 2.5 m × 10 mm, packed with the same stationary phase as column A.

Column C. A 150-ft. Golay capillary column, coated with squalane.

Preparation of pure compounds

Methylenecycloheptane (*I*). Cycloheptyl carbinol, b.p. 111–112° (20 mm), n_D^{17} 1.4771, was prepared[12] by the action of formaldehyde on cycloheptylmagnesium bromide and then esterified with acetic anhydride. The resulting cycloheptylcarbinyl acetate, b.p. 115–116° (20 mm), n_D^{17} 1.4504, was pyrolyzed at 530° in a pyrex tube reactor of 40 cm length and 25 mm diameter, filled with pyrex beads. The product

was washed dried and then distilled on a 70-plate Piros-Glover spinning band column. The best sample of compound I (> 99% pure), amounting to 45% of the total distillate, had b.p. 138.2° (760 mm); n_D^{20} 1.4615 (reported[13], 138–140°; n_D^{20} 1.4611).

1-Methylcycloheptene (II). 75 ml of 1-methylcycloheptanol (99% pure) was dehydrated by refluxing with 280 ml of dilute (1:4) sulfuric acid for two hours. The product contained about 94% of II and 6% of methylenecycloheptane (I). A pure (>99%) sample of II, b.p. 137.2° (760 mm), n_D^{20} 1.4590 (reported[14] 138°; 1.4580) was obtained by distillation on the aforementioned fractionating column, followed by preparative gas chromatography on column B.

3-Methylcycloheptene (III). Cycloheptanol (99.5% pure; b.p. 92° (25 mm); n_D^{20} 1.4760), in admixture with an equal amount of *tert.*-butyl alcohol[15], was dehydrated at 250° over Harshaw alumina[16]. Under these conditions the cycloheptene obtained (b.p. 116.4°, n_D^{22} 1.4575) was 99.5% pure and free of contaminating 1-methylcyclohexene*. The freshly distilled olefin was reacted with N-bromosuccinimide in the presence of benzoyl peroxide and the resulting 3-bromocycloheptene, b.p. 40–41° (1 mm), n_D^{20} 1.5326 (reported[17] 59° (5.2 mm), n_D^{25} 1.5304), was treated as follows:

Magnesium (4.5 g, 0.185 mole) and dry ether (170 ml) were introduced into a three-neck flask provided with a condenser, a stirrer and a dropping funnel. After starting the reaction with a few drops of methyl iodide, a mixture of 3-bromocycloheptene (30 g, 0.17 mole) and methyl iodide (24.5 g, 0.18 mole) was added dropwise over a period of 1.5 h. The reaction was carried out at room temperature and under a constant stream of dry nitrogen**. After stirring for another hour, the mixture was decomposed with ice and hydrochloric acid and the 3-methylcycloheptene obtained (16 g, 0.15 mole; yield, 88%) was purified by distillation. The best portion, amounting to 35% of the total distillate, was more than 99% pure and had b.p. 129.8° (760 mm); n_D^{20} 1.4565 (reported[19] for a sample of III of unspecified purity 130–132°; n_D^{22} 1.4562).

Analysis. Calculated for C_8H_{14}: C, 87.28% H, 12.72%. Found: C, 87.05%; H, 12.49%.

The structure of compound III was confirmed by N.M.R. and infrared spectroscopy[20].

4-Methylcycloheptene (IV). A sample of this compound was isolated in 97% purity by preparative gas chromatography (column B) of an equilibrium mixture of the methylcycloheptenes[6]. B.p. 130.6 (760 mm); n_D^{20} 1.4545.

Analysis. Calculated for C_8H_{14}: C, 87.28%; H, 12.72%. Found: C, 87.10%; H, 12.43%.

The position of the double bond in IV was confirmed by oxidation to β-methylpimelic acid, using the method of VON RUDLOFF[21]. The acid obtained had m.p. 49–50° (reported[22] for β-methylpimelic acid, 48–50°) and did not depress the melting point of a pure sample of the reference compound.

* This compound is usually formed to the extent of 2–10% when strongly acidic dehydrating agents such as β-naphthalenesulfonic acid or sulfuric acid are employed.

** No reaction takes place when the original procedure of BERLANDE[18] for preparation of 3-alkylcycloalkenes is employed in the present case.

5-Methylcycloheptene (V). A sample containing 92 % of V was prepared by gas chromatography on column B. The boiling point of the compound (Table I) was determined by extrapolation of the gas chromatography data obtained on column C.

ACKNOWLEDGEMENTS

Thanks are due to Dr. B. ALTMAN AND Mr. M. LAHMI for valuable laboratory assistance.

SUMMARY

The gas chromatographic behavior of the isomeric methylcycloheptenes and methylenecycloheptane was investigated using: (a) packed tubes containing conventional non-complex forming phases; (b) capillary tubes coated with squalane and polypropylene glycol; (c) packed columns containing complex forming silver nitrate–glycol solutions as the stationary liquid. Smooth separation of a mixture of the isomers is achieved on a silver nitrate–triethylene glycol column; by proper choice of salt concentration and working temperature the components can be quantitatively determined with an accuracy of ± 1 %. On a preparative scale, the method was conveniently employed for the isolation of the previously unknown 4-methylcycloheptene.

By comparing the relative retention volumes of the isomers, correlations between complex stability and olefin structure were deduced, taking into consideration the peculiar conformations of the seven-membered ring system.

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TERPENOIDS

LXIV. EVALUATION OF POLYESTERS AS STATIONARY PHASES IN GAS–LIQUID CHROMATOGRAPHY OF TERPENOIDS*

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Essential oils are generally intricate mixtures of a variety of terpenic and non-terpenic compounds including hydrocarbons, carbonyl compounds, alcohols, esters, acids etc. In recent years, gas–liquid chromatographic (GLC) techniques have been widely employed for the evaluation of such oils[1]. It has been observed that substrates of medium polarity are most suitable for analyses of these substances, on gas chromatography (GC) columns, considering their nature. Polyesters which fall in this category have been widely used for this purpose[2–9]. They show sufficient thermal stability and capability of resolving compounds of wide polarity and boiling range. Recently VON RUDLOFF[10] prepared a few polyesters from glycols and fatty acids, and also from dicarboxylic acids (succinic, adipic, azelaic), and evaluated their solvent capability in GLC columns.

We have been interested in the GLC evaluation of essential oils for quite some time, and to find suitable phases, a systematic study of the polyesters from different 1,ω-dicarboxylic acids $HOOC\cdot(CH_2)_n\cdot COOH$ was undertaken.

The dicarboxylic acids ranging from malonic acid ($n = 1$) to hexadecane-1,16-dicarboxylic acid ($n = 16$) were tested and analyses of several typical terpenic and non-terpenic compounds were carried out using these polyesters. The results are quite interesting and will be discussed in detail later.

The number of methylene groups separating the two carboxylic acid groups have been found to have a profound influence on the retention time, which follows a clear arithmetical relationship which holds up to brassylic acid ($n = 11$) and only deviates slightly in the case of higher dicarboxylic acids.

Thus it has been found that if $t_R{}^x$ is the retention time in minutes and x is the number of methylene groups present in the acidic component of the polyester, and if $t_R{}^y$ be the retention time on another polyester containing y methylene groups, then:

$$\frac{t_R{}^x - t_R{}^y}{x - y} = c \qquad (1)$$

where c is a constant for a particular compound at a particular temperature and is the individual contribution of a single methylene group.

* Communication No. 721 from the National Chemical Laboratory, Poona-8, India.

This relationship seems to be an integral characteristic of the number of methylene (CH_2) groups between the two carboxylic groups and is valid for non-polar terpenic and other hydrocarbons and is nearly so for the aromatic hydrocarbons.

The large difference in retention times observed for the higher polyester stationary phases (n = 9 to 16) would render them specially useful for preparative separation.

EXPERIMENTAL

A Griffin & George Model MK-II VPC apparatus, which employed a system with nitrogen under reduced pressure, was converted to a system with hydrogen under pressure, resulting in higher efficiency and sensitivity. It can now be worked with any desired flow rates. The instrument is provided with a thermal conductivity detector, which along with the injection port is heated to the same temperature as that of the oven. The recorder range is 0.0–1.0 mV and has a chart speed of 6 in. per hour.

PREPARATION OF POLYESTERS

For the preparation of the polyesters the method described by CRAIG AND MURTY[5] has been followed essentially, with minor modifications.

Diethylene glycol and the corresponding dicarboxylic acids were placed in molar proportions in a round-bottom flask and to this *p*-toluenesulphonic acid (0.2 g) and diglycerol (0.5 g) were added. This mixture was heated carefully at 135–140° (bath)/2 mm for 4 h. The resulting viscous material was dissolved in methylene chloride and washed repeatedly with water, dried (sodium sulphate), the solvent removed and the residue dried to a constant weight under vacuum at room temperature. The polyesters from different dicarboxylic acids were prepared under almost identical conditions. In the case of the malonic acid polyester preparation (n = 1), instead of heating directly at 135° (bath), the mixture was initially heated slowly below 100° (bath) for 1 h and than at the usual temperature of 135–140° (bath) for 4 h.

Polyesters up to azelaic acid (n = 7) are viscous liquids, while higher polyesters are low melting solids (m.p. below 60°). The approximate molecular weights were determined in duplicate by end group titration. The results are shown in Table I.

It is interesting to note that the polyesters containing an even number of methylene groups in the acidic component have higher molecular weights compared to those obtained from acids containing an odd number of methylene groups.

The following terpenic and non-terpenic samples were employed for the comparative evaluation of the polyesters:

(1) Tricyclene; (2) α-pinene; (3) camphene; (4) β-pinene; (5) Δ^3-carene; (6) limonene; (7) ocimene; (8) 1,8-cineole; (9) cyclohexane; (10) *p*-cymene; (11) benzene; (12) toluene; (13) cyclopentanone; (14) cyclohexanone; (15) methyl alcohol; (16) ethyl alcohol; (17) isopropyl alcohol; (18) longifolene; (19) humulene; and (20) caryophyllene.

Compounds 1 to 7 are monoterpenes and compounds 18 to 20 are sesquiterpenes.

All the samples and the requisite dicarboxylic acids were obtained in a pure state from the laboratory stock and their purity was ascertained by m.p./b.p. determinations, elemental analyses, equivalent weights and also GLC analyses in the case of volatile samples.

TABLE I

MOLECULAR WEIGHTS OF THE POLYESTERS AS DETERMINED BY END GROUP TITRATION

Number of methylene groups in the acidic fragments	*Molecular weights*
1	545, 567
2	1246, 1253
3	344, 336
4	1129, 1180
7	399, 398
8	2166, 2143
9	689, 698
11	968, 947
13	646, 633
14	953, 936
15	866, 875
16	— —

Impregnation and filling the column

The Indian fire-brick support (60–100 mesh) and the polyesters were weighed accurate to a milligram. The polyester was dissolved in methylene chloride, applied to the support and the solvent was removed by careful evaporation at 60°. It was then dried at 110° for several hours to constant weight. The impregnated material was packed in the column in the usual way. In order to have uniformity of filling every time, the same column was used and the identical amount of filling material was taken. The column was tested for gas flow rates with an identical inlet pressure in every case (within the limits of $\pm$ 3 mm pressure of mercury).

In order to compare the retention time- or volume-data from column to column, all the column variables were normalised according to LITTLEWOOD[11]. The following constants were maintained for all the columns:

(1) Column length, 6 ft.
(2) Weight of the column liquid, 2.95 g $\pm$ 0.005 g.
(3) Carrier gas flow rate (hydrogen), 4 l/h.
(4) Inlet pressure, 126 $\pm$ 3 mm of mercury.
(5) Temperature of the column, 80°, 100°, 120° and 162°.
(6) Bridge current, 150 mA.
(7) Sample size, 1 μl.

Retention times were recorded with the help of a stop-watch up to the third place of decimals and rounded off to the nearest second place as a matter of convenience. Several concordant readings were taken in each case.

TABLE IIa

RETENTION TIME IN MIN AT 80°

Compound	*Number of methylene groups in the acidic fragment of the polyester*				
	1	*2*	*3*	*4*	*7*
α-Pinene	1.38	2.73	3.92	5.40	9.67
β-Pinene	2.52	4.83	7.27	9.73	16.90
Δ^3-Carene	3.18	6.18	9.02	12.87	22.03
Limonene	4.57	8.83	13.12	17.83	30.90
Ocimene	6.71	12.53	18.55	24.80	41.73
Tricyclene	1.25	2.40	3.63	4.92	9.13
Camphene	1.73	3.70	5.63	7.50	13.13
Benzene	1.61	2.50	3.10	3.55	4.48
Toluene	2.83	4.52	5.80	7.00	9.35
p-Cymene	9.60	15.70	22.80	29.90	45.30
Cyclohexane	0.20	0.36	0.56	0.77	1.23
Cyclohexanone	26.60	34.00	39.10	41.00	47.70
Cyclopentanone	15.80	20.60	22.00	21.80	23.50
Methyl alcohol	1.77	2.22	2.63	2.36	2.25
Ethyl alcohol	1.85	2.72	3.32	3.03	3.26
Isopropyl alcohol	1.56	2.40	3.20	2.94	3.45
1,8-Cineole	7.60	12.33	18.90	22.10	42.10

TABLE IIb

RETENTION TIME IN MIN AT 100°

Compound	*Number of methylene groups in the acidic fragment of the polyester*							
	1	*2*	*3*	*4*	*7*	*8*	*9*	*11*
α-Pinene	0.75	1.43	2.18	2.75	5.26	6.04	6.73	8.82
β-Pinene	1.34	2.53	4.01	5.13	8.66	9.90	10.92	14.50
Δ^3-Carene	1.70	3.07	4.85	6.66	10.75	12.30	13.58	17.80
Limonene	2.28	4.30	6.71	8.50	14.65	16.30	17.38	24.00
Ocimene	3.08	5.53	8.30	11.10	18.54	20.20	21.50	27.50
Tricyclene	0.70	1.30	2.11	2.73	4.93	5.46	6.52	8.34
Camphene	1.23	1.93	3.17	4.03	7.10	7.82	8.60	11.53
Benzene	0.89	1.40	1.83	1.95	2.60	2.68	2.35	2.81
Toluene	1.48	2.30	3.18	3.81	4.90	5.30	4.75	5.90
p-Cymene	4.25	7.02	10.70	13.40	20.80	22.80	23.10	29.60
Cyclohexane	0.13	0.27	0.38	0.45	0.78	0.92	0.95	1.26
Cyclohexanone	11.40	17.60	18.80	18.80	20.80	21.40	20.60	22.20
Cyclopentanone	8.40	10.10	10.50	10.40	11.50	11.40	10.30	11.50
Methyl alcohol	0.97	1.23	1.48	1.27	1.32	1.15	1.01	1.05
Ethyl alcohol	1.00	1.39	1.80	1.55	1.70	1.53	1.47	1.46
Isopropyl alcohol	0.80	1.32	1.70	1.53	1.80	1.53	1.67	1.70
1,8-Cineole	3.35	5.67	9.40	10.60	18.20	19.60	22.60	25.50

TABLE IIc

RETENTION TIME IN MINUTES AT 120°

Compound	*Number of methylene groups in the acidic fragment of the polyester*							*Apiezon*
	8	*9*	*11*	*13*	*14*	*15*	*16*	
α-Pinene	3.45	3.80	4.60	4.75	4.85	5.23	5.75	9.80
β-Pinene	5.43	5.90	7.45	7.30	7.33	8.00	9.00	11.30
Δ^3-Carene	6.63	7.20	8.75	8.80	8.66	9.63	10.70	16.40
Limonene	8.30	9.10	11.30	11.10	11.33	12.50	13.40	19.40
Ocimene	9.75	10.50	12.90	12.50	13.45	13.36	15.03	18.10
Tricyclene	3.30	3.70	4.48	4.48	4.80	4.95	5.58	9.50
Camphene	4.46	4.90	5.93	5.95	6.55	6.50	7.58	11.80
Benzene	1.66	1.50	1.80	1.60	1.55	1.64	1.80	1.50
Toluene	3.47	2.80	3.86	3.10	3.15	3.20	3.60	3.30
p-Cymene	11.65	11.80	14.53	13.90	13.45	14.30	16.60	18.10
Cyclohexane	0.62	0.63	0.80	0.76	0.83	0.87	0.95	1.60
Cyclohexanone	11.50	11.10	12.20	10.90	11.00	10.63	11.10	6.40
Cyclopentanone	6.10	5.80	6.30	5.60	5.50	5.33	5.60	—
Methyl alcohol	0.66	0.64	0.60	0.54	0.55	0.52	0.50	1.10
Ethyl alcohol	0.90	0.86	0.86	0.76	0.78	0.74	0.70	1.50
Isopropyl alcohol	0.93	0.96	0.95	0.87	0.90	0.87	0.80	1.60
1,8-Cineole	9.75	11.50	12.76	12.60	13.55	13.40	14.10	19.80

RESULTS AND DISCUSSION

Retention times of monoterpenic hydrocarbons, 1,8-cineole, cyclohexane, benzene, toluene, *p*-cymene, cyclopentanone, cyclohexanone, methyl alcohol, ethyl alcohol and isopropyl alcohol were determined under identical conditions for all the polyester substrates. Results are presented in Table II (a, b, c) for the temperatures 80°, 100° and 120°, respectively. The operations were carried out at the different temperatures 80°, 100° and 120° to get good resolution of the different compounds. While examining the behaviour of monoterpenes on polyesters of higher dicarboxylic acids ($n > 7$), low temperature ranges (80–100°) were avoided as the retention time was inordinately long to be of any practical value. Only the temperature of 120° was used.

The arithmetical relationship mentioned previously and the constancy of the value of c at a particular temperature are also valid in this case.

For sesquiterpenic hydrocarbons, only the temperature of 162° was employed and the results are shown in Table III. The arithmetical relationship valid in the case of monoterpenes is also applicable in the case of sesquiterpenes.

The arithmetical pattern of the relationship of the retention time has been further indicated in Table IV, in which the average ratio of the retention time for seven monoterpenes on different polyesters (see Table IIa and IIb for actual values) have been shown against expected values. It will be seen from the tables that the observed and the calculated values agree extremely well.

The value of c which is a constant at a particular temperature for a particular compound and is the individual contribution of a single methylene group, has been calculated according to the formula (I) and tabulated in Tables Va and Vb for the two temperatures 80° and 100°.

TABLE III

RETENTION TIME IN MINUTES AT 162°

Compound	*Number of methylene groups in the acidic fragment of the polyester*		
	2	*7*	*11*
Longifolene	3.61	12.30	20.30
Caryophyllene	3.60	13.30	20.70
Humulene	5.50	17.66	26.95
Nerol	10.16	18.75	19.30
Terpineol	7.75	15.20	16.56
Camphor	5.40	9.25	10.30
Borneol	7.70	14.20	16.16
Linalool	3.40	7.07	7.70

The constant nature of the value of c is apparent from these tables.

CRAIG[12] and RUDLOFF[13] in earlier communications, based on a limited number of polyesters, felt that the number of methylene groups situated between the carboxylic groups might have some significant influence. This has now been fully justified by our present investigation.

The effect of the spacing of the methylene groups on the ester function can be appreciated up to where eleven methylene groups (brassylic acid) are involved in the acidic part of the polyesters, beyond this it seems that the retention time becomes almost constant. This is possibly due to the fact that in polyesters of higher dicarbo-

TABLE IV

RELATIONSHIP BETWEEN THE RATIO OF THE NUMBER OF METHYLENE GROUPS AND THE RATIO OF THE RETENTION TIMES ON THE RESPECTIVE SUBSTRATES

Ratio of the number of methylene groups	*Average ratio of observed retention times*	*Calculated ratio*	*Error (%)*
$^{9}/_{2}$	4.40	4.50	2.23
$^{9}/_{3}$	2.82	3.00	6.00
$^{9}/_{4}$	2.17	2.25	4.47
$^{9}/_{7}$	1.24	1.29	3.55
$^{9}/_{8}$	1.10	1.13	2.65
$^{8}/_{7}$	1.12	1.16	3.57
$^{8}/_{4}$	1.96	2.00	2.00
$^{8}/_{3}$	2.53	2.66	4.89
$^{8}/_{2}$	3.96	4.0	1.00
$^{7}/_{4}$	1.73	1.75	1.37
$^{7}/_{3}$	2.24	2.33	3.98
$^{7}/_{2}$	3.54	3.50	1.17
$^{4}/_{3}$	1.31	1.33	1.90
$^{4}/_{2}$	2.05	2.00	2.70
$^{4}/_{1}$	3.81	4.00	4.87
$^{3}/_{2}$	1.54	1.50	2.30
$^{3}/_{1}$	2.90	3.00	3.20
$^{2}/_{1}$	1.90	2.00	5.00

TABLE Va

EVALUATION OF c AT 80°

$t_R^x - t_R^y$	$x - y$	c	$t_R^x - t_R^y$	$x - y$	c
α-Pinene			*β-Pinene*		
8.29	7 — 1 = 6	1.38	14.38	7 — 1 = 6	2.39
6.94	7 — 2 = 5	1.39	12.07	7 — 2 = 5	2.41
5.75	7 — 3 = 4	1.44	9.63	7 — 3 = 4	2.40
4.27	7 — 4 = 3	1.42	7.17	7 — 4 = 3	2.39
Δ³-Carene			*Limonene*		
18.85	7 — 1 = 6	3.14	26.33	7 — 1 = 6	4.39
15.65	7 — 2 = 5	3.13	22.07	7 — 2 = 5	4.41
13.01	7 — 3 = 4	3.25	17.78	7 — 3 = 4	4.44
9.16	7 — 4 = 3	3.05	13.07	7 — 4 = 3	4.36
Ocimene			*Tricyclene*		
35.02	7 — 1 = 6	5.84	7.88	7 — 1 = 6	1.31
29.20	7 — 2 = 5	5.84	6.73	7 — 2 = 5	1.35
23.18	7 — 3 = 4	5.79	5.50	7 — 3 = 4	1.37
16.93	7 — 4 = 3	5.64	4.21	7 — 4 = 3	1.40
Camphene			*Cyclohexane*		
11.4	7 — 1 = 6	1.90	1.03	7 — 1 = 6	0.17
9.43	7 — 2 = 5	1.89	0.97	7 — 2 = 5	0.19
7.50	7 — 3 = 4	1.87	0.67	7 — 3 = 4	0.17
5.63	7 — 4 = 3	1.88	0.46	7 — 4 = 3	0.15

TABLE Vb

EVALUATION OF c AT 100°

$t_R^x - t_R^y$	$x - y$	c	$t_R^x - t_R^y$	$x - y$	c
α-Pinene			*β-Pinene*		
5.98	9 — 1 = 8	0.75	9.6	9 — 1 = 8	1.20
5.3	9 — 2 = 7	0.76	8.49	9 — 2 = 7	1.21
4.55	9 — 3 = 6	0.76	6.91	9 — 3 = 6	1.15
3.98	9 — 4 = 5	0.79	5.79	9 — 4 = 5	1.16
Δ³-Carene			*Limonene*		
11.88	9 — 1 = 8	1.49	15.1	9 — 1 = 8	1.90
10.51	9 — 2 = 7	1.50	13.08	9 — 2 = 7	1.87
8.73	9 — 3 = 6	1.46	10.67	9 — 3 = 6	1.78
6.92	9 — 4 = 5	1.40	8.88	9 — 4 = 5	1.78
Ocimene			*Tricyclene*		
18.42	9 — 1 = 8	2.30	5.82	9 — 1 = 8	0.73
15.97	9 — 2 = 7	2.28	5.22	9 — 2 = 7	0.75
13.20	9 — 3 = 6	2.20	4.41	9 — 3 = 6	0.74
10.4	9 — 4 = 5	2.08	3.79	9 — 4 = 5	0.76
Camphene			*Cyclohexane*		
7.37	9 — 1 = 8	0.92	0.82	9 — 1 = 8	0.103
6.67	9 — 2 = 7	0.95	0.68	9 — 2 = 7	0.097
5.44	9 — 3 = 6	0.91	0.57	9 — 3 = 6	0.095
4.57	9 — 4 = 5	0.92	0.50	9 — 4 = 5	0.100

xylic acids the relative proportion of the oxygen function is greatly reduced, as a consequence of which the resulting polyester behaves increasingly like a hydrocarbon phase.

Even then, the higher polyesters still show a marked difference in their behaviour from an "Apiezon" phase (compare the retention times on these polyesters with the retention time on "Apiezon", as shown in Table IIc). In some cases, the order of the compounds emerging is altered.

The spacing of these ester groups has no marked effect on the separation factor of the hydrocarbons having different structural features.

The relationship, however, deviates slightly in the case of aromatic hydrocarbons and is not applicable to oxygenated substances. The total contribution in the case of the latter is of the order of 1:2 (Table II a, b and c). The results can very well be explained in the following manner.

As the carbon content of the polyester goes on increasing with the increase of the intervening methylene groups in the acidic component, the solubility of the hydrocarbons also increases, while that for oxygenated substances may not change appreciably. Experimental values of these solubility ratios for the hydrocarbons agree well with the theoretical interpretation based on the number of methylene groups in the acidic fragment of the polyester.

This fact can be utilised practically for the analyses of essential oils. It has been noted by earlier workers[14,15] that monoterpenic oxygenated compounds often overlap sesquiterpene hydrocarbons on normal substrates. By using appropriate higher polyesters, separation of such mixtures can be achieved practically. Table III presents the retention time data under nearly identical conditions for a number of typical monoterpenic oxygenated compounds and sesquiterpenic hydrocarbons.

The concept of resolving power technique[16] has been used and the resolving powers for two of our typical polyesters are noted in Table VI. These data and the chromatogram (Fig. 1) illustrate the utility of higher polyesters.

BERNHARD in his study[17] of the terpenic hydrocarbons on capillary columns with different phases has observed that an adipic polyester column has a better separation factor as compared with "Apiezon" or silicone columns, in spite of the low number of theoretical plates.

In our study of these polyesters, it has been seen that although the retention time goes on increasing with the increase in the number of methylene groups, the

TABLE VI

RESOLVING POWERS FOR TWO TYPICAL POLYESTERS $n = 7$ AND $n = 11$

Numbers of methylene groups in the acidic fragment of the polyester	*Compound pair*	$y_2 - y_1$ *in mm*	$\frac{y_2 + y_1}{2}$ *in mm*	*Resolving power* $y_2 - y_1 \Big/ \frac{y_2 + y_1}{2}$
7	Caryophyllene–camphor	10.00	29.0	0.3448
7	Humulene–borneol	9.00	40.50	0.2222
11	Caryophyllene–camphor	26.00	40.00	0.6500
11	Humulene–borneol	28.00	55.00	0.5090

separation factor remains the same. If these higher polyesters are used in capillary columns, it may be possible that the theoretical plate number of the column will increase many fold without any loss of resolution.

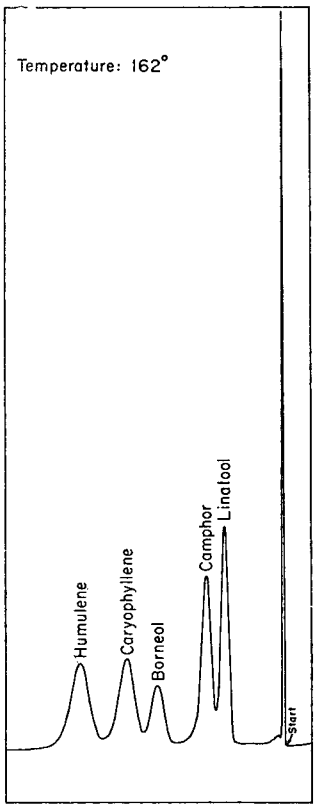

Fig. 1.

One more interesting application, though somewhat indirect, may be mentioned. In the symposium of 1957[18], the point was discussed regarding the identification of substances by using them as GLC substrates. The investigation carried out above shows that 1,ω-dicarboxylic acids can be identified with considerable accuracy by preparing a polyester of the unknown acid and running the column for some hydrocarbons under standard conditions.

It has been observed that unsaturation in the acid fraction of the polyester or a methyl group side chain does not appreciably change the retention time as compared to the difference between saturated and unbranched acid polyesters.

SUMMARY

Polyesters from different 1,ω-dicarboxylic acids $HOOC\cdot(CH_2)_n\cdot COOH$ ranging from malonic acid ($n = 1$) to hexadecane-1,16-dicarboxylic acid ($n = 16$) with diethylene glycol were prepared and evaluated as substrates for gas–liquid

chromatography. Several terpenic and non-terpenic compounds were analysed. It was observed that the number of methylene groups in the acid fragment of the polyester has a profound influence on the retention time, on the basis of which an interesting mathematical relationship has been developed. The retention time appears to be a characteristic of the number of methylene groups between the two carboxyl groups and the contribution of a single methylene group to the retention time of a particular compound at a particular temperature has been found to be a constant. This relationship holds good for aliphatic and alicyclic hydrocarbons and only slightly deviates for aromatic hydrocarbons.

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CHROMATOSTRIP ISOMERIZATION OF TERPENES*

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INTRODUCTION AND LITERATURE REVIEW

The possibility of the isomerization of terpene hydrocarbons on silicic acid has interested chemists for several years, and the literature is sometimes contradictory on this subject. ARBUZOV AND ISAEVA[1] reported that α-pinene, 3-carene and dipentene undergo isomerization when passed through a silica gel column at room temperature. RUTOVSKII AND LYUBOMILOV[2] found that neither silica gel nor aluminum sulfate alone had any appreciable effect on the isomerization of pinene to camphene. The determining factor for catalytic activity was reported to be the deposition of $Al_2(SO_4)_3$ on the SiO_2 carrier surface. RUDAKOV AND SHESTAEVA[3] used the isomerization of α-pinene as a system in studying the characteristics of a variety of silica gels. The industrial silica gels which the Russian workers examined catalyzed the isomerization of α-pinene even at 20°. These gels contained 1–10 % Al_2O_3. Silica gel freed from Al_2O_3 by the hydrolysis of $SiCl_4$ did not catalyze the isomerization at room temperature or at 100°. Their studies indicated that the activity of silica gels containing aluminum was due to the formation of alumino-silicate on the silica gel surface. HUNTER AND BROGDEN[4] made a detailed study of the isomerization and disproportionation of limonene with silica gel at 100° and 150°. Limonene initially isomerized to α-terpinene, γ-terpinene, terpinolene and isoterpinolene which subsequently disproportionated into 1-*p*-menthene, *trans*-2-*p*-menthene, 3-*p*-menthene, *trans*-8(9)-*p*-menthene and *p*-cymene. Polymerization occurred, as well as isomerization and disproportionation.

In studies of the volatiles of black pepper in the authors' laboratory, the monoterpene hydrocarbons were isolated by vacuum distillation and by thin-layer chromatography. The chromatostrip technique of STANLEY, IKEDA AND COOK[5] was used. It has advantages in that it is rapid, and that analyses can be run on minute quantities of material. With pepper oil, however, the material recovered by thin-layer chromatography possessed α-terpinene besides the compounds present in the vacuum distillate, and greater amounts of γ-terpinene, terpinolene and *p*-cymene. The compounds present in pepper oil were studied individually in an effort to determine what compounds were responsible for the observed changes. In an effort to elucidate the mechanism of sabinene isomerization with silicic acid the reaction was examined in more detail.

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EXPERIMENTAL METHODS AND PROCEDURES

Preparation of chromatostrips

The chromatostrips were prepared according to the procedure of KIRCHNER, MILLER AND KELLER[6]. "Neutral" chromatostrips were prepared by using 0.1 *N* NaOH in place of distilled water. The chromatostrips used for detecting oxygenated compounds or polymers were prepared by the same procedure except that the starch binder was omitted. Organic compounds were detected by spraying the strips with concentrated sulfuric acid and heating on a hot plate.

Analytical reagent-grade 100-mesh silicic acid was obtained from Mallinckrodt Chemical Works, N.Y. Reagent-grade silicic acid powder was purchased from Allied Chemical and Dye Corporation, N.Y. Silica gel for thin-layer chromatography was purchased from Research Specialties Co., Richmond, Calif. The silica gel was in some instances treated by a method described by MANGOLD[7] for removal of ferric ion.

Procedure for the chromatostrip isomerization experiment

The procedure described by STANLEY, IKEDA AND COOK[5] for isolating the monoterpene hydrocarbons by downward elution of chromatostrips was used. Samples of α-pinene, α-thujene, β-pinene, sabinene, 3-carene, myrcene, α-phellandrene, α-terpinene, limonene, β-phellandrene, γ-terpinene, *p*-cymene and terpinolene were analyzed. 1–10 μl of the terpene hydrocarbon was diluted with 50 μl of heptane solvent, and 25 μl of this solution was applied to the chromatostrip. The remaining solution was used as a control, and exposed to the same conditions of temperature and light as the sample on the thin-layer strip. 1 μl of the eluant was examined by gas chromatography and compared to the control. Tailing of the solvent sometimes made it difficult to calculate the percentage composition. In such instances, the majority of the solvent was removed from the 2-ml eluant by evaporation, aided by passing nitrogen gas over the surface of the sample.

Sources of terpene hydrocarbons

α-Pinene, β-pinene, *d*-limonene, α-phellandrene and *p*-cymene were purchased from commercial companies and purified by gas chromatography. The sources for the other terpenes studied are reported in another publication[8].

Gas chromatography

The starting materials, reaction products, and control samples were analyzed by gas chromatography, using an Aerograph Hy-fi with a flame ionization detector. It was fitted with a 10-ft. × 1/8 in. stainless-steel column packed with 40–60-mesh HMDS-treated Chromosorb P coated with 15 % Carbowax 20M. Operating conditions were 95° and N_2 and H_2 flow rates of 20 ml/min. A Beckman Thermotrac was also used, in conjunction with a Carle thermistor detector. It utilized a 23-ft. × 1/8-in. stainless-steel column packed with 20 % Apiezon L on Gas-Pack F.

Percent composition was estimated from the chromatograms by calculating the percentage of the total peak area.

Procedure for the sabinene–silica gel isomerization experiment

A slurry of sabinene and silica gel in a 2-ml conical test tube was stoppered and

held in a 25° water bath. The starting material was 98 % sabinene, with impurities of 1 % myrcene and less than 1 % quantities of α- and γ-terpinene. The silica gel used was Fisher Cat. No. S-157, 28–200 mesh, suitable for chromatography. Samples were withdrawn with a syringe and injected on the gas chromatograph every 2 h for the first 10 h. Analyses were also made at 14, 22, 26, 48 and 74 h.

At the end of the experiment, the presence of oxidized and/or polymerized compounds was checked by ascending thin-layer chromatography in heptane solvent.

RESULTS AND DISCUSSION

Chromatostrip isomerization experiment

α-Thujene, α-pinene, β-pinene, myrcene, 3-carene, limonene, γ-terpinene and *p*-cymene were found to chromatograph without change. The β-phellandrene sample contained small amounts of 1,8-cineole, α-phellandrene, γ-terpinene and *p*-cymene. 1,8-Cineole would not be expected to elute from the chromatostrip, because it is oxygenated. The chromatostrip eluant contained no 1,8-cineole, and 1 % increases of α-phellandrene, γ-terpinene and *p*-cymene. These increased concentrations probably resulted from abstraction of the 1,8-cineole, rather than isomerization of β-phellandrene.

The terpinolene sample contained 7 % *p*-cymene and 1 % limonene. If isomerization of terpinolene to either of those compounds occurred, it did so in quantities of less than 1 %.

The products arising from the chromatostrip isomerization of sabinene are

TABLE I

CHROMATOSTRIP ISOMERIZATION OF SABINENE

Experiment	*Starting material*	*Product*	*Percentage*
A	Sabinene (99 %)	Sabinene	47
Normal chromatostrip	Myrcene (1 %)	Myrcene	1
	α-Thujene 1 %)	α-Thujene	7
		α-Terpinene	13
		Limonene	2
		γ-Terpinene	26
		Terpinolene	6
B	Sabinene (98 %)	Sabinene	40
Normal chromatostrip	Myrcene (1 %)	Myrcene	2
	α-Thujene (1 %)	α-Thujene	3
		α-Terpinene	14
		Limonene	1
		β-Phellandrene	2
		γ-Terpinene	31
		Terpinolene	7
C	Sabinene (98 %)	Sabinene	93
"Neutral" chromatostrip	Myrcene (1 %)	Myrcene	2
	α-Thujene (1 %)	α-Thujene	1
		α-Terpinene	1
		γ-Terpinene	2
		Terpinolene	1

shown in Table I. Experiments A and B were run on different days. The differences in product composition could possibly be attributed to differences in the activity of the chromatostrips, since the strips were not of the same lot, or to differences in room temperature. The most striking difference is the presence of β-phellandrene in experiment B. Experiments B and C were run at the same time on identical samples. The chromatostrips were prepared the same day from identical materials except that 0.1 *N* NaOH was employed in place of distilled water in making the "neutral" chromatostrip. The greatly reduced isomerization of sabinene on the "neutral" chromatostrip supported the idea that the mechanism observed in experiments A and B is an acid-catalyzed isomerization of sabinene. The isomerization in experiment C could be ascribed to either acid or base catalysis. The unreactive impurity myrcene served as an internal standard and indicated the possibility that polymerization and/or oxidation reactions occurred, as well as isomerization. There was no indication of polymerization or oxidation when chromatostrips were sprayed with sulfuric acid and charred on a hot plate.

Fig. 1 is a scheme to account for the products obtained in the chromatostrip

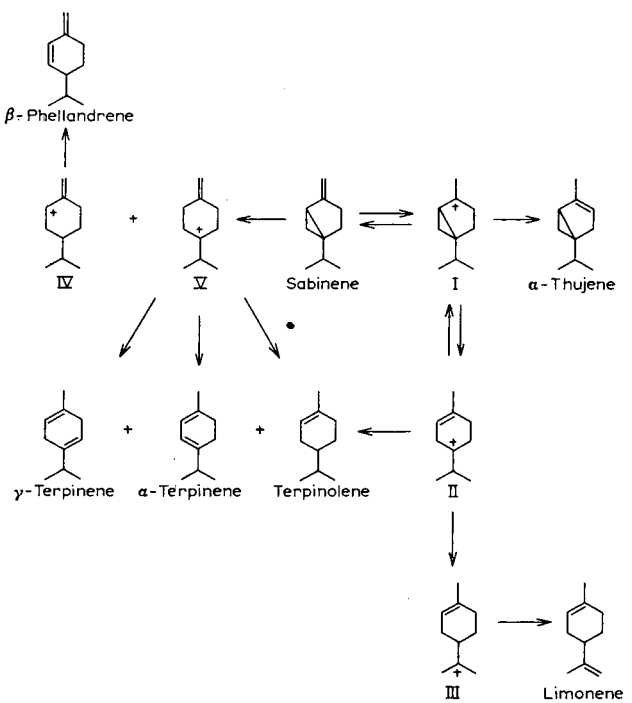

Fig. 1. Mechanism for chromatostrip isomerization of sabinene.

isomerization of sabinene. Attack of a hydrogen ion on the exocyclic double bond could result in the formation of a cyclopropylcarbonyl system (I). Loss of a hydrogen ion would lead to α-thujene, while breakage of the 3-membered ring could result in another tertiary carbonium ion (II). Dehydrogenation of II would result in the formation of terpinolene, γ-terpinene and α-terpinene. Hydrogen transfer from the

number 8 to the number 4 carbon of carbonium ion II results in the formation of carbonium ion III, from which limonene and terpinolene can be formed. β-Phellandrene was formed either in small amounts or not at all. Its presence could possibly result from breakage of the 3-membered ring of sabinene to form two carbonium ions (IV and V). Loss of hydrogen from IV would lead to β-phellandrene, while dehydrogenation of V accompanied by isomerization of the 1(7) double bond would lead to α-terpinene, γ-terpinene and terpinolene. β-Phellandrene probably does not isomerize further under these conditions, because of its more stable conjugated configuration.

Both the sample of α-phellandrene eluted from the chromatostrip and the control contained *p*-cymene. This oxidation product, however, was not detected in the starting material. Its formation amounted to 1 % of the chromatostrip eluant and less than 1 % of the control. α-Terpinene, which is also a conjugated endocyclic diene, reacted similarly. The sample of α-terpinene was only 60 % pure, 40 % of the mixture being limonene. *p*-Cymene was present in the eluant from the chromatostrip in quantities of 1 %, and was also detected in the control in quantities of less than 1 %.

The isomerization of black pepper oil observed in isolation of the monoterpene hydrocarbons by thin-layer chromatography can be accounted for by the presence of sabinene and α-phellandrene in pepper oil. α-Terpinene and increased amounts of α-terpinene and terpinolene would result from the isomerization of sabinene, and increased amounts of *p*-cymene from the isomerization of α-phellandrene.

Sabinene–silica gel isomerization experiment

The reaction scheme given for the isomerization of sabinene on the chromatostrip (Fig. 1) can be used to account for the products of silica gel isomerization. In addition, α-phellandrene could arise from isomerization of β-phellandrene, and *p*-cymene could be formed from the oxidation of α-terpinene and α-phellandrene.

Fig. 2 plots the percentage of monoterpene hydrocarbons present in the reaction mixture during the experiment. The decrease of sabinene is not shown.

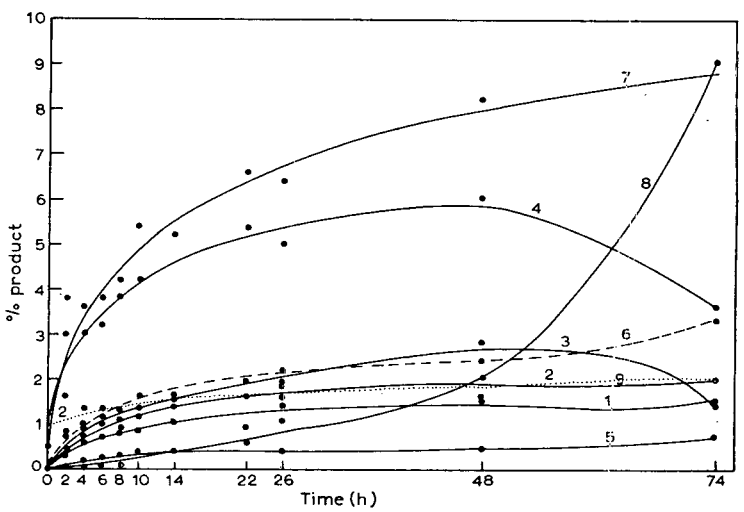

Fig. 2. Silica gel isomerization of sabinene. 1 = α-thujene; 2 = myrcene; 3 = α-phellandrene; 4 = α-terpinene; 5 = limonene; 6 = β-phellandrene; 7 = γ-terpinene; 8 = *p*-cymene; 9 = terpinolene.

The quantity of myrcene increased slightly. It was thought that myrcene might result not from isomerization, but from oxidation or polymerization of sabinene. Thin-layer chromatography of the reaction product substantiated this idea. The relative intensity of the charred spots on the chromatostrip indicated that as much as half of the reaction product could be higher-boiling material.

α-Thujene, limonene, β-phellandrene, γ-terpinene and terpinolene exhibit similarly shaped concentration curves. Initially, these products are formed quite rapidly; their rate of formation declines but their concentrations continue to increase throughout the experiment.

α-Phellandrene and α-terpinene, both conjugated endocyclic dienes, show marked decreases in concentration at the end of the experiment. *p*-Cymene is present in small quantities during the first part of the experiment and increases rapidly during the latter stages. This large increase of *p*-cymene can be accounted for by the decrease of α-phellandrene and α-terpinene. The oxidation of both these compounds to *p*-cymene observed in the chromatostrip experiment would support the possibility of this occurrence.

It is interesting that α-terpinene and not γ-terpinene appears to undergo dehydrogenation to *p*-cymene. If the oxidation mechanism shown in Fig. 3 is correct,

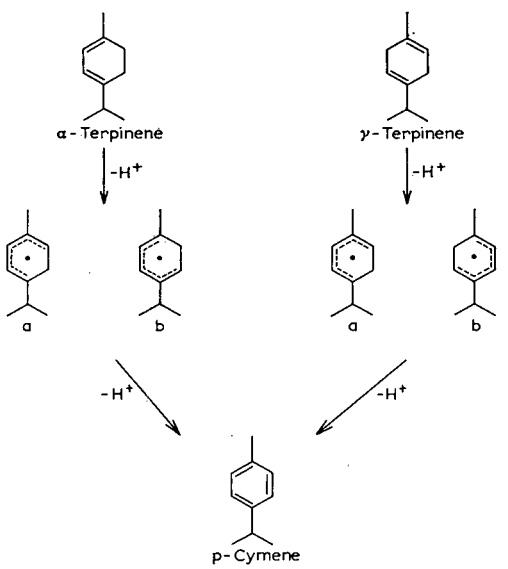

Fig. 3. Oxidation of α- and γ-terpinene.

both compounds would lead to identical intermediates. The conformation of α-terpinene must be such that the transition state a, b is more easily reached. One would not expect γ-terpinene to be more stable than the conjugated isomer α-terpinene. BATES, CARNIGHAN AND STAPLES[9] studied the relative stabilities of two analogous compounds 1,3- and 1,4-cyclohexadiene. They reported that the relative stability of the two compounds was essentially the same, the conjugated isomer being only 0.07 kcal/mole more stable than the unconjugated isomer. It was proposed that an interaction occurred between the double bonds of 1,4-cyclohexadiene, resulting in resonance

stabilization. In the most stable configuration of 1,4-cyclohexadiene as suggested by HERBSTEIN[10], the π-electrons of the two double bonds are quite close on one side of the ring, allowing for such an interaction.

The formation of larger amounts of γ-terpinene than of α-terpinene does not indicate that γ-terpinene is thermodynamically more stable. The two compounds are not existing under equilibrium conditions. It must be remembered that unidentified oxygenated and/or polymerization products were formed. The possibility cannot be overlooked that α-terpinene takes part in such reactions.

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SUMMARY

Isomerization of the monoterpene hydrocarbons of black pepper oil occurred during isolation by thin-layer chromatography. α-Thujene, α-pinene, β-pinene, myrcene, 3-carene, limonene, β-phellandrene, γ-terpinene, terpinolene and p-cymene were each eluted from chromatostrips and were all found to chromatograph without change. Sabinene isomerized to α-thujene, α-terpinene, γ-terpinene, limonene, β-phellandrene and terpinolene when so treated. A possible mechanism for this reaction is discussed. Oxidation of α-terpinene and α-phellandrene to p-cymene occurred both in the control samples and on chromatostrips.

In the silica-gel-induced isomerization of sabinene, α-phellandrene and p-cymene were formed in addition to the chromatostrip isomerization products. The results indicated that p-cymene was formed from α-terpinene and α-phellandrene.

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SEPARATION OF 2,4-DINITROPHENYLHYDRAZONES OF CARBONYL COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

When carrying out a study on the radiolysis of cyclohexanone, the need for separating and identifying some carbonyl compounds was recognized.

Various methods for separating and identifying aldehydes and ketones by paper, column or thin-layer chromatography are already known from the literature. The last-mentioned technique seems to be the most interesting, being simple and rapid.

Some authors[1-8] have already described separations of 2,4-dinitrophenylhydrazones (2,4-DNPH) of carbonyl compounds by the technique of STAHL[9]: however, many data on the compounds studied by us, and on the separation of different 2,4-DNPH mixtures obtained in our above-mentioned study, are still lacking.

The problem has been solved by thin-layer chromatography using silica gel and alumina (in some cases with $AgNO_3$) according to the technique described by BARRET, DALLAS AND PADLEY[10].

METHODS

Preparation of the 2,4-DNPH

The 2,4-DNPH were prepared by the method described by BRADY[11] and recrystallized twice from methanol.

Adsorbents

Layers of silica gel G* (I), alumina G* (II), silica gel G* + 25 % $AgNO_3$ (III) and alumina G* + 25 % $AgNO_3$ (IV) were employed.

Layer preparation

Glass plates, 20 × 20 cm, were used. The different layers, 300 μ thick, were prepared with the "Stratomat" apparatus (Chemetron, Milan).

The plates were subsequently treated as follows:

(*a*) *Layers of I and III.* After drying in air for 5 min, the plates were dried for 30 min in an oven at 110° and stored in a dry-box over $CaCl_2$ before use. The plates with layers containing $AgNO_3$ (III) have to be kept in the dark.

(*b*) *Layers of II and IV.* After drying for 5 min in air the plates were dried for 30 min in an oven at 110° and subsequently deactivated for 12 h by exposing them

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to air at room temperature (~ 60 % relative humidity). The plates carrying a layer containing $AgNO_3$ (IV) were kept in the dark.

Solvents

All the solvents used were pure compounds, chromatography grade.

For the separations mentioned in this paper the following mixtures proved to be the most useful:

(I) benzene–petroleum ether (b.p. 40–70°) (60:40),
(II) chloroform–petroleum ether (b.p. 40–70°) (75:25),
(III) benzene–*n*-hexane (50:50),
(IV) cyclohexane–nitrobenzene–petroleum ether (b.p. 40–70°) (30:15:10).

Development

The plates were placed in a Shandon chamber (type 2842) and developed up to about 14 cm from the starting point. The temperature during development was 18–22°.

RESULTS AND DISCUSSION

Since the R_F values are dependent on the adsorbent and its activity, as well as the eluant, special care was taken to standardize the techniques for layer deposition and activation.

Nevertheless the R_F values obtained show some variations, and the reproducibility of the results is better when the data are defined as a function of the R_F for a 2,4-DNPH of a compound present in the mixtures which is taken as a standard.

The results reported here are relative values, with the R_F of formaldehyde-2,4-DNPH taken as unity.

In Tables I–IV, instead of the R_F values, the R_{for} values are tabulated, where

$$R_{for} = \frac{R_F \text{ of the 2,4-DNPH of the compound listed}}{R_F \text{ of formaldehyde-2,4-DNPH}}$$

Good separations are possible for all the compounds considered by choosing the best combinations of solvents and substrates.

Figs. 1–3 show some separations of 2,4-DNPH mixtures from various aldehydes and ketones.

In the preparation of the 2,4-DNPH from carbonyl compounds in mixtures of varying composition, recrystallization was avoided, in order to avoid losing some of the more soluble 2,4-DNPH. In these mixtures some free 2,4-dinitrophenylhydrazine is present, as can be seen in Figs. 2 and 3.

Figs. 2 and 3 are chromatograms on adsorbents containing $AgNO_3$. Sharp black spots may be obtained in this case if the plates are exposed to diffuse (not direct) daylight for about 12 h. Spots containing about 0.2 γ of 2,4-DNPH are easily detectable.

By spraying the plates with a 2 % solution of NaOH in 90 % ethanol, the spots are seen more easily. In our experiments the spots changed from yellow to a more or less dark brown.

TABLE I

THIN-LAYER CHROMATOGRAPHY ON SILICA GEL G OF SOME 2,4-DNPH DERIVATIVES

2,4-DNPH from	R_{for} *(average from 5 determinations)*			
	*I**	*II*	*III*	*IV*
Formaldehyde	(1)	(1)	(1)	(1)
Propionaldehyde	1.38	1.36	1.37	1.72
Butyraldehyde	1.68	1.41	1.55	2.10
Isobutyraldehyde	1.90	1.63	2.10	2.24
Methyl ethyl ketone	1.50	1.46	1.62	1.95
n-Valeraldehyde	1.92	1.60	2.05	2.41
3-Pentanone	2.00	1.51	2.14	2.34
2-Pentanone	1.76	1.41	1.81	2.25
Cyclopentanone	0.88	1.03	0.98	1.53
n-Caproaldehyde	2.36	1.74	2.22	2.58
α-Methyl-*n*-valeraldehyde	0.84	0.98	0.90	1.43
4-Methyl-2-pentanone	1.95	1.57	2.05	2.61
2-Hexenal	2.18	1.49	2.08	2.28
5-Hexen-2-one	1.72	1.42	1.67	2.12
Cyclohexanone	1.14	1.44	1.16	2.35
Oenanthaldehyde	2.68	1.79	2.38	2.86
Benzaldehyde	1.85	1.39	1.90	1.65

* The numbers refer to the solvents used.

TABLE II

THIN-LAYER CHROMATOGRAPHY ON ALUMINA G OF SOME 2,4-DNPH DERIVATIVES

2,4-DNPH from	R_{for} *(average from 5 determinations)*			
	*I**	*II*	*III*	*IV*
Formaldehyde	(1)	solvent front	(1)	(1)
Propionaldehyde	1.23	solvent front	1.33	1.17
Butyraldehyde	1.36	solvent front	1.47	1.21
Isobutyraldehyde	1.46	solvent front	1.71	solvent front
Methyl ethyl ketone	1.46	solvent front	1.64	solvent front
n-Valeraldehyde	1.71	solvent front	1.99	solvent front
3-Pentanone	1.67	solvent front	1.95	solvent front
2-Pentanone	1.55	solvent front	1.70	solvent front
Cyclopentanone	1.20	solvent front	1.18	1.39
n-Caproaldehyde	1.54	solvent front	1.68	solvent front
α-Methyl-*n*-valeraldehyde	1.16	solvent front	1.26	1.21
4-Methyl-2-pentanone	1.68	solvent front	1.91	solvent front
2-Hexenal	1.40	solvent front	1.47	solvent front
5-Hexen-2-one	1.46	solvent front	1.54	solvent front
Cyclohexanone	1.34	solvent front	1.25	solvent front
Oenanthaldehyde	1.57	solvent front	1.79	solvent front
Benzaldehyde	0.96	solvent front	0.93	solvent front

* The numbers refer to the solvents used.

TABLE III

THIN-LAYER CHROMATOGRAPHY ON SILICA GEL G + 25% $AgNO_3$ OF SOME 2,4-DNPH DERIVATIVES

2,4-DNPH from	R_{for} *(average from 5 determinations)*			
	*I**	*II*	*III*	*IV*
Formaldehyde	(1)	(1)	(1)	(1)
Propionaldehyde	1.46	1.23	1.73	1.49
Butyraldehyde	1.81	1.22	2.11	1.92
Isobutyraldehyde	1.96	1.60	2.48	2.09
Methyl ethyl ketone	1.43	1.01	1.77	1.51
n-Valeraldehyde	1.90	1.45	2.21	2.17
3-Pentanone	1.83	1.20	2.40	1.92
2-Pentanone	1.73	1.04	1.95	1.84
Cyclopentanone	0.90	0.47	1.03	1.11
n-Caproaldehyde	2.13	1.57	2.49	solvent front
α-Methyl-*n*-valeraldehyde	0.78	0.64	0.99	0.86
4-Methyl-2-pentanone	2.12	1.07	2.17	2.09
2-Hexenal	1.90	1.16	2.25	1.98
5-Hexen-2-one	0.18	0.20	0.26	0.14
Cyclohexanone	0.98	0.35	1.24	1.15
Oenanthaldehyde	2.46	1.74	2.47	solvent front
Benzaldehyde	1.75	1.10	1.74	1.46

* The numbers refer to the solvents used.

TABLE IV

THIN-LAYER CHROMATOGRAPHY ON ALUMINA G + 25% $AgNO_3$ OF SOME 2,4-DNPH DERIVATIVES

2,4-DNPH from	R_{for} *(average from 5 determinations)*			
	*I**	*II*	*III*	*IV*
Formaldehyde	(1)	(1)	(1)	(1)
Propionaldehyde	1.10	1.25	1.20	1.08
Butyraldehyde	1.14	1.10	1.26	1.12
Isobutyraldehyde	1.19	1.40	1.26	1.19
Methyl ethyl ketone	1.12	0.65	1.17	1.08
n-Valeraldehyde	1.18	1.05	1.47	1.17
3-Pentanone	1.22	0.74	1.53	1.10
2-Pentanone	1.19	0.56	1.41	1.06
Cyclopentanone	0.86	0.30	0.75	0.75
n-Caproaldehyde	1.19	1.08	1.37	1.20
α-Methyl-*n*-valeraldehyde	0.85	0.35	0.87	0.77
4-Methyl-2-pentanone	1.20	0.53	1.35	1.13
2-Hexenal	1.11	0.53	1.25	1.10
5-Hexen-2-one	0.20	0.03	0.13	0.03
Cyclohexanone	0.74	0.15	0.70	0.69
Oenanthaldehyde	1.19	1.20	1.33	1.23
Benzaldehyde	0.95	0.47	0.90	0.99

* The numbers refer to the solvents used.

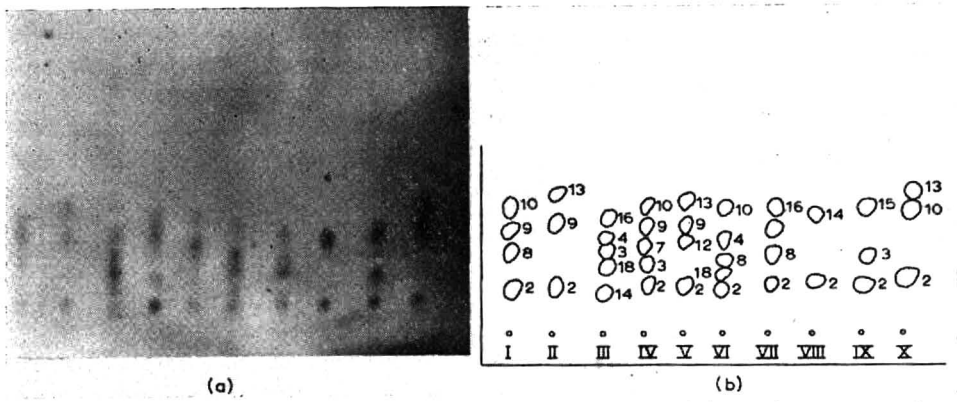

Fig. 1. Separation of 2,4-DNPH mixtures. Adsorbent: silica gel G. Solvent: benzene–petroleum ether (b.p. 40–70°) (60:40). (b). (I) Mixture of 2,4-DNPH from: formaldehyde (2), methyl ethyl ketone (8), 3-pentanone (9), *n*-caproaldehyde (10). (II) Mixture of 2,4-DNPH from: formaldehyde (2), 3-pentanone (9), oenanthaldehyde (13). (III) Mixture of 2,4-DNPH from: cyclopentanone (15), cyclohexanone (18), propionaldehyde (3), butyraldehyde (4). (IV) Mixture of 2,4-DNPH from: formaldehyde (2), propionaldehyde (3), 2-pentanone (7), 3-pentanone (9), *n*-caproaldehyde (10). (V) Mixture of 2,4-DNPH from: formaldehyde (2), isobutyraldehyde (12), 3-pentanone (9), oenanthaldehyde (13). (VI) Mixture of 2,4-DNPH from: formaldehyde (2), cyclohexanone (18), methyl ethyl ketone (8), butyraldehyde (4), *n*-caproaldehyde (10). (VII) Mixture of 2,4-DNPH from: formaldehyde (2), methyl ethyl ketone (8), 5-hexen-2-one (17), 2-hexenal (16). (VIII) Mixture of 2,4-DNPH from: formaldehyde (2), *n*-valeraldehyde (14). (IX) Mixture of 2,4-DNPH from: formaldehyde (2), propionaldehyde (3), 2-hexenal (16). (X) Mixture of 2,4-DNPH from: formaldehyde (2), *n*-caproaldehyde (10), oenanthaldehyde (13).

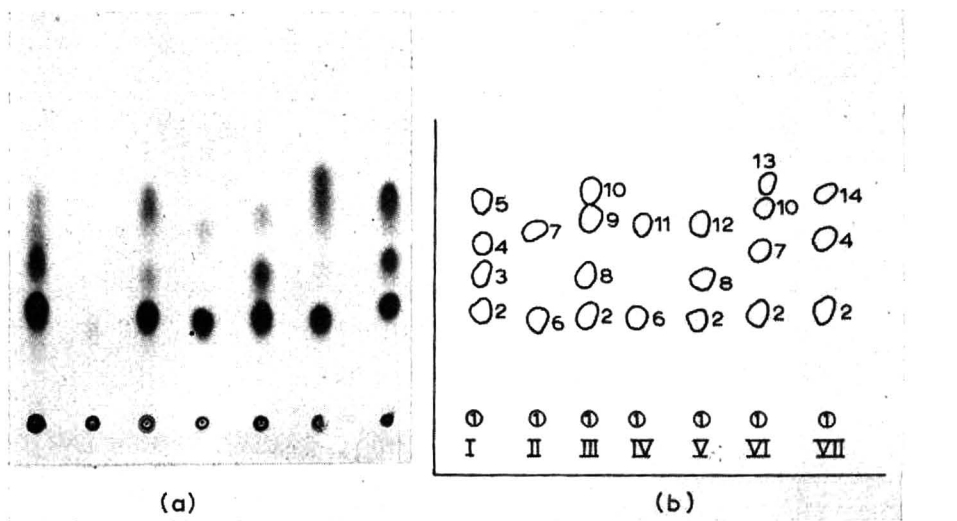

Fig. 2. Separation of 2,4-DNPH mixtures. Adsorbent: silica gel G + 25% $AgNO_3$. Solvent: benzene–petroleum ether (b.p. 40–70°) (60:40). (b). (I) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), propionaldehyde (3), butyraldehyde (4), 4-methyl-2-pentanone (5). (II) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), methyl ethyl ketone (8), 3-pentanone (9), 2-hexenal (10). (IV) Mixture of 2,4-DNPH (1), 2,4-DNPH from: α-methyl-*n*-valeraldehyde (6), benzaldehyde (11). (V) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), methyl ethyl ketone (8), isobutyraldehyde (12). (VI) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), *n*-caproaldehyde (10), oenanthaldehyde (13). (VII) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), butyraldehyde (4), *n*-valeraldehyde (14).

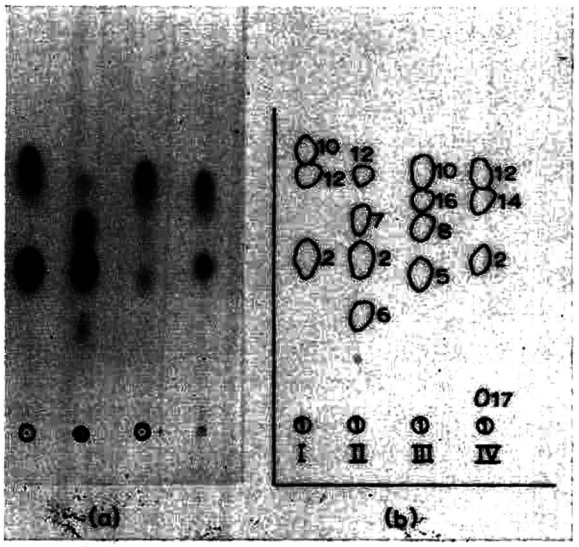

Fig. 3. Separation of 2,4-DNPH mixtures. Adsorbent: silica gel G + 25% $AgNO_3$. Solvent: benzene–petroleum ether (b.p. 40–70°) (60:40). (b). (I) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), isobutyraldehyde (12), *n*-caproaldehyde (10). (II) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), α-methyl-*n*-valeraldehyde (6), 2-pentanone (7), isobutyraldehyde (12). (III) Mixture of 2,4-DNPH (1), 2,4-DNPH from: cyclopentanone (15), methyl ethyl ketone (8), 2-hexenal (16), *n*-caproaldehyde (10). (IV) Mixture of 2,4-DNPH (1), 2,4-DNPH from: formaldehyde (2), *n*-valeraldehyde (14), isobutyraldehyde (12).

The development times of the chromatograms are usually quite short. Good separations of the compounds mentioned here were carried out in 50–150 min, the time being different for the various solvents.

SUMMARY

A method is described for the separation of 2,4-dinitrophenylhydrazones (2,4-DNPH) of aldehydes and ketones by thin-layer chromatography.

The R_F values of various 2,4-DNPH compared with the R_F value of a given compound taken as a standard are tabulated for each adsorbent–solvent system studied.

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TRENNUNG VON 2,4-DINITROPHENYL-AMINOSÄUREN DURCH HOCHSPANNUNGS-PAPIERELEKTROPHORESE

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Zur Trennung der bei der Analyse N-terminaler Gruppen von Peptiden und Proteinen anfallenden Dinitrophenyl-aminosäuren (DNP-Aminosäuren) wird neben der Papierchromatographie[1] heute mit Erfolg die Dünnschichtchromatographie[2] eingesetzt. Während DNP-Verbindungen auch durch Säulenchromatographie an modifizierten Kieselsäuren[3–5], Polyamidpulver[6–8] und Ionenaustauschern[9] getrennt werden können, scheint die Gaschromatographie z.B. der Methylester dinitrophenylierter Aminosäuren[10] bisher nur begrenzt anwendbar. Die Gegenstromverteilung wurde bei der Isolierung von DNP-Peptiden herangezogen[11,12].

Die Möglichkeit der papierelektrophoretischen Trennung bei Potentialen bis 10 V/cm wird in einer Arbeit von THORNBURG *et al.*[13] über die ionophoretische Beweglichkeit der DNP-Derivate von Aminosäuren und einigen niedermolekularen Peptiden aufgezeigt. Unter Verwendung organischer Puffer hoher Ionenstärke aber niedriger Leitfähigkeit sind die Wanderungsstrecken für pH-Werte von 3.3, 4.7, 7.2 und 9.3 in Amaranth-Einheiten angegeben (Amaranth = 1-(4-Sulfo-1-naphthylazo)-2-naphthol-3,6-disulfosäure).

Praktisch eingesetzt wurde die Niedervoltelektrophorese zur Lösung einzelner Probleme, wie der Trennung der DNP-Derivate von Glutaminsäure und Asparaginsäure, Arginin und Cysteinsäure bei pH 7.1[4], von ε-DNP-Lysin und DNP-Cysteinsäure in Phosphatpuffer pH 7.0[14] oder der wasserlöslichen mono- und disubstituierten basischen Aminosäuren in Boratpuffer bzw. Ammoniak[1,15].

Da über das Verhalten der DNP-Aminosäuren bei der Hochspannungselektrophorese bislang kaum Erfahrungen vorliegen und diese Methode mit schärfer begrenzten Zonen oder Flecken bei wesentlich verkürzter Versuchsdauer erhebliche Vorteile gegenüber einer Elektrophorese bei niederen Spannungen zeigt, soll im folgenden darüber berichtet werden.

MATERIAL UND METHODEN

1. *DNP-Aminosäuren*

Darstellung der DNP-Aminosäuren durch Umsetzung freier Aminosäuren mit 1-Fluor-2,4-dinitrobenzol bei Gegenwart von $NaHCO_3$ nach LEVY[16]. N^{ε}-DNP-Lysin wird über den Cu-Komplex nach PORTER UND SANGER[17] erhalten, N^{α}-DNP-Lysin durch Dinitrophenylierung von N^{ε}-Acetyllysin[18] und Hydrolyse mit 2 *N* HCl. N^{α}-DNP-Histidin entsteht bei der Aminolyse von Di-DNP-Histidin mit Histidin[19]. Die

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Schmelzpunkte stimmen mit den in der Literatur angegebenen Werten überein. DNP-DL-Sarkosin Schmp. 186° Zers.; DNP-L-Cysteinsäure Schmp. 174°.

Alle Verbindungen waren papierchromatographisch im System Toluol–Pyridin–Äthylenchlorhydrin–0.8 *N* Ammoniak (30:9:18:18) und in 1.5-*M* Phosphatpuffer pH 7 sowie bei der Hochspannungselektrophorese einheitlich.

2. *Puffersysteme*

Da die absoluten Wanderungsstrecken bei der Elektrophorese ausser vom pH-Wert und der anliegenden Spannung auch von der entwickelten Joule'schen Wärme

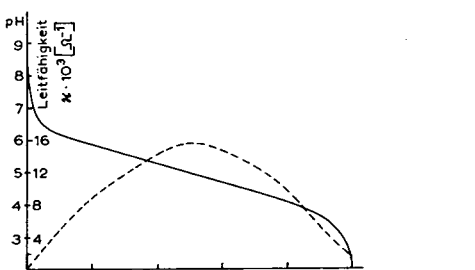
TABELLE I

FÜR DIE ELEKTROPHORESE DER DNP-AMINOSÄUREN ANGEWENDETE PUFFERSYSTEME

Die Leitfähigkeitswerte ($\varkappa \times 10^3$) liegen zwischen 2 und 6.5. In der letzten Spalte ist die Stromstärke pro cm Streifenbreite zu Beginn und am Ende einer zweistündigen Elektrophorese aufgeführt.

Pyridin (ml)	*Eisessig (ml)*	*Wasser (ml)*	*pH-Wert*	*mA/cm*
300	9	2400	6.5–6.6	1.2–1.7
40	6	2400	5.9	1.1–1.7
38	32	3000	4.8–4.9	1.6–2.6
19	47	3000	4.2	1.1–1.5
12	158	2400	3.2–3.3	0.8–1.1
Ameisensäure 85 % (ml)	*Eisessig (ml)*	*Wasser (ml)*	*pH-Wert*	*mA/cm*
150	50	2000	1.8–1.9	0.9–1.2

abhängig sind, wurden Puffersysteme annähernd gleicher Leitfähigkeit verwendet (Tabelle I). Die Ergebnisse von Leitfähigkeitsmessungen an dem für die Hochspannungselektrophorese besonders geeigneten Pyridin–Acetatpuffer sind in Fig. 1 und 2 graphisch dargestellt. Messtemperatur 17.5°.



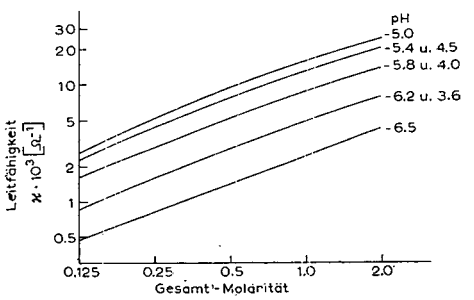


Fig. 1. 2 *M* Pyridin–Acetatpuffer. Abhängigkeit des pH-Wertes (———) und der Leitfähigkeit $\varkappa$ (– – –) von der Zusammensetzung.

Fig. 2. Leitfähigkeit $\varkappa$ als Funktion der Verdünnung für Pyridin–Acetatpuffer verschiedener pH-Werte.

3. Hochspannungs-Papierelektrophorese

Es wurde das an anderer Stelle beschriebene Gerät benutzt[20], bei dem der bis 30 cm breite Papierstreifen auf einem von Kühlsole durchströmten Metallblock aufliegt. Papier: Schleicher & Schüll 2043a, getränkt mit dem jeweiligen Puffer und mit einer Walzenpresse nach WERNER UND WESTPHAL[21] auf einen Feuchtigkeitsgehalt von 120 % des Papiertrockengewichtes gebracht. Auftragungen: kathodenseitig 12 cm vom Rand entfernt, 5 μl 0.02 *M* Lösungen der DNP-Aminosäuren in Aceton oder Dimethylformamid, N$^{\alpha}$-DNP-Lysin und N$^{\varepsilon}$-DNP-Lysin in 1 *N* HCl; Spannung: 4000 V bei 60 cm Streifenlänge $\triangleq$ 67 V/cm; Versuchsdauer: 120 Min.

ERGEBNISSE

Bei jeder Art der Elektrophorese unterliegen die absoluten Wanderungsstrecken der zu trennenden Komponenten gewissen Schwankungen. Sie sind bei gleichen Versuchsbedingungen, wie Feldstärke, Ionenstärke und pH des Puffers, abhängig von der aufgetragenen Substanzmenge infolge gegenseitiger Beeinflussung der Substanzen im Startgemisch sowie über endosmotische Effekte auch vom Auftragungsort. Recht konstante Beziehungen erhält man, wenn die Wanderungsstrecken auf eine Standardsubstanz bezogen werden. Als solche wurde das DNP-Alanin gewählt und die in Tabelle II aufgeführten relativen Wanderungswege als $R_{DNP\text{-}Ala}$-Werte ausgedrückt. Sie sind das arithmetische Mittel aus mehreren Einzelbestimmungen und zeigen Abweichungen von $\pm$ 5 %, in Acetat–Formiatpuffer pH 1.9 bis $\pm$ 10 % vom Mittelwert.

Aus dem Totalhydrolysat eines dinitrophenylierten Proteins oder Peptids wird durch Ätherextraktion neben der Gruppe der ätherlöslichen die der säure- bzw. wasserlöslichen DNP-Aminosäuren erhalten. Die chromatographische Differenzierung der DNP-Aminosäuren der wässrigen Phase (DNP-Cysteinsäure, α-DNP-Arginin, α- und ε-DNP-Lysin, α-DNP-Histidin und Di-DNP-Histidin) ist infolge des hohen Gehaltes an freien Aminosäuren schwierig und macht deren Abtrennung an einer Hyflo-SuperCel-Säule erforderlich[22]. Durch Hochspannungselektrophorese der wässrigen Phase oder deren *n*-Butanol–Essigester-Extraktes bei pH 1.9 hingegen gelingt mühelos die Abtrennung der freien Aminosäuren von den DNP-Verbindungen[23]; gleichzeitig können letztere an Hand ihrer Position im Pherogramm identifiziert werden. Di-DNP-Histidin bleibt am Start liegen, DNP-Cysteinsäure wandert anodisch heraus, während die monosubstituierten basischen Aminosäuren, bis auf das Paar α-DNP-Arginin/ε-DNP-Lysin gut getrennt, in Richtung Kathode wandern. α-DNP-Arginin und ε-DNP-Lysin, deren $R_{DNP\text{-}Ala}$-Werte von Pherogramm zu Pherogramm variieren und in erheblichem Masse von der Auftragungsstelle abhängig sind, lassen sich nach Elution durch Rechromatographie auf der Dünnschichtplatte im Toluolsystem oder in reinem Pyridin trennen[2].

Auch auf die Entfernung des in der ätherlöslichen Fraktion in beträchtlicher Menge enthaltenen Dinitrophenols durch Absublimieren oder Chromatographie an einer Aluminiumoxid-Säule kann verzichtet werden, da Dinitrophenol bei der Hochspannungselektrophorese im pH-Bereich von 1.9 bis 6.5 am Auftragungsort liegen bleibt. Somit ist eine Differenzierung gegenüber DNP-Valin, DNP-Methionin und DNP-Prolin möglich, die dünnschichtchromatographisch Schwierigkeiten bereitet. DNP-Methionin wird nach H_2O_2-Oxydation zum Sulfon durch Elektrophorese bei

TABELLE II

RELATIVE WANDERUNGSSTRECKEN VON DNP-AMINOSÄUREN, BEZOGEN AUF DNP-ALANIN = 1

Bei Wanderung in Richtung Kathode sind die *R*-Werte mit positivem Vorzeichen versehen. Experimentelle Daten siehe unter Methodik.

DNP-Alanin abs. (cm)	*13.7*	*19.0*	*15.7*	*16.3*	*14.3*	*4.2*
pH	*6.5–6.6*	*5.9*	*4.8–4.9*	*4.2*	*3.2–3.3*	*1.8–1.9*
DNP-Aminosäure						
DNP-α-AnB*	0.97	0.97	0.93	0.91	0.88	0.90
DNP-β-Ala	0.98	0.92	0.75	0.48	0.18	0.20
DNP-Asp	1.74	1.67	1.56	1.28	0.97	1.01
DNP-Asp(NH_2)	0.85	0.87	0.83	0.82	0.83	0.69
DNP-Glu	1.62	1.51	1.24	1.03	0.66–0.87	1.09
DNP-Glu(NH_2)	0.86	0.83	0.83	0.86	0.89	0.96
DNP-Gly	0.98	0.90	0.86	0.82	0.79	0.77
DNP-Hypro	0.93	1.00	1.01	1.05	1.11	1.41
DNP-Leu	0.90	0.88	0.86	0.86	0.83	1.01
DNP-Met	0.83	0.89	0.84	0.83	0.45–0.89	0.88
DNP-Met·O	0.88	0.91	0.91	0.96	1.04	1.41
DNP-Met·O_2	0.90	0.92	0.92	0.96	1.02	1.43
DNP-Phe	0.73	0.68	0.61	0.61	0.24–0.69	0.86
DNP-Pro	0.96	0.98	0.99	1.02	1.04	1.11
DNP-Sar	1.09	1.08	1.07	1.00	0.91	0.69
DNP-Ser	0.94	0.94	0.93	0.94	0.98	1.09
DNP-Thr	0.91	0.93	0.92	0.94	0.96	1.09
DNP-Try	0.40	0.37	0.26	0.24	0.27	0.31
DNP-Val	0.92	0.91	0.92	0.93	0.91	1.02
Di-DNP-CySH	0.38	0.27	0.20	0.18	0.25	0.36
Di-DNP-$(Cys)_2$	0.96	0.31–0.84	0.50	0.21	0	0
Di-DNP-Lys	0.38	0.23	0.15	0.06	0.05	0
Di-DNP-Orn	0.45	0.32	0.24	0.27	0.23	0
Di-DNP-Tyr	0.02	0	0	0	0	0
DNP-$CySO_3H$	1.88	1.90	2.07	2.13	2.01	4.30
α-DNP-Arg	+0.05	+0.02	+0.04	0.01	+0.04	+2.9
α-DNP-Lys	+0.46	+0.70	+0.62	+0.75		+11.0
ε-DNP-Lys	+0.05	+0.02	+0.03	0.01	+0.04	+2.8
α-DNP-His	+0.07	+0.40	+0.57	+0.70		+8.0
Di-DNP-His	0.45	0.36	0.25	0.19	0	0

* α-AnB = α-Amino-*n*-buttersäure.

pH 1.9 von allen anderen ätherlöslichen DNP-Aminosäuren, bis auf DNP-Hydroxyprolin, getrennt.

DNP-Tryptophan und die disubstituierten Aminosäuren, vornehmlich Di-DNP-Lysin, Di-DNP-Ornithin und Di-DNP-Cystein, ziehen vom Start weg Schwänze nach sich und erschweren die hochspannungselektrophoretische Auftrennung. DNP-Phenylalanin, DNP-Glutaminsäure und DNP-Methionin bilden bei pH 3.2–3.3 länger gezogenen Flecke, die in dem in Tabelle II aufgeführten $R_{DNP\text{-}Ala}$-Bereich liegen. DNP-Phenylalanin lässt sich jedoch besser bei pH 5 und DNP-Glutaminsäure im Neutralbereich charakterisieren. Die *R*-Werte in Boratpuffer pH 8.6 stimmen mit denen in Pyridin–Acetatpuffer pH 6.5 überein. Eine Ausnahme macht DNP-β-Alanin mit einem *R*-Wert von 0.15. Die Trenneffekte sind auf Grund lang gezogener Flecke jedoch durchweg schlechter als in den Puffersystemen der Tabelle I.

Abschliessend lässt sich sagen, dass die Papierelektrophorese der DNP-Aminosäuren bei hohen Potentialen geeignet ist, neben chromatographischen Verfahren bei der Bearbeitung spezieller Trennprobleme eingesetzt zu werden.

ZUSAMMENFASSUNG

Unter Verwendung von Pyridin–Acetatpuffer und Acetat–Formiatpuffer gleicher Leitfähigkeit wurde die elektrophoretische Beweglichkeit von 31 DNP-Aminosäuren im pH-Bereich von 1.8–6.5 bei Potentialen von 67 V/cm untersucht.

Die relativen Wanderungsstrecken sind auf DNP-Alanin bezogen und als $R_{DNP\text{-}Ala}$-Werte tabellarisch aufgeführt.

Möglichkeiten des Einsatzes der Hochspannungs-Papierelektrophorese zur Trennung der wasser- und ätherlöslichen DNP-Aminosäuren werden aufgezeigt.

SUMMARY

The electrophoretic mobilities of 31 DNP-amino acids in pyridine–acetate buffers and in acetate–formate buffer, all of approximately the same conductivity, were determined in the pH range from 1.8 to 6.5, at a potential of 67 V/cm.

The distances travelled were measured relative to that of DNP-alanine and tabulated as $R_{DNP\text{-}Ala}$ values.

Possible applications of high-voltage paper electrophoresis to the separation of water-soluble and ether-soluble DNP-amino acids are discussed.

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THE PAPER CHROMATOGRAPHY OF SOME ISOMERIC MONOSUBSTITUTED PHENOLS. I.

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INTRODUCTION

In recent years the paper chromatography of phenols has been given extensive study. Many techniques have been developed for mobile solvent systems on plain or buffered papers and for methods of detecting the spots[1,2].

The mobile solvents most frequently used consist of two or more solvents often containing an aqueous phase. EVANS *et al.*[3] used butanol–pyridine–water saturated with NaCl (1:1:2) as the mobile solvent. BATE-SMITH[4] used butanol–acetic acid–water (4:1:5) and *m*-cresol–acetic acid–water (50:2:48). RILEY[5] used *n*-butanol–benzene–water (1:9:10) and *n*-butanol–benzene–water (1:19:20) as well as *n*-amyl alcohol saturated with water. SUNDT[6] has used cyclohexane–ethyl acetate (5:1) saturated with a mixture of dimethylformamide–water (5:2). CLARK[7] has obtained good results using cyclohexane–ethyl acetate–acetic acid (5:1:1) and butanol–water–acetic acid (6:2:1) on ion-exchange paper.

The use of mixed volatile solvents presents a disadvantage in that the solvent ratios may change on standing because of the different volatilities of the components. This problem can be minimized by mixing the solvents just before using. This procedure, however, is time consuming and requires the storage and handling of larger amounts of solvents.

There are many different techniques for locating phenols on paper chromatograms. The most frequently used methods require either specialized equipment or the use of unstable reagents. Examination of the papers under short and long wave ultraviolet light is often used either before or after chemical treatment[8].

Chemical methods of spot location frequently involve spraying the paper with reagents such as diazotized sulfanilic acid, diazotized *p*-nitroaniline and diazotized benzidine[3,8]. Spraying the paper with silver nitrate followed by heating has been used[4]. Other reagents used as sprays for locating phenols are 2 % phosphomolybdic acid[5] and equal volumes of 1 % ferric chloride and 1 % potassium ferricyanide[9].

The work described in this paper was carried out to develop some simple solvent systems for the separation of isomeric monosubstituted phenols, to investigate the effect of the orientation of substituent groups on the R_F' value using the new solvent systems and to develop the use of ceric ammonium nitrate as a stable spot-locating agent for a number of phenols. Data are presented for some common phenols and for a number of phenols for which no references have been found in the paper chromatographic literature.

EXPERIMENTAL

Reagents

Toluene saturated by shaking with distilled water. Mineral spirits (Kauri-Butanol value 37–38, aniline point 56–59°) saturated with distilled water. Ceric ammonium nitrate reagent prepared by dissolving 200 g of ceric ammonium nitrate in 500 ml of 2 *N* nitric acid with heating. The solution was allowed to stand overnight and the clear supernatant liquid was decanted.

Procedure

The toluene and mineral spirits were kept saturated with water by the presence of a lower water layer in the tanks. The tanks were lined with paper sheets extending into the water layer. All chromatograms were run at 25° with Whatman No. 1 paper, using the ascending development method. The papers were allowed to equilibrate 20 min in the tank before immersion. The solvents were allowed to travel 10 cm requiring 30–45 min.

The paper sheets were sprayed with the reagent as soon as they were removed from the tanks and then washed thoroughly with water to remove the excess reagent. The papers were air dried. The R_F' values reported are the distance traveled by the spot front divided by the distance traveled by the solvent front[3].

RESULTS AND DISCUSSION

Water and toluene saturated with water have been found to be satisfactory mobile solvents for use in the paper chromatography of phenols. Mineral spirits saturated with water also has been found to be quite satisfactory. Mineral spirits is a mixture of aliphatic and aromatic hydrocarbons, but because of its low volatility (usual b.p. range 150–200°) no difficulty has been observed due to evaporation. All three solvent systems have been used for 30 days with no variation in the R_F' values observed.

A solution of ceric ammonium nitrate in 2 *N* nitric acid as a spot-locating reagent for phenylphenols has been reported[10]. This reagent has been found to be a good spot-locating material for most of the phenols tested. The reagent solution is quite stable and has been stored under ordinary laboratory shelf conditions for a year or more without loss of effectiveness. The reagent also gives easily discernible colored spots with most phenols. These spots are permanent and will not wash out with water so that the paper chromatogram may be retained as a permanent record.

A comparison of the effect of the three solvent systems on isomeric groups of phenols shows that in general the highest R_F' values are obtained with toluene. The exceptions are the hydroxyphenols, the aminophenols and the hydroxybenzoic acids. These phenols have the greatest R_F' values with water.

Phenols having substituent groups that are not readily ionized or associated have greater R_F' values with mineral spirits than with water. These include the phenylphenols, the iodophenols, the *tert.*-butylphenols and the naphthols. Groups such as the cresols, hydroxyphenols, hydroxybenzoic acids, aminophenols and diphenols have higher R_F' values with water than with mineral spirits.

The "*ortho* effect" is quite obvious among isomeric groups where any significant

difference is found in the R_F' values of the three isomers. The *ortho* isomer has the largest R_F' value and is frequently separate from the *meta* and *para* isomers. The R_F' values of the *meta* and *para* isomers are usually close together, with the *meta* isomer having a slightly greater R_F' value than the *para* isomer. The only exceptions found are resorcinol, which has a greater R_F' value in toluene than either catechol or hydroquinone, and *m*- and *p*-aminophenol, which have slightly greater R_F' values in water than the *ortho* isomer. Table I shows the R_F' values found in the three solvents and the spot colors observed.

The differences in R_F' values of various isomeric groups in a single solvent show much variation depending on the nature of the substituent group and the solvent. For example, in both water and toluene the halophenols, the nitrophenols and the cresols show only a small spread in R_F' values between the three isomers. However, in mineral spirits a large difference is apparent. With the diphenols, large R_F' differences between the isomers are present with all three solvents. The phenylphenols show little difference in R_F' values in toluene but large differences in water and mineral spirits. The aminophenols show the greatest differences in toluene.

Fig. 1 shows the effect of the orientation of substituent groups on the R_F' value of phenols with water as the mobile solvent. The use of water as the mobile solvent points to adsorption as the mechanism involved. When toluene and mineral spirits are used as the mobile solvents, water is the more polar stationary phase indicating that the mechanism is partition.

Observation of the data in Fig. 1 shows that for *meta* and *para* substituted phenols the substituents are very nearly in the same order as the R_F' values decrease. For *ortho* substituted phenols the order is altered mainly by the effect of the carboxyl, chloro and hydroxyphenyl groups.

Figs. 2 and 3 show the effect of the orientation of the substituent groups on the R_F' values of phenols with both mineral spirits and toluene as the mobile phases.

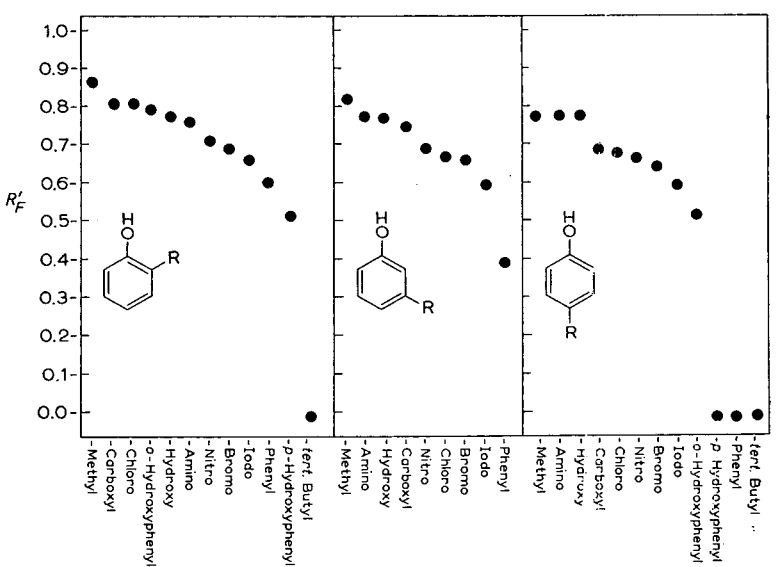

Fig. 1. Effect of the orientation of substituent groups on the R_F' values of phenols with water.

Within each table with *meta* and *para* substituted phenols, the substituents are in nearly the same order as the R_F' values decrease. The exceptions are phenyl substitution in Fig. 2 and iodo substitution in Fig. 3.

The data from both Figs. 2 and 3 show that in *ortho* substituted phenols the chloro, nitro and carboxyl groups have all advanced their relative positions on the list.

TABLE I

R_F' VALUES OF ISOMERIC MONOSUBSTITUTED PHENOLS

Phenol	R_F' *value, 25°*			*Color of spot*
	Water	*Mineral spirits*	*Toluene*	
Phenol	0.81	0.36	0.88	Brown
1-Naphthol	0.51[a]	0.71	0.99	Light purple
2-Naphthol	0.43[a]	0.53	0.97	Light yellow
o-tert.-Butylphenol	0.00	0.97	1.00	Light brown
p-tert.-Butylphenol	0.00	0.89	1.00	Light tan
o,o'-Diphenol	0.80	0.37	0.83	Brown
o,p'-Diphenol	0.52	0.08	0.53	Light tan
p,p'-Diphenol	0.09[a]	0.00	0.00	Light yellow
o-Phenylphenol	0.61	0.96	0.99	Rust red
m-Phenylphenol	0.40	0.77	0.97	Light tan
p-Phenylphenol	0.00	0.54[a]	0.95	Light gray-green
o-Cresol	0.87	0.68	0.99	Very light yellow
m-Cresol	0.83	0.60	0.99	Light yellow
p-Cresol	0.78	0.54	0.99	Very light yellow
Salicylic acid	0.82[b]	0.23[a]	0.76	Light tan
m-Hydroxybenzoic acid	0.76[b]	0.00	0.00	Tan
p-Hydroxybenzoic acid	0.69[b]	0.00	0.00	Light brown to brown
o-Aminophenol	0.77	0.00	0.21[a]	Tan to brown
m-Aminophenol	0.78	0.00	0.12[a]	Brown
p-Aminophenol	0.78[c]	0.00	0.07	Light tan
Catechol	0.78	0.00	0.00	Black
Resorcinol	0.78	0.00	0.11[a]	Brown
Hydroquinone	0.78[d]	0.00	0.00	Purple; fades rapidly
o-Bromophenol	0.70[e]	0.94	0.98	Light yellow
m-Bromophenol	0.67	0.62	0.97	Light tan
p-Bromophenol	0.65	0.60	0.97	Light brown
o-Iodophenol	0.67	0.86	1.00	Pink to tan
m-Iodophenol	0.61	0.68	1.00	Light tan
p-Iodophenol	0.60	0.65	0.98	Brown
o-Chlorophenol	0.82	0.91	1.00	Light yellow
m-Chlorophenol	0.68	0.58	0.98	Light tan
p-Chlorophenol	0.68	0.53	0.97	Gray
o-Nitrophenol	0.72[e,f]	0.94	1.00	Light tan
m-Nitrophenol	0.70[g]	0.12[a]	0.89	Tan
p-Nitrophenol	0.67[g]	0.08[a]	0.75	Yellow
p-Phenolsulfonic acid	0.95	0.00	0.00	Light yellow
p,p'-Sulfonyldiphenol	0.84	0.17[a]	0.07[a]	Light tan

[a] Spot streaks.

[b] The hydroxybenzoic acids gave two spots in water because of ionization. A 1% acetic acid solution was used to repress ionization.

[c] Tan before developing; fades to white.

[d] Bleaches spot white when developer is applied.

[e] Spot spreads out rapidly.

[f] Yellow before developing; fades to white.

[g] Yellow before developing.

Two of these three groups showing increased activity in the *ortho* position were also observed to show the same effect in the adsorption chromatograms with water. The increased activity of these phenols is thought to be caused by intramolecular hydrogen bonding in the carboxyl, nitro and hydroxyphenyl groups and by a change in polarity in the chloro group.

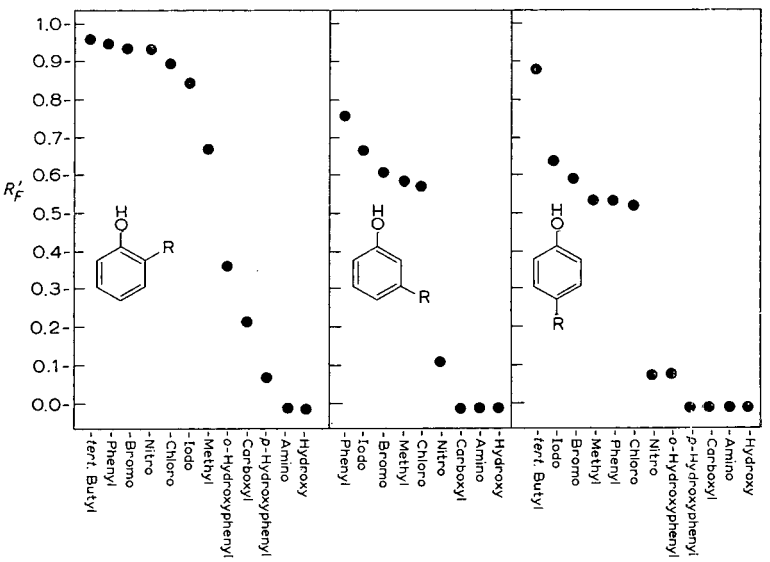

Fig. 2. Effect of the orientation of substituent groups on the R_F' values of phenols with mineral spirits.

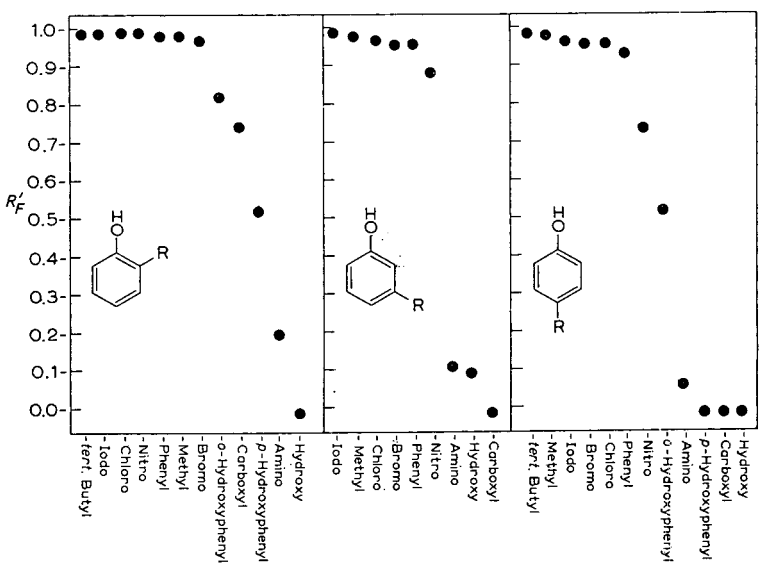

Fig. 3. Effect of the orientation of substituent groups on the R_F' values of phenols with toluene.

The great difference between the adsorption and partition systems is pointed out by the data in Figs. 1–3. The data in the partition systems show an order of decreasing activity of the substituents that approximates the reverse of the order of decreasing activity in the adsorption system. This is more nearly true with *meta* and *para* substituted phenols since other factors may influence the *ortho* substituted phenols.

Ceric ammonium nitrate has been found to be quite useful as a spot-locating reagent for phenols. This reagent has been used in the detection of organic acids on paper chromatograms[11]. However, no references to its use for detecting phenols have been found other than for the phenylphenols previously mentioned[10]. In the work reported here, this reagent has been quite useful with only a few exceptions. In using this reagent with the cresols, the spot color is very light yellow and if a small sample is used, the spot may be difficult to see because of the light color developed. Some of the phenols decolorize the reagent leaving a white spot on a yellow background before the paper is washed. This happens only when water is used as the mobile solvent. The phenols exhibiting this behavior are hydroquinone, *p*-aminophenol and *o*-nitrophenol.

From observation of the data in Table I, it is noted that only *p*,*p*-diphenol has no significant R_F' value in any of the three solvents. Work on this compound has shown that anhydrous methanol is a good mobile solvent giving an R_F' value of 0.85.

For purposes of comparison and information several phenols have been included in the data that are not presented as members of an isomeric series.

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SUMMARY

The R_F' values of a number of isomeric monosubstituted phenols have been determined for three solvent systems. The solvent systems are water, toluene saturated with water and mineral spirits saturated with water. Most of the phenols show a significant variation in R_F' values between the three solvent systems. The R_F' values of isomeric phenols vary considerably in individual solvents depending on the substituent group and its orientation. The use of ceric ammonium nitrate as a spot-locating reagent is described. This reagent has been found to be more convenient to use and store than many other spot-locating agents commonly used for phenols.

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SEPARATION OF SUGAR NUCLEOTIDES, PHOSPHORIC ESTERS AND FREE SUGARS BY PAPER CHROMATOGRAPHY WITH SOLVENTS CONTAINING BORATES OF ORGANIC BASES*

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Methods based on the formation of borate complexes have been used with some success for the separation of free sugars and sugar phosphates by paper chromatography[1–4], paper electrophoresis[5–7] and ion-exchange[8–16]. However, none of these methods will separate mixtures of some of the nucleoside diphosphate sugars, such as UDP-glucose and UDP-galactose, which differ only in the orientation of a hydroxyl group.

By using boric acid salts of organic bases, which are more soluble in the solvents used for paper chromatography, it was possible to separate glucose, galactose and mannose nucleotides of the same base. The method has also been applied with good results to the separation of free sugars or their phosphates.

EXPERIMENTAL

Materials

GDP-α-mannose, UDP-α-glucose, UDP-α-acetylglucosamine, UDP and UMP were isolated from yeast as reported by PONTIS *et al.*[17] and further purified by chromatography on Whatman No. 17 paper with ethanol–ammonium acetate as solvent[18].

UDP-α-mannose, UDP-α-xylose, UDP-α-galactose, ADP-α-glucose, ADP-β-glucose, TDP-α-mannose, ADP-α-galactose, ADP-α-mannose, GDP-α-glucose, GDP-α-galactose, deADP-α-glucose, deUDP-α-glucose and ADP-P-glyceric acid were prepared according to ROSEMAN *et al.*[19] with slight modifications.

All the sugar phosphates and free sugars employed were of the D-series, with the exception of L-arabinose. Xylose-1-P and mannose-1-P were synthesized according to MEAGHER AND HASSID[20] and POSTERNAK[21], respectively. Psicose was prepared as described elsewhere[22]. All other chemicals were commercial products.

Chromatography

In the analytical runs, Schleicher and Schüell No. 2043 B paper, cut in the manner described by MATTHIAS[23], was found to give the best resolution.

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*** Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas.

For preparative purposes, Whatman No. 3 MM paper was used, which was sewn to a piece of Whatman No. 1 in order to slow down the solvent flow[24]. Chromatograms were developed by the descending technique.

The following solvents were used:

(A) Ethylene glycol dimethyl ether–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA (70:20:30).

(B) Ethanol–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA (70:20:30).

(C) Butanol–pyridine–piperidinium borate buffer of pH 10.5 (6:4:3). The molarity of both components in the buffer is 0.35 M.

(D) Butanol–pyridine–0.05 *M* morpholinium tetraborate, pH 8.6 (7:5:2).

(E) Methanol–1 *M* ammonium acetate, pH 3.8 (70:35). This solvent is a modification of that described by PALADINI AND LELOIR[18].

0.5 *M* morpholinium borate buffer was prepared by dissolving 123.4 g of boric acid and 87 g of morpholine ($\sigma = 0.99$) in 0.01 *M* EDTA up to 1 l. For use with solvent D, EDTA was omitted and the buffer was diluted 10-fold.

Piperidinium borate buffer was prepared mixing 21.60 g of boric acid and 29.75 of piperidine ($\sigma = 0.86$) and adding water up to 1 l.

When solvents A, B, or E were used, the papers were dipped in 0.01 *M* EDTA, pH 7.0, and dried before use, as described elsewhere[25]. For use with solvent C, the papers were dipped in a 7-fold dilution of the piperidinium borate buffer.

Detection procedure

Nucleotides were detected with a Mineralight lamp. Phosphate-containing compounds were revealed according to BURROWS *et al.*[26] and sugars by the silver nitrate technique[27].

Radioactivity was located with an automatic scanner (Nuclear Chicago Corp. model D-47 gas flow counter fitted to a C-100 A actigraph II).

Recovery of the nucleotides

To free the samples from borate after chromatography with solvent C or D, the procedure described by ZILL *et al.*[10] was followed. In the case of solvents A or B, two different methods were used:

(1) The sample was eluted with water and brought to pH 6.0 with acetic acid; Norit A was added (50 mg/μmole), the suspension was shaken occasionally and filtered. After washing with water, the nucleotides were eluted from the charcoal with ethanol–concentrated ammonia–water (25:0.5:75). The total recovery was about 80 %.

(2) Alternatively a simpler method was used: the eluted sample was neutralized with acetic acid using bromothymol blue as internal pH indicator, and chromatographed with solvent E to remove borate.

RESULTS AND DISCUSSION

Nucleoside diphosphate sugars

Small differences in the mobilities of sugar nucleotides were obtained with the system of HARRAP[1] employed by NIKAIDO[28] to resolve a mixture of galactose-1-P and glucose-1-P. This solvent (methyl cellosolve–methyl ethyl ketone–3 *N* ammonia

(70:20:30), saturated with boric acid), however, has the disadvantage of changing its composition during the chromatographic run, due to precipitation of borate. To overcome this difficulty, different bases, more soluble in the organic phase, were tested instead of ammonia. Morpholine gave reproducible results and better resolution. Another improvement was obtained when methyl cellosolve was replaced by ethylene glycol dimethyl ether or ethanol.

The pH was found to be critical. At high pH values (above 10), galactose- and xylose-nucleotides decomposed almost completely, while low pH values cannot be used because no borate complexes are formed. A compromise was reached with a buffer of pH 8.6.

Solvent B gives a clear separation between galactose- and glucose-containing nucleotides; the running time necessary for the resolution depends upon the base involved (Table I). The corresponding mannose nucleotide runs in an intermediate position and it is difficult to obtain a complete separation among the three compounds, especially in the case of guanosine derivatives.

Deoxyribonucleotides run much faster than the other substances tested. This is understandable because the elimination of one of the hydroxyl groups decreases the ability to form a borate complex.

TABLE I

MOBILITIES OF SUGAR NUCLEOTIDES AND SUGAR PHOSPHATES IN SOLVENT B*

Running time: 70 h		*Running time*: 60 h		*Running time*: 20 h	
Compound	$R_{glucose\text{-}1\text{-}P}$	*Compound*	$R_{glucose\text{-}1\text{-}P}$	*Compound*	$R_{glucose\text{-}1\text{-}P}$
GDP-galactose	0.20	ADP-galactose	0.30	deADP-glucose	1.07
GDP-mannose	0.23	ADP-P-glyceric		TDP-mannose	1.28
GTP	0.25	acid	0.33	TTP	1.36
GDP	0.27	ADP-mannose	0.35	de-UDP-glucose	1.40
GDP-glucose	0.28	ATP	0.36	TDP-glucose	1.49
GMP	0.32	ADP	0.42	TMP	1.52
		ADP-α-glucose	0.46	cyclic 3′,5′-AMP	1.90
		ADP-β-glucose	0.46		
		AMP	0.49		

Running time: 50 h			
Compound	$R_{glucose\text{-}1\text{-}P}$	*Compound*	$R_{glucose\text{-}1\text{-}P}$
UDP-galactose	0.32	Fructose-1,6-P_2	0.33
UDP-mannose	0.43	2,3-P_2-glyceric acid	0.51
UTP	0.45	Glucose-6-P	0.72
UDP	0.48	Galactose-1-P	0.75
UDP-glucose	0.51	Mannose-1-P	0.80
UDP-xylose	0.59	PP_i**	0.93
UMP	0.62	P_i	0.98
UDP-acetylglucosamine	0.71	Glucose-1-P	1.00
		Xylose-1-P	1.04
		3-P-glyceric acid	1.05

* Ethanol–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA (70:20:30). Mobilities are referred to glucose-1-P.

** Tailing of this compound is often observed.

The highest mobility was observed with cyclic 3′,5′-AMP, which has only one acid group and one hydroxyl group.

With solvent A the separation pattern is similar although the spots are smaller and sharper than with solvent B. Solvent A has the disadvantage of requiring longer development times (Fig. 1).

Mixtures of sugar nucleotides and nucleoside mono-, di- or tri-phosphates of the same base cannot be resolved with these systems. Good results were obtained by first submitting the sample to preliminary separation with the neutral ethanol–ammonium acetate solvent.

To counteract the tendency of several compounds to give diffuse spots with solvents A or B, the paper was impregnated with 0.01 *M* EDTA.

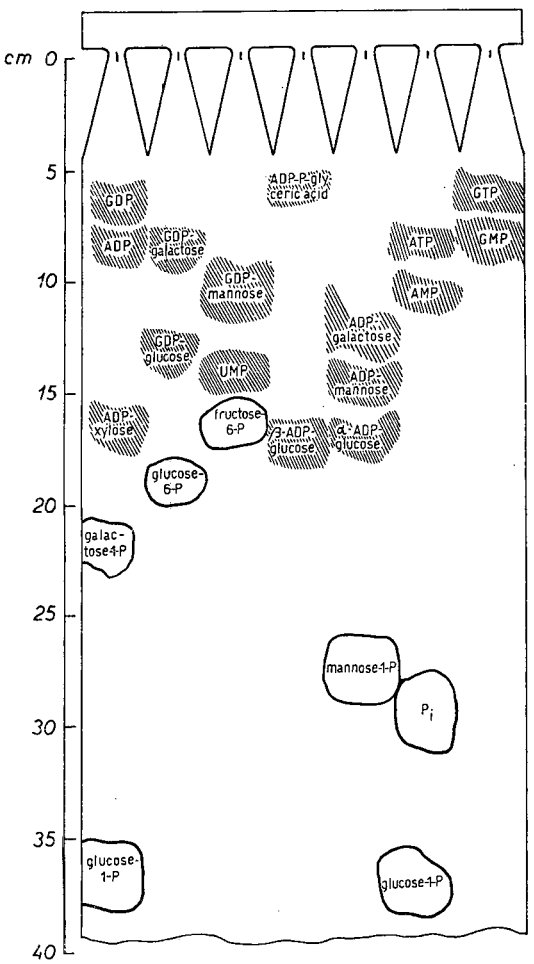

Fig. 1. Separation of nucleotides and phosphoric esters by paper chromatography for seven days in solvent A (ethylene glycol dimethyl ether–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA, 70:20:30). Shaded areas correspond to ultraviolet-absorbing substances.

Solvent systems A and B have been successfully used to resolve a mixture of ADP-glucose, ADP-mannose and ADP-galactose[29] as well as UDP-acetylglucosamine and UDP-acetylgalactosamine from corn grains.

Another application of this chromatographic technique was the separation of a mixture of UDP-^{14}C-glucose and UDP-^{14}C-galactose prepared with an enzymatic extract from *Saccharomyces fragilis*[30] as shown in Fig. 2.

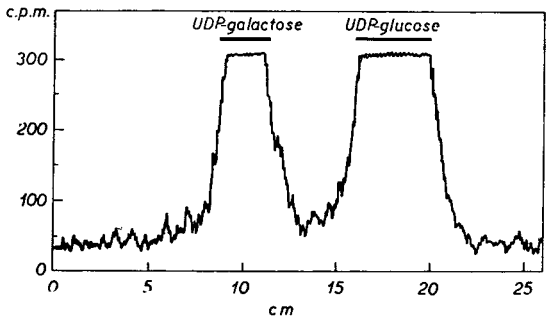

Fig. 2. Distribution of radioactivity in a chromatogram with solvent A of a mixture of labeled uridine nucleotides prepared with an enzymatic extract from *Saccharomyces fragilis*[30].

Sugar phosphates

With the exception of xylose-1-P, which runs very close to glucose-1-P, both solvents A and B gave a good resolution of the different sugar 1-phosphates tested.

The separation pattern is similar to that obtained with the sugar nucleotides: mannose-1-P running between galactose-1-P and glucose-1-P.

Free sugars

Some groups of sugars are very difficult to separate in a single run with the usual solvents; this is the case of glucose, fructose, mannose and arabinose.

TABLE II

MOBILITIES OF SUGARS IN SOLVENT D*

Sugar	$R_{glucose}$	*Sugar*	$R_{glucose}$
Glucuronic acid	0.18	Glucosamine	0.90
Psicose	0.36	Erythrose	0.95
Sorbitol	0.37	Glucose	1.00
Mannitol	0.42	Mannose	1.15
Lactose	0.43	Arabinose**	1.22
Sorbose	0.44	Ribose	1.29
Trehalose	0.51	Acetylglucosamine	1.36
Tagatose	0.60	Xylose	1.38
Maltose	0.62	Acetylgalactosamine	1.52
Fructose	0.64	Fucose	1.59
Galactosamine	0.69	Dihydroxyacetone	1.82
Sucrose	0.73	Deoxyglucose	1.98
Galactose	0.89	Deoxyribose	2.22

* Butanol–pyridine–morpholinium tetraborate 0.05 *M* (7:5:2). Mobilities are referred to glucose.

** Produces a diffuse spot.

The principle of complex formation with borate led to the development of solvent D, which allows the separation of glucose, fructose and mannose after an 18 h run[31] (see Table II).

TABLE III

MOBILITIES OF SUGARS IN SOLVENT C*

Sugar	$R_{mannose}$	*Sugar*	$R_{mannose}$
Galactosamine	0.21	Fructose	0.58
Glucosamine	0.28	Trehalose	0.68
Glucose	0.38	Arabinose	0.71
Sorbose	0.38	Maltose	0.78
Sorbitol	0.42	Psicose	0.78
Xylose	0.48	Sucrose	0.98
Galactose	0.48	Mannose	1.00
Lactose	0.49	Ribose	1.05
Tagatose	0.54	Acetylgalactosamine	1.75
		Acetylglucosamine	2.40

* Butanol–pyridine–piperidinium borate buffer, pH 10.5 (6:4:3). See "Methods". Mobilities are referred to that of mannose.

A more alkaline solvent (solvent C), obtained by substituting morpholine by the stronger base piperidine, was also found useful. It has the advantage of separating the four above-mentioned sugars, but it needs about 70 h of development (Table III). In addition, the buffer concentration is very critical as it is shown in Fig. 3. Slight changes of its molarity produce significant modifications in the relative mobilities of the compounds.

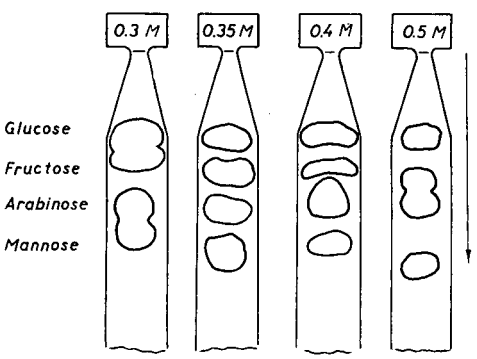

Fig. 3. Changes in the mobilities of glucose, fructose, arabinose and mannose, using solvent C prepared with increasing molarities of both components of the buffer, piperidine and boric acid.

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SUMMARY

Four different chromatographic solvents containing borates of organic bases are described. Two of them permit the separation of sugar nucleotides differing only in the sugar moiety and give also a good resolution for some sugar 1-phosphates. The other two systems permit, in a single run, the separation of a group of sugars which is usually difficult to achieve.

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THE *IN SITU* IDENTIFICATION OF PHOSPHATE SPECIES IN MULTI-SPOT CHROMATOGRAMS BY NEUTRON ACTIVATION ANALYSIS

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As a preface to a theoretical treatment of multiple zones and spots, *i.e.*, more than one zone for what is commonly accepted to be a single species, KELLER AND GIDDINGS[1] presented an extensive review of the phenomenon. Among the examples cited was one reported by CURRY[2], who attributed multiple spots on paper chromatograms of sodium orthophosphates to proton exchanges of the type:

$$H_2PO_4^- + B^{n-} = HPO_4^{2-} + HB^{(n-1)-}$$

where B^{n-} is some Bronsted base. In paper chromatography the water present as the immobile phase and, normally, as a component of the mobile phase, can act in this capacity. It need not be the only base present. ERDEM[3] employed CURRY's suggestion to explain the multiple spotting of orthoarsenates on paper. As KELLER AND GIDDINGS remarked, the proposal is not wholly acceptable for kinetic reasons. The most serious experimental omission is the verification that the two spots do indeed contain different phosphate species. CURRY based his identification of the spots on their relative size and intensity when the different sodium salts of phosphoric acid were chromatographed. What is needed is an independent, nonchromatographic identification of the species *in situ*. The availability of a nuclear reactor suggested neutron activation analysis.

THEORY

The naturally occurring stable isotopes of sodium and phosphorus, when bombarded with thermal neutrons, accept a fraction of these neutrons to produce radioactive species. The relationship valid during activation is[4]:

$$A_t = N\phi\sigma\,(1 - e^{-0.693t/T}) \qquad (1)$$

where A_t is the activity in disintegrations per second at time t after start of the irradiation, N is the total number of nuclei present in the sample, ϕ is the neutron flux in neutrons/cm^2·sec, σ is the activations cross section in cm^2, and T is the half life of the radioactive isotope. Here, t is the period of irradiation and A_t is the activity at the instant of removal of the sample from the reactor. The equation must be further

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modified to include the fact that the radiation observed or the apparent activity, R_t, *i.e.*, the radiation admitted to a Geiger tube through an aperture, is some fraction of A_t. The proportionality factor is the detection coefficient or counting yield, Y (ref. 4), *i.e.*:

$$R_t = YA_t \tag{2}$$

which may be resolved into some eleven contributions. On introduction into eqn. (1):

$$R_t = YN\phi\sigma\,(1 - e^{-0.693t/T}) \tag{3}$$

In this particular problem the species in the solute spots are judged to be the same or different by a determination of the ratio of the number of atoms of each, *i.e.*:

$$\frac{R_t(\mathrm{Na})}{R_t(\mathrm{P})} = \frac{Y_{\mathrm{Na}}N(\mathrm{Na})\phi_{\mathrm{Na}}\sigma_{\mathrm{Na}}(1 - e^{-0.693t_{\mathrm{Na}}/T_{\mathrm{Na}}})}{Y_{\mathrm{P}}N(\mathrm{P})\phi_{\mathrm{P}}\sigma_{\mathrm{P}}(1 - e^{-0.693t_{\mathrm{P}}/T_{\mathrm{P}}})} \tag{4}$$

The paper strips holding the spots, each of which contain both sodium and phosphorus, were irradiated simultaneously at the same position in the reactor so that $\phi_{\mathrm{Na}} = \phi_{\mathrm{P}}$ and $t_{\mathrm{Na}} = t_{\mathrm{P}} = t$. The combined radioactivity of the two species present in a single spot was determined so that it seemed reasonable to assume that the detection coefficient was identical for both, *i.e.*, $Y_{\mathrm{Na}} = Y_{\mathrm{P}}$. The counter geometry was identical in both cases and both isotopes lose β-particles of nearly the same energy. Eqn. (4) becomes:

$$\frac{R_t(\mathrm{Na})}{R_t(\mathrm{P})} = \frac{N(\mathrm{Na})\sigma_{\mathrm{Na}}(1 - e^{-0.693t/T_{\mathrm{Na}}})}{N(\mathrm{P})\sigma_{\mathrm{P}}(1 - e^{-0.693t/T_{\mathrm{P}}})} \tag{5}$$

The nuclear properties of interest appear in Table I. Geiger-Müller counters are approximately 100 times more efficient for β-particles than for γ-rays[5]. The error in assuming sodium to be strictly a β-emitter is about 1 %, which is much less than the error in the nuclear cross section. The outstanding advantages existent with this pair of elements are: (1) there is one naturally occurring isotope for each species so there is only one nuclear reaction to produce one daughter, (2) the nuclear cross sections are both sufficiently large and comparable that convenient activities can be produced in both species in a reasonable irradiation time, (3) the half lives are sufficiently different to give a sharp change in slope of the plot of the activity *vs.* time as the sodium decays away and (4) the half lives are sufficiently short to make the time required by the experiment reasonable. This last factor is also convenient in disposal and contamination problems, *i.e.*, one may decontaminate by sealing off the radiation area for a reasonable period of time.

KOCH[6] suggests some possible interferences. These are listed in Table II along with other pertinent data. The threshold energy, *i.e.*, the kinetic energy of the neutron just capable of making the reaction energetically possible[5], is 1 MeV or above for all reactions except the last. Thermal neutrons, the kind encountered in the TRIGA reactor, are those with energies of $2.5 \cdot 10^{-8}$ MeV[7] to $3.5 \cdot 10^{-8}$ MeV[5]. Thus the only feasible interference is from silicon. A measure of the probability of the reaction is best given by the product of the relative isotope abundance and the cross section, *i.e.*,

TABLE I

NUCLEAR PROPERTIES OF THE ISOTOPES OF THE REACTIONS ^{23}Na (n,γ) ^{24}Na AND ^{31}P (n,γ) ^{32}P

	^{23}Na	^{31}P
Per cent abundance[4]	100	100
Nuclear cross section for thermal neutrons (barns)[4] (Probable error = 20%[5])	0.536 ± 0.01	0.19 ± 0.01
Daughter	^{24}Na	^{32}P
Mode of decay[5]	β^-, 1.39 MeV γ, 1.38 MeV 2.76 MeV	β^-, 1.712 MeV
Half life[4]	14.97 h	341.3 h

0.003 (compared with 0.19 for phosphorus, a factor of 63 in difference). This coupled with the extremely small amount of silica in paper as contamination reduces the likelihood of interference from this reaction to an infinitesimal.

The mixture of radioactive ^{24}Na and ^{32}P is classified as a mixture of independent radionuclides with stable daughters (^{24}Mg and ^{32}S). Its total activity, which is the observed activity, R_t, as a function of time is:

$$\log R_t = \log[R_t(\mathrm{Na})]_0 + \log[R_t(\mathrm{P})]_0 - (\lambda_{\mathrm{Na}} + \lambda_{\mathrm{P}})t^*/2.303 \qquad (6)$$

Quantities $[R_t(\mathrm{Na})]_0$ and $[R_t(\mathrm{P})]_0$ are the activities at the time of the first count which here is the instant of removal from the reactor ($t^* = 0$). In actuality this activity was not determined either because the sample was judged dangerous by the radiation officer or because of time consumed in transfer of the sample from the reactor site to the counter. The rates at $t^* = 0$ are the extrapolated values obtained from the activity–time plot and the known elapsed time from termination of irra-

TABLE II

POSSIBLE INTERFERING NUCLEAR ACTIVATIONS

Interfering reaction	*Per cent abundance*	*Threshold energy (MeV)*	*Nuclear cross section of parent* (barns)*
^{23}Na analysis			
$^{27}Al(n,\alpha)^{24}Na$	100	3.3	0.14 (13) 0.116 (14)
$^{24}Mg(n,p)^{24}Na$	78.6	4.9	0.22 (13) 0.19 (14.5)
^{31}P analysis			
$^{32}S(n,p)^{32}P$	95.02	~ 1	0.30 (6–10) 0.31 (15)
$^{35}Cl(n,\alpha)^{32}P$	75.4	1.0	0.19 (14.5)
$^{30}Si(n,\gamma)^{31}Si \downarrow -\beta^-$ $^{31}P(n,\gamma)^{32}P$	3.05	Thermal	0.110

* The number in parentheses is the neutron energy for the cross section listed.

diation to the first count. The constants λ_{Na} and λ_P are characteristic of the radioactive species, *i.e.*, $0.693/T$ where T is the half life. The graphical method of resolution of eqn. (6) into the separate activities is sufficiently well known so that it is not repeated here[4,5].

Fig. 1 is typical of the data and demonstrates the ease of resolution of the activities and the subsequent almost utopian character of the method. The dashed lines are the extrapolations of the linear portions of each curve to $t^* = 0$. The values of $[R_t(Na)]_0$ and $[R_t(P)]_0$ (found from the intercepts at $t^* = 0$) were used in eqn. (5) to calculate $N(Na)$ and $N(P)$ present in the solute zone.

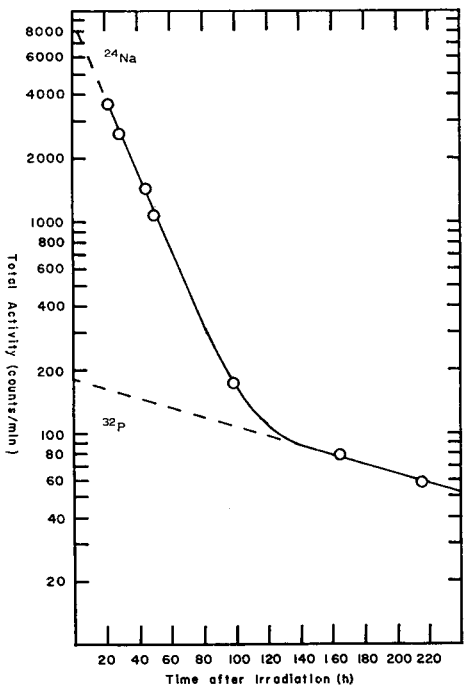

Fig. 1. Observed activity, R_t, of solute zones containing ^{24}Na and ^{32}P as a function of time.

EXPERIMENTAL

The TRIGA reactor[8,9] is a thermal, heterogeneous, enriched uranium reactor normally of 10 kW power level. The 12 in. thick graphite reflector holds a rotary specimen rack of forty 1-in. diam. × 4 in. long, 50 cm³ capacity specimen tubes 3 in. from the core of enriched uranium–zirconium hydride rods. The unit is situated at the bottom of a 6 ft. diam. × 20 ft. deep water filled tank. Specimens are transferred to and from the sample rack or a 1 in. diam. thimble in the center of the core by a pneumatic tube. The approximate neutron flux is 10^{11} neutrons/cm²·sec in the core (average value), $0.7 \cdot 10^{11}$ neutrons/cm²·sec at the specimen rack and $1.5 \cdot 10^{11}$ at the central thimble. Operation at 30 kW yields $8 \cdot 10^{11}$ neutrons/cm²·sec[10]. The University of Arizona reactor is currently licensed to operate at 100 kW for 2 h at a flux of about 10^{13} neutrons/cm²·sec.

The paper strip with the materials for activation was coiled with another unused strip of identical size to prevent transfer of material from one position on the formed strip to another (a potential source of double spots). The coiled paper was inserted in a polyethylene reactor sample cylinder which had been washed with detergent and rinsed with distilled water followed by acetone and dried. Careful cleaning removes contaminating substances which can activate to give a dangerously active container. Container and samples may be confiscated for storage for indefinite periods. This was delivered to the reactor operator for activation. The radiation officer monitored the container after activation and returned it if radiation was at a sufficiently low level. The reactor operator furnished the neutron flux, activation time, and the time of removal from the reactor.

Examination of the radioactivity of the strip chromatogram was performed with a Tracerlab (Waltham, Mass., U.S.A.) model SC-55S Auto/Step Chromatogram Scanner. The paper strip, affixed to a geared wand by cellophane tape, advanced under a slit collimator in discrete steps. The step length and slit width were both adjustable from 0 to 7 mm. Radiation passed through the slit to a TGC-2 end-window Geiger tube of 1.9 mg/cm^2 window thickness, 200 μsec recovery time, and 1400 V operating potential. The signal passed to a model SC-71 Compu/Matic II Scaler. This could be programmed to determine the number of counts in a preselected time interval or the time interval required for a present number of counts or whichever came first. On completion of the count the data passed to a model SC-88 Auto/Computer consisting of a Translator and Lister. This unit printed out on a tape the sample number, count, time, and computed the counts per minute. On completion of the printing step, the counter reset itself and the scanner advanced the strip to the next position. The scanner was set to advance the strip 5.0 mm per counting site and the slit width set at 5.1 mm to allow some overlap of sites. The counting time was varied to compromise between random errors of short counting periods and the half life which made long counting periods undesirable. In general, periods of one minute were satisfactory. The performance of the equipment depended upon the line voltage. This was metered and controlled by a Metered Variac, Type W5MT3AW (General Radio Co., Concord, Mass.) and kept at 115 to 125 V.

The circumstances which lead to multiple spot formation are complicated and will be described in detail in another publication. One circumstance seems to be the sodium content of the paper which must be reduced by washing. Routinely the strips to be used were immersed in a four liter beaker of 2 *M* acetic acid for 5 min followed by three consecutive 10 min immersions in distilled water. If the last rinse showed no difference in pH from distilled water as measured with a short-range pH paper, the papers were termed acid free. The paper strips were hung from a glass rack and each side washed with 20 ml of 95 % ethanol delivered from a polyethylene wash bottle. This was repeated with ethyl acetate. The papers were allowed to dry in air. This procedure removed any traces of grease and oil introduced during cutting and handling. Polyethylene gloves were worn while manipulating papers to further prevent contamination. The sodium content of fingerprints is sufficient to give a high activity on irradiation.

Chromatography was performed on 55 cm $\times$ 2.8 cm paper strips cut in the direction of machining from 47 $\times$ 55 cm sheets of Whatman Filter Paper No. 1, Acid Washed. Two different packages of paper were used which are designated B-1 and B-2.

RESULTS

To test the reliability of the method, known sodium salts of orthophosphoric acid were placed on a paper strip and the Na/P ratio determined by neutron activation. Table III presents the results of two independent determinations.

TABLE III

SODIUM/PHOSPHORUS RATIO IN SODIUM ORTHOPHOSPHATES AS DETERMINED BY NEUTRON ACTIVATION ANALYSIS

Salt	*Na/P*	
	Trial 1	*Trial 2*
NaH_2PO_4	1.0	1.2
Na_2HPO_4	2.0	2.0
Na_3PO_4	3.1	3.0

These same salts and phosphoric acid were chromatographed with 15% water/85% (pyridine–ethyl acetate, 45:100 v/v) as the forming solvent (mobile phase). Table IV presents the results of six chromatograms of the various solutes formed simultaneously in the same chamber for the same length of time. After radiocounting, the spots were sprayed with 0.4% ammonium molybdate in 8% nitric acid, dried, sprayed with 0.05% benzidine hydrochloride in 10% acetic acid and exposed to ammonia vapors as is outlined by BLOCK, DURRUM, AND ZWEIG[11]. Regions containing phosphate species developed a blue color. The Na/P ratios were determined at the point of maximum radioactivity in each phosphate solute zone. All of the chromatograms were formed with the goal of producing widely separated solute spots. This required that the forming solvent should over-run the chromatogram, *i.e.*, drip off the paper strip. Thus the distance of the spot from the origin can be reported but not the R_F value. Fig. 2 is a photograph of the revealed chromatogram.

For phosphoric acid, there is sufficient evidence of sodium in the zone but nothing near the 1/1 ratio required by the primary salt. The species is predominately

TABLE IV

DATA OF THE CHROMATOGRAMS OF THE SODIUM ORTHOPHOSPHATES (FIG. 2)

Paper No	*Solution applied*	*Distance of phosphate zone from origin (cm)*	*Na/P*
176	0.01 *M* H_3PO_4	17.5	0.03
177	0.05 *M* H_3PO_4	17.5	0.14
178	0.10 *M* H_3PO_4	16	0.05
179	0.01 *M* NaH_2PO_4	18.5	0.24
180	0.01 *M* Na_2HPO_4	5	0.75
		18	0.19
181	0.01 *M* Na_3PO_4	4.5	1.2
		18	0.19

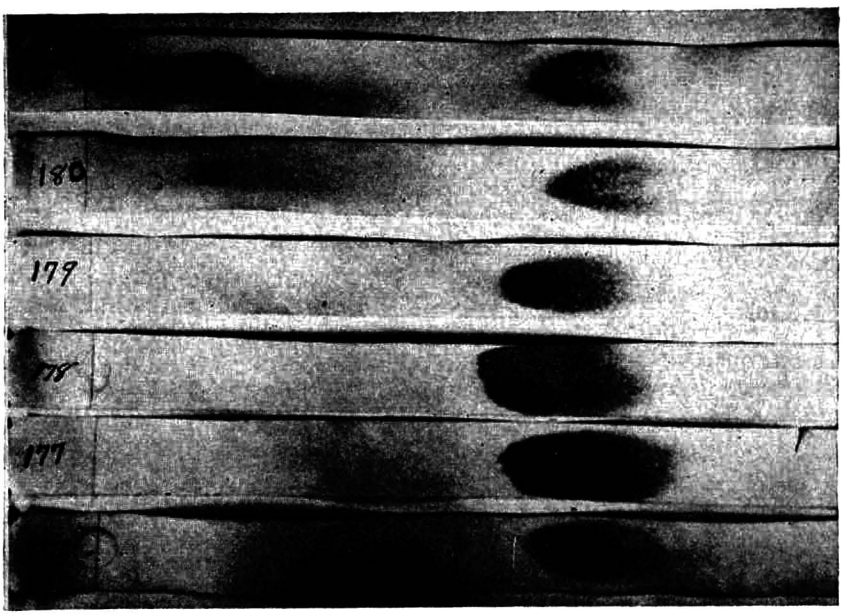

Fig. 2. Chromatograms of the sodium orthophosphates formed with pyridine–ethyl acetate/water (see Table IV).

H_3PO_4. There is evidence in support of the contention that the sodium came from the paper. With the primary salt, the single spot has a higher Na/P ratio than the phosphoric acid but it is sufficiently less than unity that the conclusion is that sodium was extracted from the solute zone and protons introduced. The same conclusion applies to the secondary and tertiary salts. In addition, the second or slow spot in both papers 180 and 181 has a Na/P ratio much less than expected from the composition of the salt applied. In the sense that the Na/P ratios of the solute zones in multiple spot chromatograms differ, the species are different as proposed by CURRY. The two zones do not originate in a discontinuity in the mobile phase[1]. However, the composition of these zones cannot be explained by a simple proton exchange. The production of the fast spot of low Na/P ratio, particularly significant in the case of the tertiary salt which contains no protons, strongly indicates a source of protons and sodium ions in the system and their participation in the chromatographic process. This source can only be the paper itself and the impurities therein.

A valid criticism of the foregoing discussion is that it is an error to assign a degree of protonation to the phosphate ion on the basis of the Na/P ratio since the pyridine can act as a base to accept a proton and behave as a cation, *i.e.*, the fast zone may be a pyridinium phosphate rather than phosphoric acid. This is very possible if one wishes to use this language of recognized stoichiometric formulas. However, chromatograms were also performed with butanol–water. The investigation with this solvent was not extensive because the time required for formation of the chromatograms was impractical (useful results were obtained in some cases *only* after 100 h of chromatography). Multiple spots were produced but the circumstances were different. Two spots were obtained from tertiary sodium phosphate and one

spot from the primary salt when formed with pyridine–ethyl acetate/water. Fig. 3 shows these salts chromatographed with 10% water in *n*-butanol; No. 323 is 0.01 *M* tertiary sodium phosphate (one spot) while No. 324 is sodium dihydrogen phosphate (two spots). The presence of pyridine in the forming solvent affects the multizoning but is not responsible for it. Table V gives the distance of the spots from the origin and the Na/P ratio as determined by neutron activation analysis. It is difficult to conceive that the sodium deficiency of the fast spot in No. 324 is compensated by any species other than protons.

Fig. 3. Chromatograms of the sodium orthophosphates formed with *n*-butanol–water (see Table V).

The occasion arose to employ eqn. (3) in an estimation of the sodium content of the paper. This required an estimate of Y and ϕ, the first of which is the greater problem. Unfortunately the facilities for direct calibration of Y were unavailable and a geometrical calculation had to suffice. This involved the distance from the paper to the Geiger tube window and the slit dimensions combined with the assumption that the sample is a point source spherical radiator at the center of the slit area. The oversimplified model showed that the fraction of the radiation sphere collected by the slit is at best 0.40 and it can be as low as 0.15. Thus the error in the detection coefficient is as much as 70% which overwhelms the 20% error in nuclear cross section and the estimated error in the neutron flux.

TABLE V

DATA OF THE CHROMATOGRAMS OF THE SODIUM ORTHOPHOSPHATES (FIG. 3)

Paper No.	*Solution applied*	*Distance of phosphate zone from origin (cm)*	*Na/P*
323	0.01 *M* Na_3PO_4	5	0.80
324	0.01 *M* NaH_2PO_4	5	1.2
		12	0.38

Samples of both batches of paper, acetic acid washed and unwashed, were analyzed for sodium by neutron activation. Table VI reports the sodium content. The per cent by weight sodium is based on $Y = 0.40$; the upper limit for the content in μequiv. is based on this same Y and the lower limit on a 70% error. The third column reports the sodium content of a section of a chromatogram, solute free, over which the forming solution had passed during chromatography. ULTEE AND HARTELL[12]

TABLE VI

SODIUM CONTENT OF CHROMATOGRAPHIC PAPER

Source	*Unwashed*		*Washed*		*Chromatographed*	
	Per cent by wt.	*μequiv.*	*Per cent by wt.*	*μequiv.*	*Per cent by wt.*	*μequiv.*
B-1	0.33	42–140	0.06	8–26	0.005	1–5
B-2	0.74	96–320	0.08	11–35		

report a carboxyl content of 8 μequiv./g for Whatman No. 1. The more extensive study of ACKERMAN AND KRÜGER[13] reports ion exchange capacities of 2.2 to 9.0 μequiv./g for a variety of German papers. This seeming agreement is deceiving for the two groups differ by a factor of ten for capacities of papers from the same manufacturers (they apparently did not duplicate grades since the numerical designations for the papers do not agree). If one presumes a capacity of 8 μequiv./g, and this is a very tenuous assumption, then the unwashed paper has a sodium content far above the exchange capacity of the paper, is reduced to slightly above this capacity on washing, and is slightly below capacity after chromatography. In view of the suspicion which must be attached to the published values of the exchange capacity of paper, a better estimate of Y would not provide any more reliable conclusion. One can calculate the relative reduction in sodium content brought about by these procedures. In this case, eqn. (4) is employed and the doubtful terms, *i.e.*, detector coefficient and nuclear cross section, cancel. Had the irradiation of the three paper samples been carried out simultaneously, the right-hand side of eqn. (4) would involve the ratio of N-values only. This was not the case, however, so the flux and irradiation time had to be retained. Nonetheless it can be said with a great deal of confidence that washing the paper reduces the sodium content by a factor of 7 to 8 and chromatography reduces the sodium content by a factor of 12 below that of the washed paper. This labile sodium of the paper must play a part in the chromatographic process, most likely as an exchangeable ion, and the paper, the various phosphate anions, and the bases in the forming solvent, including water, must all complete in some complex fashion for protons.

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SUMMARY

The solute zones obtained on paper chromatography of phosphates were analyzed for the sodium to phosphorus ratio by neutron activation analysis. Where

double spots were formed, the analysis indicated different phosphate species in the two zones. The work also indicated that the sodium in the paper plays an active role in the phenomenon, probably through some ion exchange mechanism.

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AN APPROACH TO THE ION EXCHANGE CHROMATOGRAPHY OF POLYELECTROLYTES

I. A MODEL BASED ON THE LAW OF MASS ACTION

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INTRODUCTION

It is recognized that at the present state of development of the theory of polyelectrolyte solutions no entirely satisfactory theory for the ion exchange of polyelectrolytes can be worked out. However, it would be convenient to have a relatively simple theoretical tool, if it would help to explain most, even if not all, of the peculiarities of the ion exchange chromatography of homologous polyelectrolytes. Such an approach is presented below. It accounts reasonably well for the experimental results which have been obtained with polylysines on carboxymethylcellulose[1], though it only takes into consideration ion exchange as interaction of electrostatic forces, while purposefully ignoring for the moment several other factors which undoubtedly do play a role in the chromatography of polyelectrolytes on ion exchangers.

However, we feel justified in publishing this approach, because its rather close agreement with the experimental data[1] indicates that it might have practical value in suggesting the optimal conditions for chromatography of polyelectrolytes on ion exchangers, and that this treatment may be taken as a basis for future developments, in which other factors will also be considered.

THE MODEL

Only the case where those groups of the polyelectrolyte that take part in the exchange and the groups on the exchanger's surface have the same affinity for each other is considered. This means that in the present model: (1) the charges of the groups of the polyelectrolyte, which take part in the exchange, are equal; (2) the charges on the groups of the exchanger are also equal; (3) no mutual interaction exists among the exchanger's groups, likewise among the polyelectrolyte's groups; (4) only negligible conformational changes (if any) accompany the ion exchange. That is, the steric fit between the charges of the exchanger and the charges of the polyelectrolyte only involves conformational changes corresponding to changes in free energy which are negligible in comparison to those of the exchange proper.

It is obvious that this model corresponds to a highly idealized case. Conditions

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(1), (3) and probably (4) do not hold in the case of proteins, for example. Condition (3) certainly does not hold with highly substituted synthetic ion exchangers, where a large pH- and ionic strength-dependent swelling obviously shows some interaction of the charged groups. Even in the case of ion exchange of polylysines on low-substituted carboxymethyl-cellulose[1] some interactions among the polylysine groups and among the exchanger's groups certainly occur. It is clear, however, that the ion exchange of polylysines on carboxymethyl-cellulose is closer to the idealized case than any ion exchange chromatography of proteins.

It is compatible with the proposed model that some groups of the polyelectrolyte are in an unfavourable steric position. Thus, n, the number of charges which take part in the exchange, may be smaller than the total number of charges of the polyelectrolyte and is not necessarily equal to the degree of polymerization of, say, polylysines or polyglutamic acids. However, except for the lower mers, it is reasonable to assume that n is proportional to the degree of polymerization. For the same reason the "monovalent ion" M (see next section) does not necessarily correspond to the monomer of the series considered.

It is assumed that a monolayer is formed on the exchanger's surface. This is likely to be the case with polyelectrolytes carrying only one type of charge, but it is probably not so with proteins.

As it has been pointed out in the introduction we are not attempting here to treat the effect of changes of the ionic strength on activity coefficients, on counter ion distribution, on conformation, on solvation etc. A change in counter ion distribution as well as a change in tertiary structure will, for example, affect n (the number of charged groups taking part in the exchange), and therefore also K_P (see later) Finally, we assume that the ion exchange is reversible.

SYMBOLS

(P) = concentration of the polyelectrolyte P.
(E) = concentration of the eluent E, monovalent.
(M) = concentration of the monovalent ion, M, having a mol. wt. equal to W_P/n.
Its only charged group is identical with one of the charged groups of P, which take part in the exchange.
n = number of the charges on P's surface which take part in the exchange.
They are all identical (see above).
Z = total number of charges on the surface of 1 g exchanger (in equivalents).
W_P = molecular weight of the polyelectrolyte P.
θ = fraction of the groups on the surface of the exchanger, which are bound to the polyelectrolyte, and to the eluent: $\theta_P + \theta_E = 1$.
α = sorption velocity constant.
f = activity coefficient.
K_P = $\alpha_P n/\alpha_E$.
K_M = α_M/α_E.
q = g of polyelectrolyte (q_P), and of monovalent ion (q_M), respectively, which are fixed by 1 g of exchanger.
Other symbols are used with their usual meanings.

THE ISOTHERM

A Langmuir treatment has already been applied to ion exchange of ions of equal charge[2]. Within the limits pointed out in the section on the model we will try to apply it to ion exchange of polyelectrolytes.

Let us consider the exchange reaction:

$$P^{n\pm} + AE_n \rightleftharpoons AP + n\,E^{1\pm} \tag{1}$$

The probability that a group of the exchanger is bound to E and is thus available for exchange with one group of the polyelectrolyte, is equal to θ_E. The probability that n groups of the exchanger should occur in sterically favourable positions, so that the corresponding groups of the polyelectrolyte could bind to them is, of course, $\theta_E{}^n$. The velocity of sorption of the polyelectrolyte is thus:

$$\alpha_P \theta_E^n f_P(P) \tag{2}$$

Similarly, since n E ions will take the place of one P ion on the exchanger's surface (*cf.* above), the velocity of sorption of E will be:

$$\alpha_E \theta_P f_E^n (E)^n \tag{3}$$

At equilibrium:

$$\alpha_P \theta_E^n f_P(P) = \frac{1}{n} \alpha_E \theta_P f_E^n (E)^n \tag{4}$$

By introducing symbols already defined and by rearranging, one obtains the sorption (ion exchange) coefficient:

$$\frac{q_P}{(P)} = \frac{K_P Z W_P (1 - \theta_P)^n f_P}{n(E)^n f_E^n} \tag{5}$$

This equation, which is an obvious consequence of what has been said previously, agrees well with what is found experimentally. The very high dependence of R_F values of polyelectrolytes on (E) is a common observation and is shown by the fact that (E) enters into the formulation of the sorption coefficient (Eqn. 5) at the nth power, n being by definition higher (and probably much higher) than 1. TISELIUS[3] and BOARDMAN AND PARTRIDGE[4] and others have already pointed out that every equation for the sorption coefficient of polyelectrolytes, if based on the law of mass action, should indicate this. The simple derivation given above is essentially an application of the law of mass action to surface phenomena.

It is also well known that this "all or nothing" chromatographic behaviour of polyelectrolytes becomes less and less evident as their groups are titrated—and this is actually one of the reasons why pH gradients in the isoelectric range are extensively used in protein chromatography[5,6]. This fact is also shown in Eqn. (5): a decrease of

n will decrease this strong dependence of the sorption coefficient on (E). (K_P also will be affected, see later).

Equation 5 also indicates that the isotherms of a polyelectrolyte increase in curvature as n increases. In fact, the higher n, the more sensitive the sorption coefficient towards small changes of θ_P. It is known that the chromatographic behaviour of proteins on ion exchangers corresponds to that of substances having strongly curved isotherms (see *e.g.* ref. 7): where tailings or mutual displacement phenomena are very frequent. This fact is also very clearly indicated by the chromatographic behaviour of polylysines on carboxymethyl-cellulose (ref. 1, Fig. 2). At constant eluent concentration the zones become more skew as n increases. Since equilibrium conditions were approached, this observation has to be referred to an increased curvature of the isotherms[8–11].

BOMAN[12] has reported that the displacement of proteins depends not only on the concentration of the displacer[8], but also on that of the small "eluting" ions. A likely explanation is provided by Eqn. (5) (and probably by other formulations[13] as well). At low (E) the isotherm of the displacer D is too high for a displacement to take place at the chosen (D) (Fig. 1a). An increase in (E) brings about a flattening of the isotherms, which is more evident for D than for P (Fig. 1b). Thus at this (E), a displacement can take place. At still higher (E), both isotherms become essentially linear and therefore displacement is no longer possible.

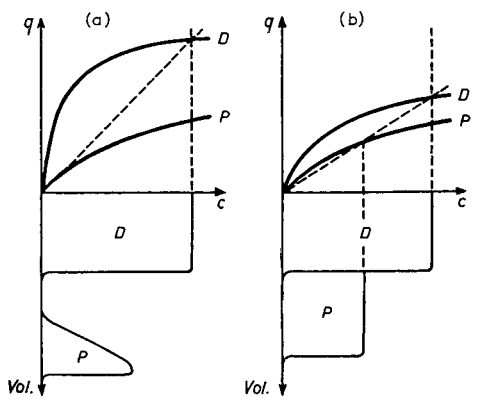

Fig. 1. Effect of (E) on the ion exchange isotherms of a polyelectrolyte P and of its displacer D. (a) at low (E); (b) at higher (E). For explanations of this suggested mechanism, see the section THE ISOTHERM.

Equation (5) is therefore in good agreement with the experimental observations available. It is unfortunate that not more than a qualitative check of this equation is possible at present for the limited amounts of polylysines available[1]. A quantitative check has been carried out, however, for the dependence of the chromatographic behaviour of polylysines on their polymerization number[1]. Since the equations tested (see later) are based on the same assumptions (see the Section MODEL), the fact that they fit the experimental data well is also indirect evidence that Eqn. (5) is essentially correct.

THE DEPENDENCE OF K_P ON n

In the reaction (1) the chemical potentials at the equilibrium are given by:

$$\frac{\mu^\circ_{AEn} + \mu^\circ_P - \mu^\circ_{AP} - n\mu^\circ_E}{RT} = \ln \frac{\theta_P(E)^n f_E^n}{\theta_E^n f_P(P)} = \ln K_P \tag{6}$$

Similarly, for the exchange reaction:

$$M^{I\pm} + AE \rightleftharpoons AM + E^{I\pm} \tag{7}$$

the chemical potentials are given by:

$$\frac{\mu^\circ_{AE} + \mu^\circ_M - \mu^\circ_{AM} - \mu^\circ_E}{RT} = \ln \frac{\theta_M f_E(E)}{\theta_E f_M(M)} = \ln K_M \tag{8}$$

From the assumptions made in the section MODEL

$$\mu^\circ_{AEn} = n\mu^\circ_{AE} \qquad \mu^\circ_{AP} = n\mu^\circ_{AM} \qquad \mu^\circ_P = n\mu^\circ_M$$

Thus,

$$n \ln K_M = \ln K_P \tag{9}$$

At constant (E), for $(E) \gg (P)$ and for $(E) \gg (M)$ (*i.e.*, at the initial part of the isotherm, where θ_P and θ_M can be neglected), by assuming that:

$$\frac{f_P}{f_M f_E^{n-1}} = 1 \tag{10}$$

one obtains, by comparison between the sorption coefficients of P and M at the same (E), that they are related as follows:

$$\ln \frac{q_P}{(P)} = \ln \frac{q_M}{(M)} + (n-1) \ln \frac{K_M}{(E)} \tag{11}$$

Within a homologous series of polymers $\ln[q_M/(M)]$ is, of course, constant. Since the retention volumes Δ are directly proportional to the sorption coefficients[9–11], one obtains:

$$\ln \Delta = a + (n-1) \ln \frac{K_M}{(E)} \tag{12}$$

where a is a constant, which depends on the type of polymer series, on the exchanger, on the column and on the initial conditions.

Equation (11) has been checked in batchwise isotherms and equation (12) in column experiments with elution at constant (E) (ref. 1, Fig. 1). In each set of ex-

periments the agreement with the theory was excellent. Moreover, similar values of K_M were found in both cases, in spite of the somewhat different experimental conditions.

These equations also provide an explanation for the common observation that in ion exchange chromatography of polyelectrolytes (*e.g.*, proteins, nucleic acids, etc.) it is usually not possible to elute more than a few components with a single concentration of the eluent. It is apparent, in fact (Eqn. 12), that at high values of n the retention volumes reach unworkable values.

Elution of polyelectrolytes is often accomplished by increasing (E) gradually. The individual polyelectrolytes travel more slowly than the gradient until they are reached by the lowest (E) in which they have an R_F of 1. From this moment on all polyelectrolytes have the same sorption coefficient and travel at the same speed as the gradient. Thus, if the column is long enough for the gradient used, a simple correlation exists between n and the (E) in which the individual polyelectrolytes emerge from the column. At $(E) \gg (P)$ and $(E) \gg (M)$, from the formulations of the adsorption coefficients of P (Eqn. 9) and of M (from Eqn. 9), and assuming the validity of Eqn. (10), it is easy to show that such a relation is:

$$\frac{K_M{}^n}{(E_P)^n} = \frac{K_M}{(E_M)}, \textit{ i.e. } \ln (E_P) = \ln K_M - \frac{1}{n} \ln \frac{K_M}{(E_M)} \tag{13}$$

where (E_P) and (E_M) are the lowest (E) at which P and M, respectively, have an R_F of 1.

This equation also has been tested with polylysines on carboxymethyl-cellulose (ref. 1, Fig. 3): it fits well the experimental data, except for the higher mers, which require a higher (E) than predicted. This is probably due to salting out effects, which are likely to be more evident with higher mers than with low ones. It is interesting to note that the value of K_M obtained from Eqn. (13) agrees well with those obtained from Eqns. (11) and (12), in spite of the different experimental approach.

CONCLUSIONS

The present equations should not be expected to hold quantitatively in ion exchange of proteins. In fact, in the latter case, some of the stated or understated assumptions of the present treatment are not likely to hold: (1) The charged groups on the protein molecule are of different types and often show evident interactions; (2) proteins, being polyfunctional, may well form multiple layers on the exchanger surface; and (3) it is doubtful whether equilibrium conditions are ever attained in the chromatography of very large molecules; etc.

The combined effects of these and other factors are that the sorption isotherm is more curved, and it may not reach a saturation level, the "elution isotherms" do not necessarily coincide with the sorption isotherms, etc. In the case of proteins, the present treatment can therefore only be regarded as semiquantitative. However, the equations obtained can be used as a guide to establish the conditions for protein chromatography, as they point out some of the main parameters affecting the sorption coefficient; in changing the conditions care should of course be taken that no opposing effects influence the sorption coefficient.

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SUMMARY

Some considerations on the ion exchange of polyelectrolytes are presented. The approach is limited by the several assumptions. Nevertheless, the equations presented describe reasonably well the ion exchange of polylysines on carboxymethyl-cellulose (described in the following paper) and they agree, also, at first approximation, with the chromatographic behaviour of proteins on ion exchangers.

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AN APPROACH TO THE ION EXCHANGE CHROMATOGRAPHY OF POLYELECTROLYTES

II. EXPERIMENTAL EVALUATION OF THE MODEL

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INTRODUCTION

In the preceding paper[1] one of us has suggested a model for the chromatography of polyelectrolytes by ion exchange. The model is based essentially on the law of mass action, as applied by LANGMUIR to surface phenomena. Although the validity of the treatment is certainly limited by the assumptions made, it did bring out most of the peculiarities observed in the chromatography of polyvalent ions when compared with monovalent ions. Reviews have appeared which summarize some of the most important concepts of ion exchange as applied to separations of complex polyelectrolyte mixtures[2–6]. An ideal system for reducing the above model to experimental verification would be the chromatography of a family of polyelectrolytes of increasing charge and size. Such a family or homologous series can be obtained from partial hydrolysis of macro ions such as polylysine[7,8], polyglutamic acid[9], polyadenylic acid[10], and polythymidylic acid[11]. As was initially shown by STEWART AND STAHMANN[12], the first 20 members of the lysine homologous series can be fractionated on carboxymethyl-cellulose[13]. The chromatographic properties of the members of this series, under various elution conditions, have been used to test certain relationships predicted by the theory. Exchange isotherms of lysine polypeptides of various concentrations have been determined, and they give reasonable supporting evidence to the model used.

EXPERIMENTAL

Column packing

All of the experiments described in this manuscript were carried out at room temperature on carboxymethyl-cellulose[13], CM-C, with a capacity of 0.8 mequiv./g**, which had been sieved on U.S. standard mesh sieves, and the fraction between 200–325 mesh washed and prepared according to the method of PETERSON AND SOBER[4]. The sodium form of the exchanger was used. Columns were packed by pumping a dilute

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** Brown Company, Berlin, N. H., Lot No. 1222.

slurry into Pyrex cylinders, initially filled with the slurry[14]. These cylinders were stoppered at either end with special adapters, designed to keep mixing of effluents at a minimum.

Column elutions

Three types of elution were used depending on the experiment being performed. The first made use of an exponential gradient, the second of a linear gradient, and the third was an elution at constant eluent concentration. The exponential gradient mixing device consisted of a one liter mixer[12], and the linear gradient was produced by using two of the three 500 ml chambers of a modified multichambered gradient mixing device[14].

All of the chromatograms were monitored at 220 mμ, which is a convenient wavelength for peptide bond detection[15,16]. Except for the batchwise determinations, effluents were continuously monitored in 1 cm quartz flow-cells in either the Zeiss PM Q II or Beckman DB spectrophotometer. The Cary 11 was used for determining concentrations from the batchwise experiments. A peristaltic finger pump was convenient for pumping eluents at about 1.5 ml/h and faster, however, some difficulties were experienced using this pump at slower flow rates.

Preparation of hydrolyzates

Forty milligrams of high molecular weight polylysine* (mol. wt. 110,000) was suspended in 6 ml of 6 *N* HCl at 70°, and 2 ml aliquots removed at 20, 40 and 80 min. After excess HCl was removed by rotary evaporation, each aliquot was analyzed by column chromatography as previously described[12]. It was found that the 80 min hydrolyzate gave a size distribution which was suitable for the large scale isolation and purification of peptides containing from 1–15 lysine residues. Material prepared in this manner was also used in experiments which were designated to test the chromatographic relationships predicted by the theory.

Preparation of lysine polypeptides

Five hundred milligrams of the polylysine was hydrolyzed as above and suspended in 50 ml of distilled water and chromatographed in 40 mg portions on a 0.9 × 34 cm column containing about 3.5 g of CM-C at 2 ml/min with an exponential gradient[12]. Peptides of equal size were pooled, diluted about 10 fold, added directly to the same column at the same flow rate, and rechromatographed. All peptides probably did not require this 10-fold dilution, but the lower limit for quantitative recovery was not determined. Volatile buffers were not used because of their absorption in the region used for monitoring column effluents.

Determination of exchange isotherms

Concentration isotherms of lysine peptides containing from 3–13 residues were determined batchwise in 10 ml polyethylene disposable syringes, containing a filter disc and 0.1 g of CM-C. These syringes provided a convenient method for adsorbing the chromatographically purified peptides from high dilutions, for determining peptide concentrations at various levels of the experiment, and for mixing the slurry during each equilibration period.

* Pilot Chemicals, Inc., Watertown, Mass., Lot No. L-22.

The high dilution of the peptides was a consequence of diluting out the NaCl to such a level that each peptide could be quantitatively adsorbed by percolating the same through the CM-C bed, which was allowed to settle in each syringe. Following this adsorption each bed was flushed with distilled water, which was allowed to drain freely from the syringes. An additional 0.5 ml was removed by compressing the bed with the plunger. At this point 3.0 ml of 0.43 *M* NaCl was added through the filter disc of each syringe. Buffer was not used because it did not prove to be necessary during the column chromatography of lysine peptides by gradient elution[7].

The concentration of NaCl used during the isotherm determinations was chosen because at this concentration a representative family of peptides could be obtained by constant salt elution of a partial acid hydrolyzate of polylysine. A control syringe was used to determine non-peptide absorbing material which washed from the CM-C during the experiment. This absorption was subtracted from that obtained during all syringe experiments.

The exchange isotherms were calculated from the effluent histories of each batch experiment. The amount of peptide in the stationary phase at any one mobile phase concentration was calculated by subtracting the total amount of peptide in the latter phase from that remaining in both phases. The volume of the mobile phase was about 3.2 ml. Concentrations were determined by using a molar extinction coefficient of 600 for the peptide bonds. Absorbancy measurements provided the information required for determining the individual peptide concentrations.

Symbols

The symbols used in this paper have been taken from the previous publication where the theoretical considerations are reported[1]. DP is the abbreviation for degree of polymerisation.

DISCUSSION AND RESULTS

As mentioned earlier, this publication is directed toward the experimental verification of certain relationships predicted by the mass action law for polyelectrolyte exchange in chromatography. The model used has been discussed in some detail in the preceding publication[1], and it is the purpose of this paper to check the theory with a homologous series of lysine polypeptides.

Establishment of equilibrium

One of the basic assumptions is that local equilibrium be established between the solid and mobile phases in the ion exchange column at every stage of the chromatographic development. Under ordinary elution conditions equilibrium is seldom established[17], however, it can certainly be approached. One of the effects of non-equilibrium is the broadening of the solute bands as they move down the column[17]. The criteria used for determining the flow rate at which equilibrium was approached was the height and sharpness of peptide zones as they emerged from the column. Such zones seemed to reach a maximum height and sharpness about 10 ml/h/cm^2.

Retention volume predictions

If for a first approximation the assumptions made in the theoretical treatment

are reasonably good[1], then during constant salt elutions a plot of the log Δ versus n-1 should give a straight line, the slope being log $K_M/(E)$. Because n is unknown, Δ was plotted as a function of the degree of polymerization, DP, as in Fig. 1. The degree of polymerization was obtained from the order of emergence of each peptide from the chromatographic column[12], and it is probably proportional to n. The value of K_M as calculated from the slope was 705.

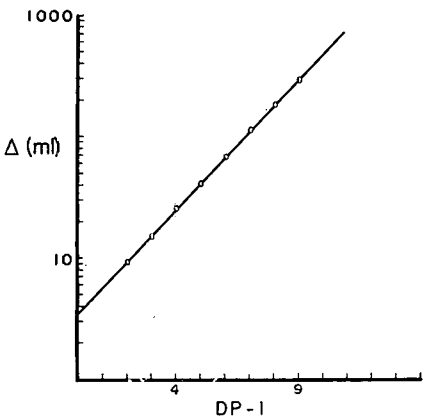

Fig. 1. Relationship between the log Δ and DP. Δ = retention volume. DP = degree of polymerization. The experimental points were calculated from the chromatogram shown in Fig. 2.

The experimental points used in calculating the above retention volumes were obtained from a chromatogram in which 10 mg of hydrolyzate was eluted with 0.43 M NaCl, at 6 ml/h as shown in Fig. 2. A similar experiment performed at one-third this flow rate gave comparable retention volumes. The fact that the zones emerging from the column became increasingly more asymmetric as DP increased is in good agreement with the theory[1].

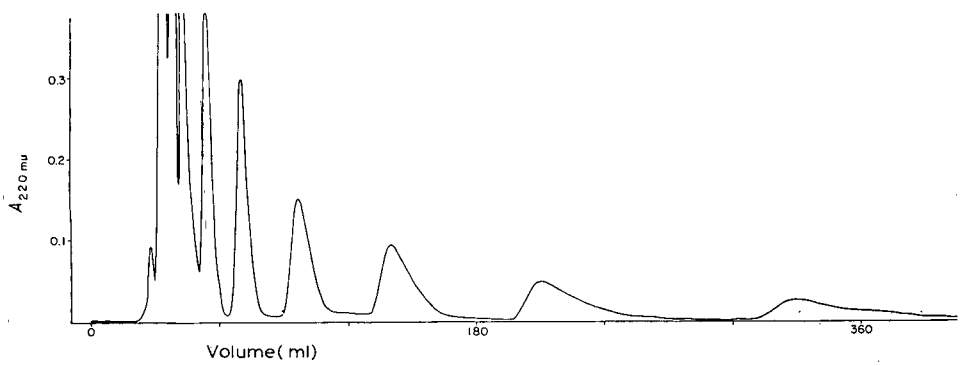

Fig. 2. Elution at constant eluent concentration of a partial acid hydrolyzate of poly-L-lysine hydrobromide. Ten milligrams of polylysine was added to a 0.9 × 34 cm column packed with about 3.5 g of carboxymethyl-cellulose 0.8 mequiv./g and eluted with 0.43 M NaCl at 6 ml/h. The salt front emerged with the front shoulder of the first peak, lysine. Hydrolyzate was prepared by reacting high molecular weight polylysine with 6 N HCl for 80 min at 70°.

The derivation of the above and subsequent relationships required that $\theta_E \gg \theta_P$. This assumption is probably valid in that the total peptide in any one zone on the column was always considerably less than 1.0% that present during isotherm determinations at high peptide concentrations.

Gradient elution predictions

If one accepts the approximations mentioned in the previous section, and operates the column under gradient conditions such that all of the peptides reach an R_F of 1, or nearly 1, before they emerge from the column, a plot of log (E) versus 1/DP should give a straight line. The intercept at 1/DP = 0 is equal to log K_M. To obtain such a system, a series of chromatograms were run such that the eluent volume was decreased proportionally with the flow rate, the maximum concentration of the eluent and overall time of the experiment remaining constant. A plot representing the data can be seen in Fig. 3, in which the concentration of the effluent at the point of emergence of each peptide was plotted as a function of 1/DP. If the linear portion of each curve is extrapolated to 1/DP = 0, values for K_M between 690 and 800 are obtained. The point to which all of the curves have been extended represents high molecular weight polylysine (110,000), 860 residues per molecule.

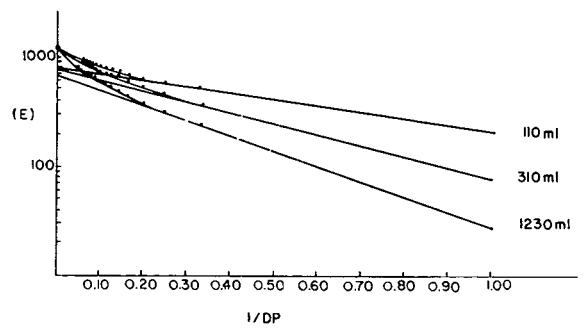

Fig. 3. Relationship between log (E) and 1/DP. (E) = elution concentration of various lysine peptides, DP = degree of polymerization. The experimental points used for plotting the curves were taken from chromatograms in which 10 mg of polylysine hydrolyzate were eluted with a constant gradient, 0–1.0 M NaCl, of 1230, 310 and 110 ml, at 6, 1.6 and 0.6 ml/h, respectively. The point to which the curves are extended is the elution concentration of unhydrolyzed high molecular weight poly-L-lysine hydrobromide, containing an average of 860 residues per molecule.

The fact that one obtains a curve and not a straight line can probably be attributed to the effect of increasing NaCl concentrations on the activity coefficients. As DP increases, the effect of this is felt more than for low DP. The change in activity coefficients would turn up essentially as a "salting out" effect which is known to appear much before the substance actually precipitates[18]. This effect, by increasing the affinity of the polylysines for the adsorbent, would make them emerge at a later point of the gradient, *i.e.*, at higher salt concentrations. Such an effect should be less apparent in constant salt experiments.

Determination of exchange isotherms

The isotherms were determined batchwise according to the method described above (Fig. 4). The fact that some of the curves do not fit well in the family of curves

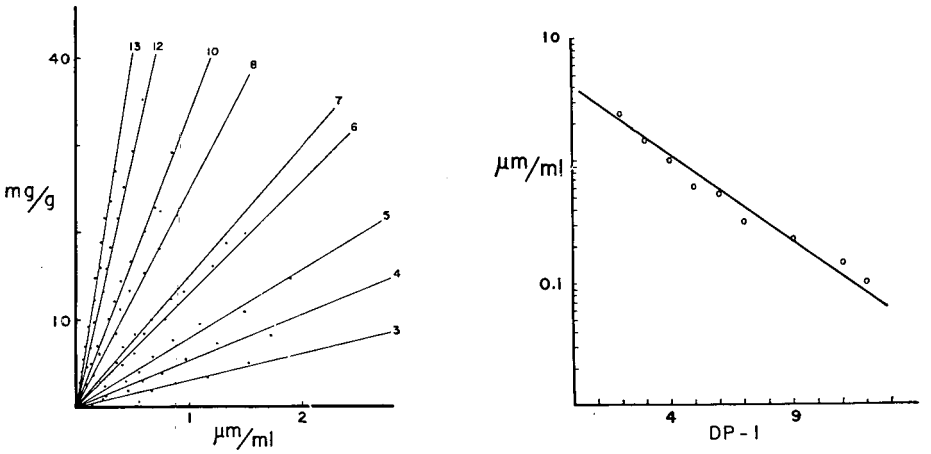

Fig. 4. Isotherms of a series of lysine peptides as determined from batch experiments. Each isotherm was determined on 0.1 g of carboxymethyl-cellulose, 0.8 mequiv./g, at 0.43 *M* NaCl. The ordinate represents mg of peptide per g of exchanger, and the abscissa the concentration of peptide in the mobile phase, 3.2 ml. The degree of polymerization is given above each curve.

Fig. 5. Concentration relationship between isotherms of a series of lysine peptides. Ordinate gives peptide concentration in mobile phase at a constant stationary phase concentration, 7.7 mg/g (Fig. 4). DP = degree of polymerization.

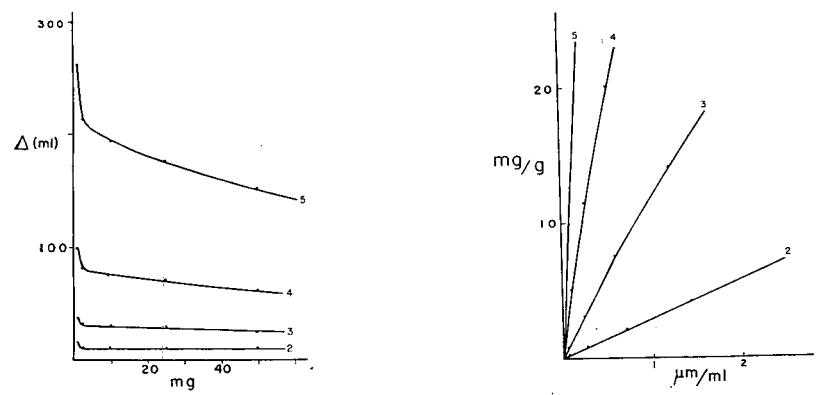

Fig. 6. Relationship between the retention volume and mg of poly-L-lysine hydrolyzate used during one step elutions. Experimental points were taken from chromatograms similar to Fig. 2, the difference being that they were determined at 0.25 *M* NaCl at 60 ml/h with 1–50 mg hydrolyzate. The degree of polymerization is given to the right of each curve.

Fig. 7. Isotherms of a series of lysine peptides as calculated from retention volumes, Fig. 6, using 0.72, a value calculated from Δ intercept at DP = 0 (Fig. 1), as the proportionality constant. Peptide concentrations corresponding to particular retention volumes were taken as a fraction, mg hydrolyzate added to column/50 mg, of the maximum concentration of each peptide obtained during the 50 mg elution.

set by the others may be explained by differences in the hold up volumes of the various syringes. Variations of 0.1 ml were shown to have a significant effect on the slope of the isotherms eventually obtained. The release of 220 mμ absorbing material from columns to which no peptide had been adsorbed also added some uncertainty.

Equation (11) of the preceding paper[1] states that a plot of log (P) against ($n-1$) should give, at constant q_P, a straight line with a negative slope ($-\log [K_M/(E)]$). The experimental points obtained do, in fact, lie on a line (Fig. 5). The value of K_M calculated from this plot, 586, is of the same order of magnitude as that obtained from constant salt elutions at the same NaCl concentration.

During the experiments reported above and carried out under conditions of gradient or constant salt elution, it was observed that the relative position of each peptide was not a function of flow rate. In another set of experiments we have determined the relative distribution coefficients, and therefore the isotherms, under non-equilibrium conditions. Chromatograms were made at 60 ml/h, with increasing amounts of hydrolyzate (1–50 mg) in 5 ml of distilled water, and eluted at 0.25 M NaCl. The data are represented in terms of retention volumes (Fig. 6). In this figure, Δ, and therefore the distribution coefficient, is indeed a curved function of polyelectrolyte concentration and DP.

If, however, the data are represented in terms of mobile and stationary phase peptide concentrations, comparable with Fig. 4, such non-linearity is difficult to detect (Fig. 7).

CONCLUSIONS

The mass action theory of ion exchange chromatography, as presented in an earlier paper[1], has been tested with a homologous series of lysine peptides. These experimental tests were based on the elution characteristics of this polylysine series under various chromatographic conditions. There was good correlation between the theoretical predictions and the experimental data. Isotherms of these peptides were shown to be related in a manner predicted by the theory. Moreover, data were also obtained on the isotherms of polylysines under non-equilibrium conditions.

Although the relationships used in testing the theory were derived by making a series of assumptions and approximations which are only rarely approached in real systems, they do seem to describe adequately the ion exchange process involved in the chromatography of lysine peptides, which presumably is dominated by electrostatic type forces.

ACKNOWLEDGEMENTS

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SUMMARY

This publication is directed toward the experimental verification of certain relationships predicted by a mass action theory for polyelectrolyte exchange in chromatography. These relationships describe the ion exchange process as determined with a family of lysine polypeptides of increasing size and charge. It is concluded that the theory, in addition to explaining certain peculiarities observed in protein chromatography, provides a mathematical treatment which, as a first approximation, was experimentally verified.

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ZONE ELECTROMIGRATION OF SOME GALLIUM AND INDIUM COMPLEXES

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INTRODUCTION

Zone electromigration is a simple technique for obtaining information about complex ions in solution. The sign of the charge on the moving species is immediately evident from the direction of motion so that this technique is particularly useful for studying complexes of metal ions with anionic ligands. In addition, data may be obtained on the conditions under which a given species can exist in solution.

The observed zone mobility of a metal ion in a complexing medium is actually the weighted mean of the zone mobilities of all of the metal-containing species which are present in equilibrium with one another[1]. Thus it is influenced by the formation constants of the system. The variation of zone mobility with pH or ligand concentration is expected to be abrupt only in those regions where the predominating complex species is being replaced by another of different charge. Experimental results often show the character of a roughly constant zone mobility changing more or less sharply to another constant value over a narrow range of pH. This has been used in the estimation of complex formation constants[2,3]. The relation between mobility and charge or structure of a given species is complicated by such factors as partial ionization and absorption by the paper. However, complex ions with the same charge and similar structure would be expected to have similar mobilities, although the molecular weight may enter into this as well[2].

If a metal ion forms two species in solution which exchange with one another very slowly, two distinct zones may be observed on electromigration. An intermediate exchange rate may lead to an elongated, or comet-shaped spot. This may be the cause of part of the trailing sometimes observed in complexing media, although other factors also lead to this[1].

We have applied zone electromigration to the study of gallium(III) and indium(III) ions in a number of organic acid solutions. It is known that these ions are complexed by ligands of this type, although only slight attention has been given to their study.

EXPERIMENTAL

A closed strip technique was used in which the paper was sandwiched between two metal plates insulated with polyethylene and cooled with tapwater. Four strips could be run in parallel. Electrolyte reservoirs were not used; the ends of the paper

were in direct contact with platinum foil electrodes. By using suitable buffer solutions of the acid and its salts at the ends, pH changes arising from electrolysis were localized. Measurements showed that the pH of the strip where migration took place remained constant. This technique minimized electroosmosis, and separate experiments showed that it was negligible.

The solutions of complexing agents were made up to 0.1 *M* in free acid and the pH adjusted shortly before use with 1 *M* NaOH or HNO_3. Fumaric, phthalic, and salicylic acids were not soluble to the extent of 0.1 mole/l, so that saturated solutions were used.

Whatman No. 3 MM chromatography paper (4 cm × 25 cm) was used as supporting medium; in each experiment, spots of Ga and In were run side by side on the same strip. Migration times of about 1 h at a voltage gradient of about 15 V/cm were generally suitable. The absorbance was maintained near 0.025 ml/cm^2. Roughly 0.01 ml of migrant solution at concentrations less than 0.05 *M* were used.

The gallium(III) and indium(III) were in solution as the nitrates, with an excess HNO_3 content just sufficient to prevent precipitation of the hydroxides. Too high an acidity interfered with migration and led to non-reproducible results. The migrant zones were detected with alcoholic alizarin solution, followed by exposure to NH_3 vapor when necessary.

Paper chromatograms were obtained using the ascending technique.

Mobilities and R_F values were determined by measuring from the center of the zone to the starting point. When comet-shaped or trailing zones occurred, measurement was made from the center of the most intensely colored region.

RESULTS AND DISCUSSION

Zone mobilities are collected in Table I, and chromatographic R_F values in Table II. Comet-shaped zones are indicated by c, and precipitation at the starting point with no motion, by p. The R_F values labelled c refer to spots that streaked back to the starting point. The systems in which strong complexing was indicated by electromigration gave R_F values close to unity, but for some of the others lower values were found. With gallic and salicylic acids at low pH values, two solvent fronts were indicated by zones of different pH on the chromatogram. The R_F values were measured with respect to the first front, but the spots actually moved with the second, more acidic, front.

These R_F values are included to give an indication of possible absorption by the paper. The mobility values could be corrected using these results, but this would not change the basic arguments which follow.

The behavior of gallium and indium is similar in each complexing medium investigated. The only notable difference lies in the appearance of anionic gallium complexes at a lower pH than those of indium, implying a stronger complexing tendency for the former. Anionic species are formed with all ligands which are likely to form chelate structures utilizing carboxyl or hydroxyl groups. Thus, oxalato and malonato complexes with relatively high anionic mobilities are formed, while succinic acid, which could chelate only with the formation of an unstable seven-membered ring, gives almost no anionic motion. With the latter, precipitation takes place at pH 5–6, while in more strongly complexing media there is none until pH 9 or greater.

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TABLE I

ZONE MOBILITIES OF GALLIUM AND INDIUM COMPLEXES

U = Mobility × 10^3 $cm^2 \cdot V^{-1} \cdot min^{-1}$; — = motion toward cathode; + = motion toward anode.

Acid											
Oxalic	pH	0.3	1.0	1.3	2.0	3.0	4.0	5.0	6.0	7.0	9.5
	U Ga	—1.5	+4.5	+8	+11	+11.5	+11	+11c	+10.5c	+11; +5.5	+12; +7
	U In	—4	+1	+2	+10	+10	+10	+10.5	+9.5	+9.5; +3.5	+9.5; +5
Citric	pH	0.3	1.0	2.0	3.0	4.0	4.3	5.0	6.0	7.0	9.0
	U Ga	—4	+3	+4.5c	+7	+6.5	+7	+7.5c	+7.5c	+7.5c	+6.5c
	U In	—4	+1	+2	+6.5	+7c	+7.5	+7.5	+8	+8.5	+8
Malonic	pH	0.3	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	10.3
	U Ga	—7	—6	+6.5	+7	+8	+9.5c	+7.5c	+9	+8.5	+9
	U In	—6.5	—7	+2.5	+5	+7.5	+9	+7.5	+9.5	+9c	+9
Tartaric	pH	0.3	1.0	2.0	3.0	4.0	5.0	5.4	6.0	7.0	9.0
	U Ga	—7	—1	—0.5	+5	+6c	+5.5c	+5.5c	+8c	+8c	+7.5c
	U In	—8	—2.5	—2.5	+4	+6	+5.5	+5.5	+8	+7.5	+7
Malic	pH	2.0	3.0	4.0	5.0	5.5	6.0	7.0			
	U Ga	+1.5c	+4c	+5.5c	+5.5c	+5.5c	+5.5c	+5.5c			
	U In	—2.5c	+2	+5.5	+6.5	+6	+9	+7			

Gallic	pH	2.0		3.0		4.0		5.1		6.0		7.0
	U Ga	−1c		+1c		+2c		+2.5c		+2.5c		+2.5c
	U In	−3		−1		+1.5		+2c		+1.5c		+2.5c
Lactic	pH	2.0		3.0		4.0		5.0		6.0		7.0
	U Ga	-5		−2.5		+3c		+3c		+2c		+3c
	U In	−5c		−5		+2.5		+2c		+1.5c		+1.5c
Salicylic	pH	2.0		3.0		4.0		5.0		6.0		7.0
	U Ga	−2.5c		−3c		+1c		+1.5		+2c		+1c
	U In	−5		−3.5c		−0.5c		−2c		−1c		−2c
Succinic	pH	2.0		3.0		4.0		5.0		6.0		
	U Ga	−8c		−3.5c		−1.5c		p		p		
	U In	−6.5		−4		−1		+2.5c		p		
Phthalic	pH	2.0		3.0		3.9		5.0		6.0		7.0
	U Ga	−6c		−1c		−0.5c		+1c		p		p
	U In	−5.5		−2		−1c		+1c		+1c		p
Fumaric	pH	2.0		3.0		4.0		5.0				
	U Ga	−6.5		−2.5c		p		p				
	U In	−6		−2.5		p		p				

c = comet-shaped zone.
p = precipitation at the starting point.

TABLE II

R_F VALUES OF GALLIUM AND INDIUM COMPLEXES

Acid								
Oxalic		pH	0.3	1.0	3.0	5.0	7.0	9.5
	R_F	Ga	1	1	1	1	1	1
		In	1	1	1	1	1	0.9
Citric		pH	0.3	1.0	3.0	5.0	7.0	
	R_F	Ga	1	1	1	1	1	
		In	1	1	1	1	1	
Malonic		pH	0.3	1.0	3.0	5.0	7.0	10.3
	R_F	Ga	1	1	1	1	1	0.8
		In	1	1	1	1	0.9	0.9
Tartaric		pH	0.3	1.0	3.0	5.0	7.0	9.0
	R_F	Ga	1	1	0.9	0.9	0.9	0.9
		In	1	1	1	1	1	1
Malic		pH	2.0	4.0	5.5	7.0		
	R_F	Ga	1	1	1	1		
		In	1	1	1	1		
Gallic		pH	2.0	3.0	4.0	7.0		
	R_F	Ga	0.6*	0.8*	1	0.9		
		In	0.6*	0.8*	0.9	0.9		
Lactic		pH	2.0	4.0	6.0	7.0		
	R_F	Ga	0.9	0.8	1.0	1.0		
		In	0.9	0.7	0.5c	0.3c		
Salicylic		pH	2.0	3.0	4.0	7.0		
	R_F	Ga	0.6*	0.5*	0.5c	0.5c		
		In	0.6*	0.5*	0.3c	0.3c		
Succinic		pH	2.0	3.0	4.0	6.0		
	R_F	Ga	0.9	0.6	0.3c	p		
		In	0.9	0.7	0.8	p		
Phthalic		pH	2.0	3.0	3.9	5.0		
	R_F	Ga	0.8	0.6	0.4c	0.2c		
		In	0.8	0.7	0.6	0.3		
Fumaric		pH	2.0	3.0	5.0			
	R_F	Ga	0.9	0.4	p			
		In	1	0.5	p			

* Two solvent fronts.
c = spot streaks back to the starting point.
p = precipitation at the starting point.

Malic and tartaric acids which can form 5- or 6-membered chelate rings also lead to high anionic mobilities.

Possible species would be $[MA_3]^{3-}$ with the bidendate oxalate and malonate ligands, and $[MA_2]^{1-}$ with the tartrate and malate ions which can be tridendate. The relative mobility values would be in accord with such formulas, but there is no real proof that the completely chelated species are formed here. In all likelihood the citrate

ion is also tridendate; this would lead to an anionic bis-complex. The three carboxyl groups here lead to a possible charge of 3– on the citrate ion, although they need not all be ionized in the complex.

The slight anionic motion observed with the monocarboxylic acids, lactic, gallic and salicylic, would imply at least partial ionization of the hydroxyl groups involved in chelation if tris-complexes are formed. Species with coordinated OH^- groups, or with more than three acid ions coordinated through only one position, may be contributing.

Phthalic and fumaric acids, which are unlikely to chelate well, lead to no significant anionic motion. The low mobilities observed do imply the formation of complexes, although the observed precipitation at comparatively low pH values indicates that these are weak.

The zone mobilities of gallium and indium in KNO_3 solution where complex formation is unlikely are -2.9 and $-4.0 \cdot 10^{-3}$ $cm^2 \cdot V^{-1} \cdot min^{-1}$, respectively at low pH. These comparatively low values imply strong interactions with the supporting medium as would be expected for tervalent species. The mobility increases when a neutral hydroxyl ligand such as glycol is added, but the zones show considerable trailing. The much higher cationic mobilities at low pH values in some of the complexing media (malonic, tartaric, and succinic acids, in particular) may then be caused by the existence of cationic complex species, which, while having a reduced charge, interact much less strongly with the paper.

The results of this work cannot be related directly to other studies, most of which have been made at a constant (high) ionic strength. A comparison is of interest in several cases, however.

COZZI AND VIVARELLI[4] studied indium complexes in tartrate, malate and succinate solutions by electrode potential measurements. For all of these complexing agents they proposed the species $[In(HA)(A)]$ at pH < 3, and $[In(OH)(A)_2]^{2-}$ at pH > 3. (The neutral acid would be represented by H_2A.) Moreover, the formation constants were the same for all three ligands. Our results indicate that the behavior of indium in succinic acid solutions is markedly different from that in the other two acids; the formation of anionic species is much reduced. In addition, there is no indication in any case of a predominating neutral species at low pH.

The gallium tartrate complexes proposed by SAVCHENKO AND GONCHAROV[5] from potentiometry and conductance were $[Ga(A)]^+$ at pH 1.5, $[Ga(A)_2]^-$ at pH > 3, and a mixture of species, chiefly $[Ga(HA)_2(A)]^{1-}$ and $[Ga(HA)(A)_2]^{2-}$, at intermediate values. Our results do not disagree with these proposals.

DUTT AND BOSE[6] have found that the mono-, bis-, and tris-malonato complexes of gallium exist in solution; the first predominates at pH < 2.25, the second, between pH 2.25 and 3.5, and the last, at higher pH's. Again, while our results indicate that the cationic species ceases to be important at a lower pH, there is otherwise no disagreement.

ZELYANSKAYA AND BAUSOVA[7] have polarographic evidence for $[Ga(C_6H_4OCOO)_3]^{3-}$ in salicylate solutions at pH 2.5–4.5. Under our conditions, we have a cationic species changing to an anionic one at pH 3–4. The low anionic mobility makes an ion with charge -3 unlikely as the predominating species.

At pH 7 and above there is definite evidence for the formation of two oxalato complexes with both gallium and indium. Evidently these do not exchange with one

another rapidly here, as the two zones observed for each metal were distinctly separated. The new species has a lower mobility, indicating either a lower negative charge or a very considerably different structure. DUTT AND SUR[8] find that the bis-oxalato complex of gallium is very stable at low pH values, while the tris-complex exists at higher values. Polarographic studies of KUZNETSOVA[9] show that the tris-complex $[Ga(A)_3]^{3-}$ is most stable at pH 8.6–10. It is probable that one of the species responsible for the "double spots" observed is $[M(A)_3]^{3-}$, the other may be $[M(A)_2(H_2O)_2]^{1-}$, or perhaps an hydroxo compound.

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SUMMARY

The zone electromigration of gallium and indium ions was carried out as a function of pH in 11 organic acid solutions. Results show the variation of complex formation with pH, and permit a comparison to be made of the complexing properties of the various acids.

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ELECTROPHORESIS OF LANTHANIDES, Sc, Th, U AND Zr IN GLYCOLIC ACID

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Hydrocarboxylic acids in general, and particularly lactic acid, have been extensively used as eluants in lanthanide separations using cationic exchangers[1–3]. Several papers on formation constants of glycolate, lactate and α-hydroxybutyrate complexes of lanthanides have appeared recently[4–6]. KONRAD-JAKOVAC AND PUČAR[7–9] describe a paper electrochromatographic technique to separate lanthanides using lactic acid as eluant.

CHOPPIN *et al.*[6,10] have proved that anionic complexes of the type ML_4^- (L = glycolate, lactate, α-hydroxybutyrate) are formed in solutions with high concentrations of ligand. Based on this fact the present paper describes the influence of pH and glycolate ion concentration of the electrolyte in the separation of lanthanides. Further work will deal with a similar study with lactic and α-hydroxybutyric acids.

EXPERIMENTAL

The technique used has been described elsewhere[11]. Solutions of 0.1 *M* lanthanide perchlorate were used. Alcoholic solutions 0.005 % of 8-hydroxyquinoline and 0.1 % of xylenol orange were employed as reagents for detection.

In the preliminary experiments only La, Gd and Yb were used as representatives of light, medium and heavy lanthanides.

RESULTS

To determine the most suitable voltage for use, experiments were carried out at 2, 6, 9 and 12 $V \cdot cm^{-1}$. As had already been observed in a former paper[11], the mobility (expressed as $cm^2 \cdot V^{-1} \cdot h^{-1}$) is dependent on the voltage used.

Fig. 1 shows the electrophoregram obtained with 9 $V \cdot cm^{-1}$, which was the value selected for all experiments. Neutral spots of Gd and Yb (same mobility as hydroquinone) are observed. The composition possible of the species present is indicated for gadolinium in the figure. The separation of these species increases as applied voltage increases.

In a second series of experiments the influence of electrolyte concentration was determined. The results are shown in Fig. 2 and the following conclusions can be drawn:

(a) The spots are always cationic whatever the electrolyte concentration.
(b) The spots are more elongated with low concentrations of electrolyte.

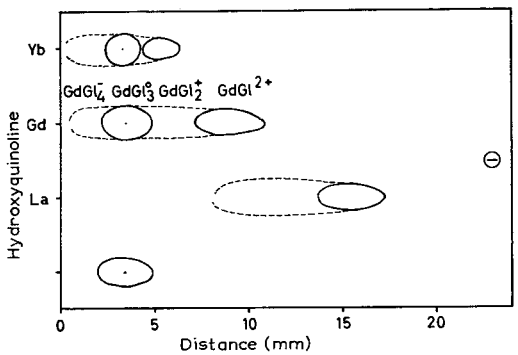


Fig. 1. Electrophoregram obtained under the following conditions: Electrolyte: glycolic acid (1 %) neutralized with NaOH to pH 5; voltage: 9 V·cm^{-1}; time: 3 h.

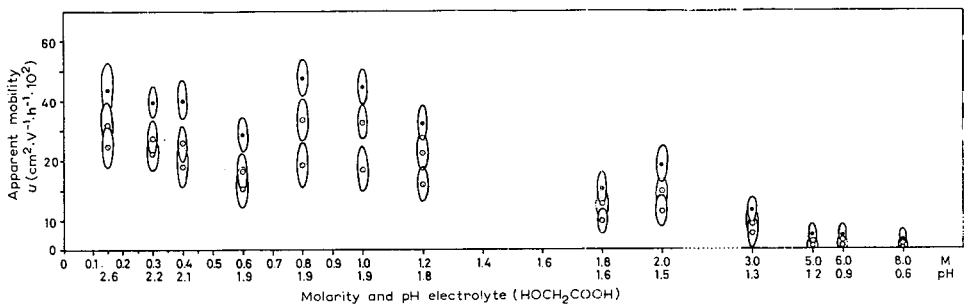


Fig. 2. Variation of mobility of La, Gd and Yb with electrolyte molarity (glycolic acid). Cationic spots: ● = La; ⊙ = Gd; ⊘ = Yb. Voltage: 9 V·cm^{-1}; time: 3 h.

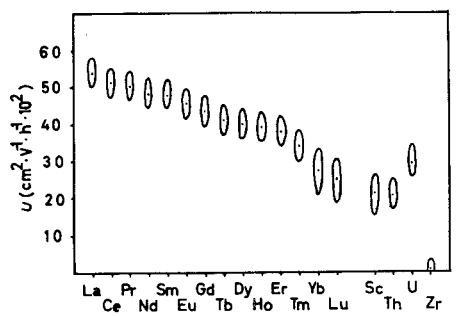


Fig. 3. Mobilities in 1 *M* glycolic acid. Voltage: 9 V·cm^{-1}; time: 3 h.

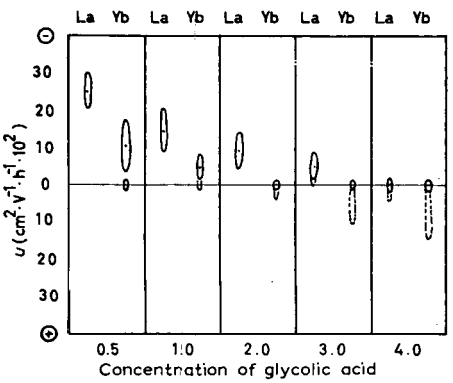
(c) The separation factor of the three spots has a maximum between 0.8 and 1.0 *M* glycolic acid.

(d) Up to a concentration of 2.0 *M* the separation of La from Yb is always possible and complete. Between 0.8 and 1.0 the separation of the three elements is also complete.

The mobilities of all the lanthanides, and also of Sc, Zr, Th and U, were determined using 1.0 *M* glycolic acid as electrolyte. Fig. 3 shows the results obtained and thus the possibility of separations.

The influence of the concentration of electrolyte with a fixed pH was studied in the experiments represented in Fig. 4, where glycolic acid solutions varying from 0.5 to 4.0 % and neutralized to pH 5.0 were used as electrolytes.

Experiments represented in Fig. 5 were made using 1.0 *M* glycolic acid, partially neutralized with NaOH, in order to determine the minimum concentration of glycolate ion necessary to change the charge of La.



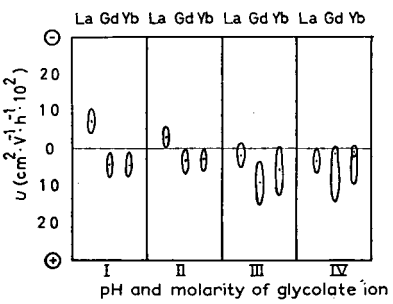


Fig. 4. Variation of mobility of La and Yb with concentration of glycolic acid partially neutralized to pH 5.

Fig. 5. Variation of mobility with concentration of glycolate ion. (I) 0.10 *M*, pH 2.55; (II) 0.18 *M*, pH 2.90; (III) 0.26 *M*, pH 3.20; (IV) 0.33 *M*, pH 3.40.

DISCUSSION

The experimental results indicated in Fig. 1 are in accordance with the stability constants of the glycolates, which increase with the atomic number of the lanthanides[6] and so greater concentrations of neutral and anionic species will exist in heavier lanthanides.

Lanthanum is cationic when $[HOCH_2COO^-] \ll 0.2\ M$ and anionic at higher concentrations. Gadolinium and lutetium are anionic when $[HOCH_2COO^-] \geqslant 0.1\ M$.

Several experiments were performed to determine the possibility of separation by a gradient of glycolate ion concentration in the electrolyte. But the irregularity of the neutralization front, the lengthening of anionic species (presumably due to the simultaneous presence of neutral and anionic species) and the formation of insoluble glycolates of heavier lanthanides (Yb and Lu) when glycolate concentrations increase, did not permit satisfactory separations.

Except when the signs of the electrical charges of the ionic species are different, the conditions of Fig. 3 (1.0 *M* glycolic acid without neutralization) are to be preferred when separating lanthanides by electrophoresis.

SUMMARY

The influence of the concentration of glycolic acid and glycolate ion in the electrophoresis of lanthanides is studied. The best conditions for lanthanide separation are with 1.0 *M* glycolic acid without neutralization. The sign of the predominant ionic species depends on the concentration of glycolate ion. Above 0.3 *M* glycolate all the lanthanides are anionic.

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SEPARATION OF IODINE ANIONS BY GLASS FIBRE PAPER CHROMATOGRAPHY

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The study of isotope-exchange reaction kinetics can be made much easier by use of radiochromatography as demonstrated by MORÁVEK, NEJEDLÝ AND FILIP[1]. ARNIKAR AND TRIPATHI[2] used paper chromatography in their study of the iodide–iodate isotope exchange reaction. In connection with our experiments on isotope exchange between different oxidation states of iodine it was decided to use paper chromatography for the separation of the reaction products.

Several authors[3–8] have demonstrated the separation of the iodine anions I^-, IO_3^-, and IO_4^- on cellulose papers with various eluants. The purpose of this investigation was to study the method and to establish the best conditions for the separation.

Preliminary experiments with various cellulose papers and eluants invariably produced two spots for periodate. This phenomenon has also been reported by GRASSINI AND OSSICINI[5] for the system isopropanol–1.5 *F* ammonia, and by SERVIGNE[8] for the system isopropanol–water (3:1), but not for the many systems studied by other authors[3,4,6,7].

Since periodate is a strong oxidizing agent in both acid and alkaline medium and since it also forms complexes with polyhydroxy compounds such as cellulose, which are believed to be the first stage in the reduction of periodate, these multiple spots are to be expected. DOBICI AND GRASSINI[9] consider cellulose to be *a priori* unsatisfactory for trace-scale separations of periodate and iodate.

Glass fibre paper was then tried and produced satisfactory separations with several of the eluants tried. On account of the easy detection of small amounts, radioactive iodine compounds were used and the spots identified by their radioactivity as well as by chemical development. By comparison some separations were run on cellulose papers parallel to the separations on glass fibre paper.

EXPERIMENTAL

(*a*) *Synthesis of radioactive compounds*

Radioactive ^{131}I was delivered as essentially carrier-free sodium iodide in a dilute basic solution of sodium thiosulphate.

Potassium iodide. To a solution of 10 mg potassium iodide in 2 ml water 0.1 mC ^{131}I is added and the iodide is oxidized to iodine by adding 1 ml 6 *M* nitric acid and a few drops of 1 *M* sodium nitrite solution. The iodine is extracted into 2 ml carbon tetrachloride and back-extracted into water by shaking with 2 ml water containing a few drops of a 1 *M* solution of potassium bisulfite. The solution is acidified with 0.5 ml

of 6 *M* nitric acid, boiled to expel sulfur dioxide and subsequently made strongly basic (pH 14) with potassium hydroxide and diluted to 5 ml.

Potassium iodate and potassium periodate. These are prepared by a modification of WILLARD's method[10].

100 mg potassium iodide is dissolved in 3 ml water containing 1 mC ^{131}I, and 100 mg potassium chlorate is added to the resulting solution. The solution is warmed to boiling and made acid with 10 drops 6 *M* nitric acid. The reaction is finished when the iodine has completely disappeared. The resulting iodate solution is divided in two equal parts. One part is made basic with potassium hydroxide and diluted to 25 ml.

The other half is made basic with potassium hydroxide and 1 ml of a freshly prepared solution of 20% potassium hypochlorite is added. The solution is warmed to nearly boiling, and after 30 min heating, chlorine is passed in until all of the alkali is neutralized and insoluble potassium metaperiodate precipitates. The precipitate is centrifuged off and washed with cold water. In order to purify the preparation the metaperiodate is dissolved as soluble $K_4I_2O_9$ in 2 ml dilute potassium hydroxide solution, and subsequently the solution is neutralized with 6 *M* nitric acid which reprecipitates insoluble potassium metaperiodate. The precipitate is centrifuged off and washed with cold water. The dissolution, precipitation and washing is repeated twice more and leaves a product essentially free from other iodine compounds. Finally, the precipitate is dissolved in dilute potassium hydroxide solution and diluted to 25 ml.

In the following experiments aliquots of the three base solutions were measured with a NaI scintillation well counter, and larger portions were mixed to yield solutions with known specific activities of iodide, iodate and periodate.

(b) Chromatography and detection

The paper was cut in strips 40 cm by 3 cm and *ca.* 50 μl containing *ca.* 10 μg each of radioactive KI, KIO_3 and KIO_4 was applied as a thin line on to the paper. The papers were allowed to stand overnight to reach equilibrium with the saturated atmosphere in the jars. The elution was descending and the solvent front was allowed to advance *ca.* 25 cm before the elution was stopped. The radioactivity was measured by means of a radiochromatogram scanner coupled to a ratemeter and recorder. This allowed continuous scanning of the radioactive chromatograms. The recorder was set to the same speed as the scanner, usually 60 mm/h. To determine the total activity under the peaks, the spots were cut out of the paper and measured in the well counter. This allowed the calculation of the per cent recovery of the anions. Better than 95 % recovery was obtained in all runs executed on glass fibre paper.

The chemical development of the spots was made according to FEIGL[11].

(1) *Iodide.* The paper is sprayed successively with a starch solution in 2 *M* acetic acid and a solution of 0.1 *M* potassium nitrite. A blue coloration indicates the presence of iodide.

(2) *Iodate.* The paper is sprayed successively with 2 *M* acetic acid and a solution of pyrogallol in acetone. A pink to brown spot indicates iodate. Periodate gives a similar colour.

(3) *Periodate.* Equal parts of a filtered solution of tetrabase (*p,p*-tetramethyl-diamino-diphenylmethane) in 2 *M* acetic acid and a 10% solution of manganous chloride are mixed and sprayed on the paper. A blue coloration indicates periodate. Iodates and chlorates do not interfere with the test.

RESULTS

The R_F values obtained with Schleicher & Schüll glass fibre paper No. 6 are shown in Table I.

TABLE I

R_F VALUES OF I^-, IO_3^- AND IO_4^- ON GLASS FIBRE PAPER WITH VARIOUS SOLVENT SYSTEMS

No.	*Eluent composition*	*Reference*	*R_F values*		
			IO_4^-	IO_3^-	I^-
1	Ethanol–H_2O–15 F NH_3 (6:2:7)	4	0.01	0.84	0.93
2	Ethanol–H_2O–15 F NH_3 (12:2:7)		0.01	0.81	0.98
3	Ethanol–H_2O–15 F NH_3 (18:2:7)		0.01	0.54	0.95
4	Ethanol–H_2O–15 F NH_3 (24:2:7)		0.01	0.43	0.86
5	Ethanol-H_2O–15 F NH_3 (30:15:5)	3	0.00	0.70	0.97
6	Isopropanol–1.5 F NH_3 (7:3)	5	0.04	0.74	0.98
7	Butanol–1.5 F NH_3 (1:1)*		0.01	0.08	0.80
8	Butanol–ethanol–5 F NH_3 (1:1:1)		0.00	0.72	1.00
9	Butanol–ethanol–7.5 F NH_3 (2:1:1)		0.01	0.56	0.97
10	Acetone–1 F NH_3 (4:1)	6	0.01	0.46	0.96
11	Methyl isobutyl ketone–1.5 F NH_3 (1:1)*		0	0	0
12	Ethyl acetate–ethanol–1.5 F NH_3 (1:1:1)		0.01	0.82	0.98
13	Isopropanol–H_2O (3:1)	8	0.01	0.28	0.94
14	Butanol–acetone–H_2O (5:2:3)	6, 7	0.01	0.37	0.95

* Equal volumes were shaken. The organic phase was used for the elution, the water phase for saturating the chamber atmosphere.

The solvent fronts were somewhat indistinct and are best observed against a strong light. The positions of the spots were determined by measuring the distance from the starting line to the abscissa corresponding to maximum intensity of the recorded peaks. Nearly all the systems investigated produced essentially higher R_F values for iodate and periodate than reported for cellulose papers. The effect is particularly pronounced for the iodate and sometimes leads to an unsatisfactory separation between the iodate and iodide spots. This is the case for the systems ethanol–water–15 F NH_3 reported by LEDERER[3] and HALPERN[4]. An increase of the volume of ethanol in proportion to the volume of water and ammonia results in better separations, however, the iodate peaks are considerably broadened (see Fig. 1). Isopropanol–1.5 F NH_3 (7:3) produced very satisfactory separations. Acetone–1 F NH_3 (4:1) produced satisfactory R_F values, and the speed of elution is fast (25 cm in *ca.* 100 min) compared with the systems ethanol–water–15 F NH_3 (12:2:7) (25 cm in *ca.* 210 min) and isopropanol–1.5 F NH_3 (7:3) (25 cm in *ca.* 360 min). As a general rule a slow elution always seems to produce better defined spots than a fast one. The systems butanol–acetone–water (5:2:3)[6,7] and isopropanol–water (3:1)[8], are noteworthy because in some isotope exchange studies a strongly alkaline medium is contradicted in the subsequent separation of the exchanging species. Butanol–acetone–water (5:2:3) yields excellent separations on glass fibre paper.

It was never possible to detect any reduction products of periodate in the runs executed on glass fibre paper. Some runs were performed on cellulose papers parallel to

the separations on glass fibre paper. Whatman papers No. 1 and 2, and Schleicher & Schüll No. 2043 were tried. This always resulted in a reduction of the bulk of the periodate, probably to iodate, because the new spot always has almost the same R_F value as iodate and yields a positive test with pyrogallol and a negative test with tetrabase. Fig. 2 shows the results obtained when periodate is chromatographed on

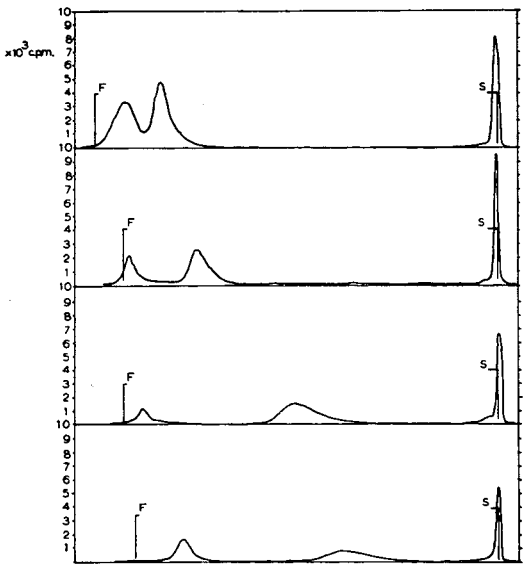

Fig. 1. Separation of iodide, iodate and periodate on Schleicher & Schüll glass fibre paper No. 6 with ethanol–water–15 *F* NH_3. From top to bottom: ethanol–water–15 *F* NH_3 (6:2:7); ethanol–water–15 *F* NH_3 (12:2:7); ethanol–water–15 *F* NH_3, (18:2:7); ethanol–water–15 *F* NH_3 (24:2:7). Scanning speed 60 mm/h; slit width 1.5 mm; time-constant ratemeter: RC = 45 sec. S = starting line; F = solvent front.

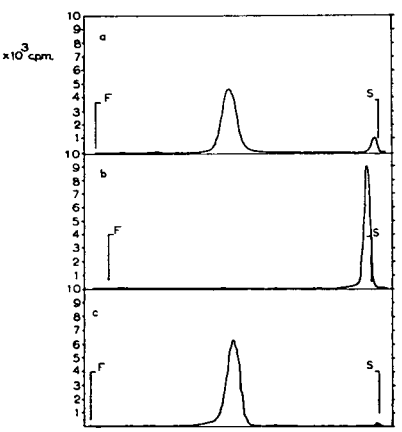

Fig. 2. (a) Periodate developed on Whatman cellulose paper No. 1. (b) Periodate developed on Schleicher & Schüll glass fibre paper No. 6. (c) Iodate developed on Whatman No. 1. Solvent: ethanol–water–15 *F* NH_3 (12:2:7). Scanning speed 60 mm/h; slit width 1.5 mm; time-constant ratemeter: RC = 45 sec; S = starting line; F = solvent front.

Whatman cellulose paper No. 1, and Schleicher & Schüll glass fibre paper No. 6 with ethanol–water–15 *F* NH_3 (12:2:7). For comparison a parallel run with iodate on Whatman No. 1 is included.

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SUMMARY

The separation of iodide, iodate and periodate by chromatography on glass fibre paper with different eluants is investigated. Cellulose papers are unsatisfactory for trace-scale separations of periodate because the bulk of the periodate is reduced, probably to iodate.

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Short Communication

Gas chromatographic separation of deuterated methanes

In this communication, the separation of deuterated methanes by gas–solid chromatography with an adsorption glass capillary column is described.

Fig. 1 shows a chromatogram of CH_4, CH_3D, CH_2D_2 and CD_4 resolved at low temperature (—188°). The samples were obtained by exposure to X rays (tungsten, 50 kV, 30 mA) of a mixture containing 45% deuterium, 45% xenon and 10% methane.

The column (0.30 mm internal diameter, and 35 m long) was prepared by passing a 20% sodium hydroxide solution through it for 6 h at 100°, in order to etch the internal surface of the glass[1]. The column was then washed to neutrality, dried and deactivated to a constant activity by passing pure nitrogen saturated with water at

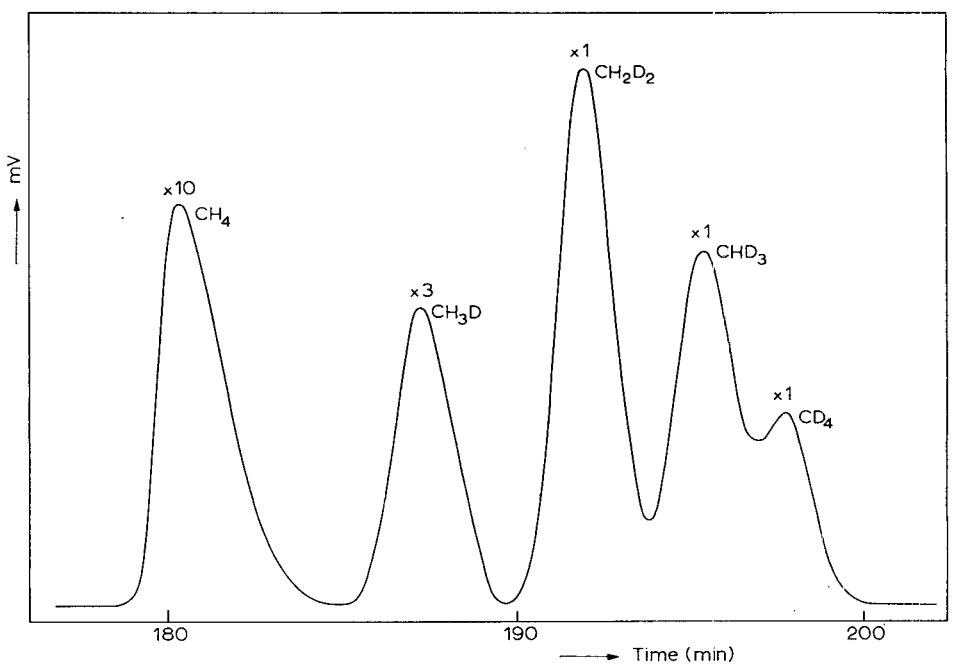

Fig. 1. Separation of deuterated methanes on an etched glass capillary column.

0° through it. An efficiency of about 55,000 theoretical plates was obtained for the methane peak. The working conditions for such a chromatogram are: column temperature: —188°; carrier gas: nitrogen with an inlet pressure of 32 mm Hg, and a flow rate of 0.62 ml/min. A home made gas chromatograph was employed; it was equipped with a flame ionization detector and a liquid nitrogen cryostat.

At the investigation temperature, methane has the lowest retention volume of the compounds in the mixture, since the "normal" isotopic effect is the predominant one. The separation between CH_3D and CH_4 is better than between CH_3D and CH_2D_2 and the same is observed for the other consecutive pairs of isotopically substituted molecules.

This behaviour could be connected with the dependence of the ratio of retention volumes (for each adjacent pair of isotopic compounds) on the ratio of their masses[2]; by plotting the log of the retention volume ratios *versus* $1 - m_1/m_2$, where m_1 and m_2 are the masses of the corresponding isotopic molecules, a straight line was obtained.

Similar relationships were found for the vapour pressure of different isotopes[3]. By gas chromatography it is possible to investigate these effects and to compare the values of the retention volumes with the vapour pressure measurement.

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Notes

Substituted hydrazones as derivatives of ketones in gas chromatography

It has recently been demonstrated that a variety of reactive ketones may function as reagents for primary amines, with the formation of the so-called Schiff bases or eneamines; these substances possess excellent gas-chromatographic properties[1]. In an analogous manner, compounds with reactive amino groups may function as reagents for ketones; thus N,N-dimethylhydrazine has been found to be useful in gas-chromatographic work with keto-steroids[2]. Other hydrazines and hydrazine-related compounds should also prove to be of value as reagents for ketones. We have investigated the gas-chromatographic properties of a number of these derivatives, including compounds obtained by reaction with N-aminopiperidine, N-aminohomopiperidine, pentafluorophenylhydrazine and phenylhydrazine[3,4].

Table I gives the retention data for the derivatives of di-*n*-hexyl and di-*n*-heptyl ketones, and for those of androstan-17-one.

The derivatives were prepared by reaction of the ketone with the appropriate reagent in ethyl acetate solution (acetic acid catalyst); complete condensation occur-

red in less than an hour in all cases, and the rate of the reaction could be followed by injection of aliquots of the reaction mixture directly into the chromatographic column. The mass spectrum of each derivative was obtained as the peak was eluted from the gas-chromatographic column, following the method of RYHAGE[5]; in each case the mass of the molecular ion corresponded exactly with the molecular weight of the

TABLE I

RETENTION BEHAVIOR OF KETONE DERIVATIVES

Compound	*Relative retention time*	
	F-60-Z[a]	*CNSi*[b]
n-Eicosane	1.00	1.00
Di-*n*-hexyl ketone	0.13	0.15
N-aminopiperidine derivative	0.64	0.66
N-aminohomopiperidine derivative	1.04	1.12
pentafluorophenylhydrazone	1.47	2.92
phenylhydrazone	3.92	6.77
Di-*n*-heptyl ketone	0.27	0.36
N-aminopiperidine derivative	1.38	1.51
N-aminohomopiperidine derivative	2.24	2.54
pentafluorophenylhydrazone	3.24	6.48
phenylhydrazone	6.59	14.7
	F-60[c]	
Cholestane	1.00	
Androstan-17-one	0.16	
N-aminopiperidin derivative	0.85	
N-aminohomopiperidine derivative	1.44	
pentafluorophenylhydrazone	2.00	
phenylhydrazone	4.06	

[a] Column conditions: 6 ft. × 4 mm glass U-tube; 7% F-60 (methyl siloxane polymer containing a small percentage of *p*-chlorophenyl groups; Dow-Corning Corp.)–1% EGSS-Z (ethylene glycol succinate-phenyl methyl siloxane copolymer; Applied Science Laboratories) on 80–100 mesh Gas Chrom P; 189°; 15 p.s.i. argon. Retention time of *n*-eicosane: 17.0 min.

[b] Column conditions: 6 ft. × 4 mm glass U-tube; 5.5% 20-mole % β-cyanoethylmethylsiloxane (General Electric Co; we are grateful to Dr. A. MARTELLOCK for a gift of this polymer) on 100–120 mesh Gas Chrom P; 170°; 15 p.s.i. argon. Retention time of *n*-eicosane: 22.5 min.

[c] Column conditions: 6 ft. × 5 mm glass U-tube; 1% F-60 on 60–80 mesh Gas Chrom P; 203°; 14 p.s.i. argon. Retention time of cholestane: 13.5 min.

expected derivative. In several cases (the N-aminopiperidine derivatives of di-*n*-heptyl ketone and androstan-17-one, and the N-aminohomopiperidine derivative of di-*n*-heptyl ketone) the derivatives were isolated by ordinary techniques and found to possess satisfactory elemental analyses. A sample of the N-aminopiperidine derivative of androstan-17-one was collected after chromatography and found to be unchanged (infrared).

The retention times of the aliphatic ketones are relative to that of *n*-eicosane, those of the steroid derivatives are relative to that of cholestane.

The retention times observed for the derivatives are greater than those of the parent ketones. They show very satisfactory gas-chromatographic behavior, as illustrated in Fig. 1. Not unexpectedly, the condensation products from N-aminohomo-

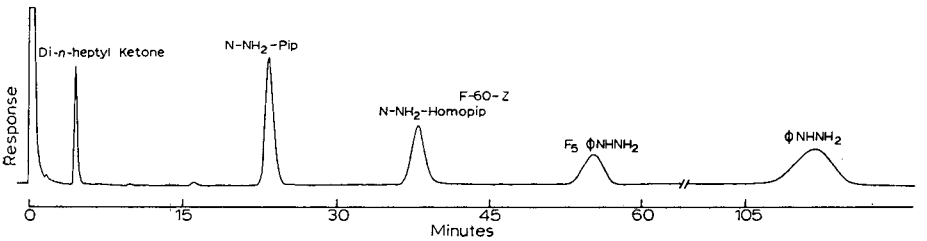

Fig. 1. Gas-chromatographic separation of di-*n*-heptyl ketone and four of its derivatives. The compounds are di-*n*-heptyl ketone and its condensation products with N-aminopiperidine (N-NH_2-Pip), N-aminohomopiperidine (N-NH_2-Homopip), pentafluorophenylhydrazine (F_5 $\phi NHNH_2$) and phenylhydrazine ($\phi NHNH_2$). Column conditions are given in Table I.

piperidine are eluted considerably later than the corresponding compounds derived from N-aminopiperidine. The reduced volatility of phenylhydrazones, when compared to the derivatives from N-aminohomopiperidine, would not be expected on the basis of molecular weight alone, and the effect must be ascribed to properties of the aromatic system. This selective retention is especially pronounced with the polar phase CNSi, which is known to have pronounced selective retention properties for unsaturated compounds[6]. Although possessing significantly greater molecular weights, the pentafluorophenylhydrazones are eluted much more rapidly than the corresponding phenylhydrazones. This striking result follows the pattern observed earlier for fluoro-substituted esters[6].

The availability of a variety of derivatives for use in the characterization and identification of organic compounds by gas chromatographic methods is highly desirable. Compounds not normally considered as useful reagents for derivative formation may be of considerable value in such work, since it is retention behavior rather than the classical criteria (melting point, color, solubility) which is of significance. Further, problems associated with hydrolysis or instability in solution may not be relevant; for example, both eneamines and trimethylsilyl ethers are excellent derivatives for work in gas phase analytical technology, but they are unsuitable for classical work requiring purification by recrystallization.

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The analysis of straight-chain aliphatics by urea partition chromatography and gas–solid chromatography

C_{15} through C_{35} *n*-paraffins and α-olefins from a low-temperature coal tar have been analyzed by a combination of urea adduction in a highly efficient liquid–liquid partition chromatography process followed by gas–solid chromatography on alumina-coated capillary columns operated up to 400°.

In previous work *n*-paraffins and α-olefins in the range C_{10} through C_{16} were successfully analyzed by gas–liquid chromatography on a polyphenyl ether column[1]. However, this column could not be used for the characterization of pitch oils because the maximum operating temperature was 220° and even at this temperature there was some bleeding. In the present work it has been found that gas–solid chromatography with alumina-coated columns can be used at least as high as 400° for the analysis of aliphatic hydrocarbons at least through C_{36}. Standard urea adduction procedures failed to work on complex, high-boiling pitch oils, but it was found that a modification of a columnar process described in a patent[2] gave complete recovery of the straight-chain material.

A mixture of 40 parts by weight of urea and 60 parts of 20–100 mesh silica gel was packed dry in a 19 mm internal diameter, 23-in. length glass column. About 20 ml of a urea-saturated solution, in 90 volume % methanol and 10 volume % water, was passed through the column, and the excess was allowed to drain through. About 1 g of

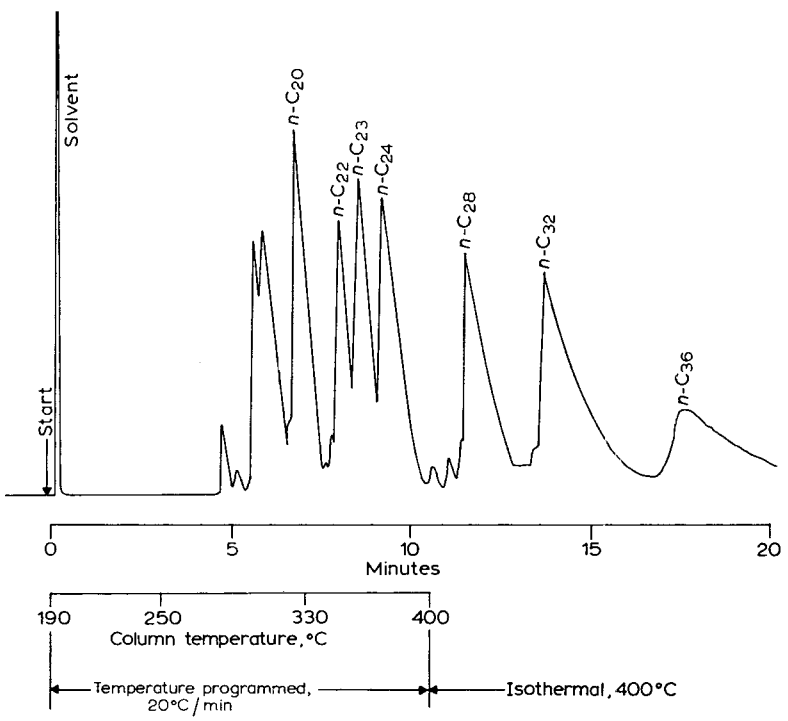

Fig. 1. Gas–solid chromatography of C_{20} through C_{36} *n*-paraffins on an alumina-coated column.

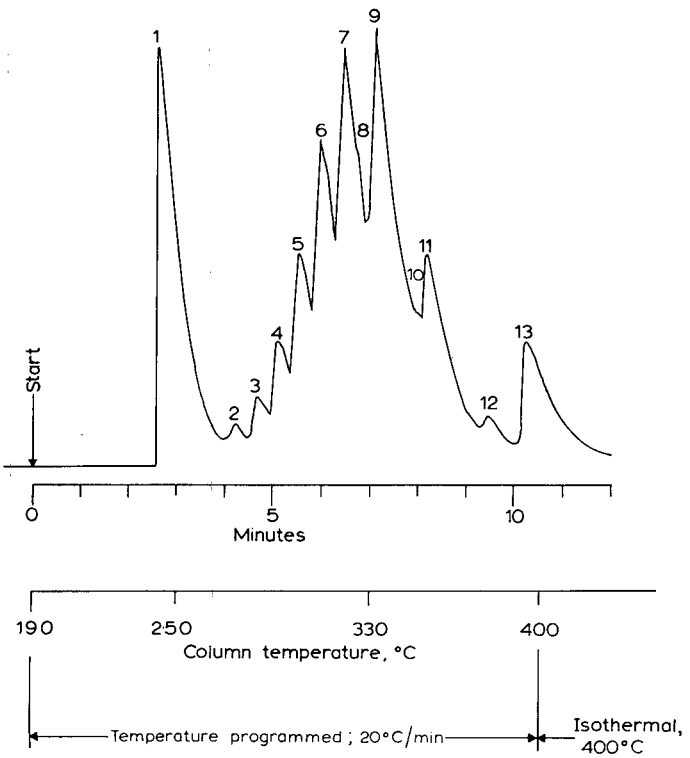

Fig. 2. Gas–solid chromatography of straight-chain aliphatic pitch oil fraction 1 (C_{15}–C_{21}).

the aliphatic pitch oil fraction was charged. If the sample was not entirely liquid, a little *n*-pentane was added to it. These fractions, containing the highest carbon number *n*-paraffins and α-olefins, had been obtained from the pitch of a low-temperature West Virginia bituminous coal tar. The uncomplexed, branched material was eluted from the column with about 20 ml *n*-pentane. About 40 ml water was passed through the column to destroy the complex. This was followed by about 20 ml *n*-pentane to dissolve out the released straight-chain material. The *n*-pentane solutions were each washed with equal volumes of water in a separatory funnel, and the *n*-pentane was removed with dry nitrogen. The yields of straight-chain material ranged from 41 to 61 weight %, and the yields of branched-chain material ranged from 39 to 59 weight % for these various fractions. Infrared analysis[3] of the pitch oil fractions was in agreement with the results of the urea fractionations, and synthetic test mixtures yielded pure compounds as shown by subsequent gas chromatography. Infrared analysis[3] of the urea adducted material indicated that it was free of branched paraffins and olefins. A small amount of *trans*-olefins was present but not sufficient to affect the quantitative analysis of the α-olefins.

A matched pair of 50 ft. by 0.02 in. internal diameter γ-alumina-coated aluminum capillary columns was used with a dual flame detector; temperature programming was 190° to 400° at 20° per min. The results with a synthetic mixture containing C_{20}, C_{22}, C_{23}, C_{24}, C_{28}, C_{32}, and C_{36} *n*-paraffins are shown in Fig. 1. Of

interest are the six or more unlabelled peaks in this chromatogram due to impurities present in some of these high carbon number samples. The straight-chain pitch oil fractions were analyzed in this manner, using a charge of from 0.4 to 1.6 microliters and an injection chamber temperature of 400°. The results for the lowest carbon number fraction, C_{15} to C_{21}, are shown in Fig. 2. This fraction had been obtained from the pitch oil by a spinning band distillation at 0.02 mm Hg. These results are of interest because they demonstrate the presence of rather low carbon number aliphatics in the pitch and also show the carbon number distribution in this distillate fraction, with a maximum at C_{17} to C_{18}. Peaks 1 and 13 are due to the internal standards *n*-tridecane

TABLE I

GAS–SOLID CHROMATOGRAPHIC ANALYSIS OF STRAIGHT-CHAIN ALIPHATICS FROM PITCH OIL FRACTION 1

Peak No.	*Compound*	*Relative retention to* n-C_{13}	*Weight %*
1	*n*-Tridecane	1.00	(internal standard)
2	*n*-Pentadecane	1.61	0.5
3	1-Pentadecene	1.79	1.3
4	*n*-Hexadecane	1.96	2.1
5	1-Hexadecene	2.11	3.9
6	*n*-Heptadecane	2.27	7.6
7	1-Heptadecene	2.46	8.4
8	*n*-Octadecane	2.58	4.1
9	1-Octadecene	2.73	16.0
10	1-Nonadecene	3.06	1.2
11	*n*-Eicosane	3.15	6.9
12	1-Heneicosene	3.63	1.1
13	*n*-Tetracosane	3.94	(internal standard)

TABLE II

GAS–SOLID CHROMATOGRAPHIC ANALYSIS OF STRAIGHT-CHAIN ALIPHATICS FROM PITCH OIL FRACTION 4

Peak No.	*Compound*	*Relative retention to* n-C_{20}	*Weight %*
1	*n*-Eicosane	1.00	(internal standard)
2	*n*-Pentacosane	1.46	0.1
3	1-Pentacosene	1.50	0.8
4	*n*-Hexacosane	1.56	1.4
5	1-Hexacosene	1.61	1.5
6	*n*-Heptacosane	1.64	4.2
7	*n*-Octacosane	1.70	8.9
8	1-Octacosene	1.78	2.2
9	*n*-Nonacosane	1.80	6.8
10	1-Nonacosene	1.89	2.0
11	*n*-Triacontane	1.91	4.2
12	1-Hentriacontene	2.02	1.7
13	*n*-Dotriacontane	2.07	3.2
14	*n*-Tritriacontane	2.20	1.1
15	1-Tritriacontene	2.28	1.7
16	1-Pentatriacontene	2.56	1.4

and *n*-tetracosane, respectively. The complete results for this fraction, and the highest carbon number fraction, are summarized in Tables I and II, respectively.

The individual *n*-paraffins and α-olefins were identified by means of relative retentions of pure compounds and correlation charts. It was found that the relative retentions of all α-olefins were equal to the relative retention of the *n*-paraffin of the same carbon number plus 5/8 of the difference between the relative retention of that *n*-paraffin and the next higher *n*-paraffin. The resolution was not sufficient to obtain separate peaks for trace constituents so that an occasional carbon number in a series appeared to be missing. Studies with synthetic mixtures showed that if a significant quantity of a straight-chain compound was present a distinct peak was always obtained.

A variety of pitch oil fractions was analyzed by these procedures; *n*-paraffins in the range C_{15} through C_{33} and straight-chain olefins in the range C_{15} through C_{35} were identified and their amounts were determined. The results of the gas–solid chromatographic analysis of straight-chain pitch oil fractions on alumina-coated columns has demonstrated that the method will work on this high-boiling material, not more than 20 minutes being required per run. This particular use of these columns has apparently never been described.

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Zur Radiogaschromatographie von weissem Phosphor und Phosphorverbindungen

Weisser Phosphor und korrodierende Phosphorverbindungen wie PCl_3, $POCl_3$ und Alkyl-Phosphorhalogenide lassen sich gaschromatographisch bestimmen, wenn die Teile des Gaschromatographen aus V2A-Stahl gefertigt sind. Bei Verwendung von Säulen aus 10% Emulphor (Badische Anilin- und Sodafabrik AG) oder 10% Silikonelastomer (Wacker Chemie GmbH) auf Kieselgur und Wasserstoff als Trägergas werden symmetrische Banden erhalten. Bei einer Säulenlänge von 2 m betrug die Zahl der theoretischen Böden *ca.* 3000.

Die in Fig. 1 gezeigte Messanordnung hat sich zur Radiogaschromatographie ^{32}P-markierter Verbindungen sehr bewährt. Mit Hilfe dieser Vorrichtung, die sich unmittelbar hinter der Wärmeleitfähigkeitszelle (Hersteller Fa. GowMac) befindet, wird die β-Aktivität des ^{32}P enthaltenden Gasstroms kontinuierlich mit Hilfe eines Zählrohrs gemessen. Das Zählrohr befindet sich hierbei auf Zimmertemperatur und

kommt nicht mit dem Gasstrom in Berührung, so dass es nicht kontaminiert wird. (Versuche mit einem elektrisch beheizten Durchflusszählrohr der Fa. Frieseke und Höpfner, Typ FHZ 45, zeigten, dass auf diese Weise hochsiedende Verbindungen nicht bestimmt werden können, weil das Zählrohr oberhalb 70–80° bereits spontan Impulse anzeigt.)

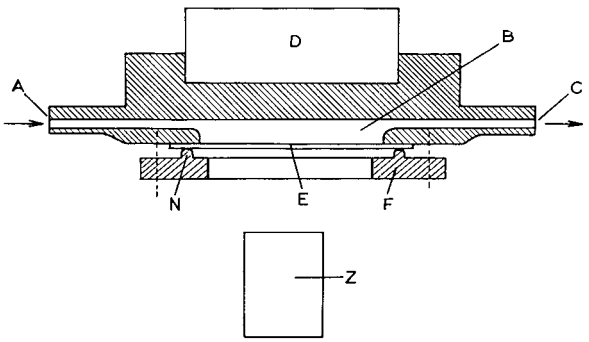

Fig. 1. Schema der Messanordnung.

Bei der in Fig. 1 gezeigten Anordnung tritt das von der Wärmeleitfähigkeitszelle kommende Substanz-Trägergasgemisch bei A in den Innenraum B ein und verlässt diesen über C. Die Aussparung D dient zur Aufnahme der elektrischen Heizung. Die Temperatur in der Zelle B wird durch einen bis dicht an die Zellenwandung reichenden Thermofühler kontrolliert. Die harte β-Strahlung des ^{32}P gelangt durch das Aluminiumfenster E (0.1 mm Dicke), das durch den Verschlussring F mit der Nut N gasdicht an den Block gedrückt wird, in das Endfensterzählrohr Z (Typ FHZ 15 a der Fa. Frieseke und Höpfner). Dieses ist von der Kammer K in einem Abstand von ca. 2 cm vom Aluminiumfenster getrennt montiert und befindet sich daher auf Raumtemperatur. Zur automatischen Registrierung wurden die Impulse einem Ratemeter zugeleitet und die Signale auf einen Schreiber gegeben.

Der Kammerraum B hat die Form eines flachen Hohlzylinders von 7 mm Höhe und 4.5 cm Durchmesser, sein Volumen von 3.1 cm^3 ist grösser als das Volumen der Wärmeleitfähigkeitszelle. Dies bedingt (zusammen mit der strömungstechnisch ungünstigen Form) eine grössere Aufenthaltsdauer als in der Wärmeleitfähigkeitszelle. Deshalb sind die Banden des Aktivitätschromatogramms etwas breiter als die des normalen Chromatogramms, jedoch wird durch das relativ grosse Volumen und die Form des Raumes B in Fig. 1 eine hohe Nachweisempfindlichkeit der Radioaktivität erreicht.

Herrn K. JURISCHKA danken wir für die Hilfe beim Aufbau der Apparatur.

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Volatile oxygenated constituents of hops

Identification by combined gas chromatography and mass spectrometry

The oxygenated constituents of hops have been studied by several authors[1–5] but identification has generally been limited to the major constituents. The present work reports a rather detailed study using the powerful analytical method of the direct combination of capillary gas–liquid chromatography and mass spectrometry (Cap–MS)[5–8].

Experimental

Authentic samples of compounds were obtained from reliable commercial sources or synthesized by well-established methods. They were purified by gas–liquid chromatography (GLC) separation before use.

Hop oil was obtained by the steam distillation of Brewers Gold hops. The oxygenated fraction was separated from the hydrocarbons on silica gel as described previously[4].

The capillary column used for the analysis was 192 ft. long by 0.01 in. I.D., stainless steel, coated with Silicone SF 96 (50)* containing the tail-reducing agents Carbowax 20 M and Alkaterge T (1 % of each in the silicone). The GLC conditions were: sample, 20 μl, injected into a 1/300 split stream injector having a temperature of 200°; carrier gas, helium at a velocity of 25.4 cm/sec for both mass spectral and flame ionization work; column temperature programmed non-linearly from 50–180°.

For the mass spectral analysis, approximately half of the capillary effluent was led into the ionization chamber of a Bendix Time-of-Flight mass spectrometer as described in previous work[5–8]. In order to obtain a chromatogram comparable to that obtained with a flame ionization detector and concurrent with the mass spectral analysis, the rest of the effluent was passed through a vacuum ionization gauge, which served as the GLC detector.

For retention time measurements the end of the capillary was led into a flame ionization detector. To determine accurately if an authentic sample had the same GLC retention time as a particular peak, it was mixed with a 20-fold amount of the oxygenated fraction and chromatographed, using the same GLC conditions as for Fig. 1. An appreciable increase in the particular peak would confirm that the retention time of the authentic compound was the same.

Results

Fig. 1 shows the capillary GLC analysis obtained using a flame ionization detector. Table I shows the results of the analysis; column 2 lists constituents whose mass spectral patterns and GLC retention times were identical with those of authentic samples. In several cases authentic samples were not available but the mass spectral pattern indicated the structure and molecular weight. Such identification can be only tentative but because it has some value it is listed in column 3.

A study using conventional packed column separation was carried along with the Cap–MS study and the identity of many of the constituents was confirmed by

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

comparison of their infrared absorption spectra with those of authentic samples (Table I).

TABLE I

IDENTIFICATION OF CONSTITUENTS IN OXYGENATED FRACTION OF BREWERS GOLD HOP OIL USING CAPILLARY GLC AND MASS-SPECTRAL ANALYSIS

Peak number in Fig. 1	*Identification confirmed by comparison of mass spectrum and GLC retention time with those of authentic sample*	*Mass spectral prediction where authentic sample not avaible*
4	2-Methylpropyl isobutyrate*	—
5	Methyl hexanoate	—
6	—	Methyl thio-2-methylbutyrate
7	Butyl isobutyrate	—
8	2-Methylbutyl propionate*	—
9	Methyl 5-methylhexanoate*	—
10	—	—
11	2-Methylpropyl 2-methylbutyrate	—
12	3-Methylbutyl isobutyrate	—
13	2-Methylbutyl isobutyrate*	—
14	Methyl heptanoate* and methyl 4-methylhex-2-enoate*	—
15	—	—
16	Pentyl isobutyrate	—
17	—	2-Methylpropyl branched pentenoate and methyl thiohexanoate (branched)
18	—	Pentenyl isobutyrate
19	—	Methyl 2,5-dimethylhexanoate
20	Methyl thiohexanoate*	and methyl 6-methylheptanoate**
20a	Ethyl heptanoate	—
21	2-Nonanone	and 2-methyl-5-pentenylfuran**
22	Hexyl propionate*	—
23	2-Methylbutyl isovalerate	—
24	2-Methylbutyl 2-methylbutyrate* and linalool*	—
25	Methyl octanoate*	—
26	Hexyl isobutyrate*	—
26c	—	Methyl thioisoheptanoate
27	—	Oxygenated terpene
28	Methyl thioheptanoate* and 2-decanone	—
29	—	Methyl nonanoate (branched)
30	Heptyl propionate*	—
31	Octyl acetate*	and methyl nonenoate**
32	Methyl nonanoate*	—
33	Heptyl isobutyrate	—
34	2-Methylbutyl hexanoate	—
35	—	Branched 2-undecanone
35a	—	Methyl 2-methylnonanoate
36	—	Mol. wt. 204
37	—	—
38	—	Methyl 8-methylnonanoate
39	2-Undecanone*	—
40	—	—
41	Methyl dec-4-enoate***	—
42	Methyl deca-4,8-dienoate***	—
43	Methyl geranate*	—
44	Methyl decanoate*	—
45	—	Methyl decenoate

(*continued on p. 401*)

TABLE I (*continued*)

Peak number in Fig. 1	*Identification confirmed by comparison of mass spectrum and GLC retention time with those of authentic sample*	*Mass spectral prediction where authentic sample not avaible*
46	Octyl isobutyrate	—
47	—	2-Methylbutyl heptanoate
48	—	2-Dodecanone (branched)
48a	Neryl acetate	—
48b	—	Branched nonanyl isobutyrate
48c	—	Methyl undecenoate (branched)
49	Geranyl acetate*	—
50	—	Methyl 9-methyldecanoate
51	—	—
52	—	Methyl undecenoate
53	—	Methyl undecadienoate
54	—	Methyl undecenoate
55	Methyl undecanoate	—
56	—	Methyl undecenoate
57	Neryl propionate	—
58	—	2-Tridecanone (branched)
59	—	—
60	Geranyl propionate*	—
61	—	—
62	Neryl isobutyrate	and methyl dodecanoate (branched)
63	2-Tridecanone*	—
64	—	Methyl dodec-8-enoate**,+ and methyl
65		dodecadienoate Mol. wt. 208
66	—	Linalyl propionate
67	Geranyl isobutyrate*	—
68	—	—
69	Methyl dodecanoate	—
70	—	Methyl dodecenoate
71	—	Branched 2-tetradecanone
72	—	Tetradec-9-en-2-one**,+
73	—	Sesquiterpene, mol. wt. 204
74	—	Mol. wt. 204
75	—	Terpene ester
76	—	—
77	2-Tetradecanone	
78	—	Methyl tridecenoate
79	—	Methyl tridecenoate
80	—	Sesquiterpenoid, Mol. wt. 220
81	—	Sesquiterpenoid, Mol. wt. 222
82	—	—
83	—	—
84	—	Pentadeca-6,9-dien-2-one**,+
85	—	Mol. wt. 220
86	—	Mol. wt. 222
87	2-Pentadecanone	—

* Infrared absorption spectrum identical with that of authentic sample.
** Identification supported by infrared absorption spectrum.
*** Identity previously proved by NMR and ozonolysis[4].
\+ Identification supported by ozonolysis.

Little information could be obtained from the mass spectral patterns of peaks 70–86. Many of these peaks appeared to be sesquiterpenoids but their mass spectral patterns did not match those of any of the limited number of sesquiterpenoids available. Peaks 75 and 80 had retention times corresponding to those of caryophyllene epoxide and humulene epoxide respectively[3].

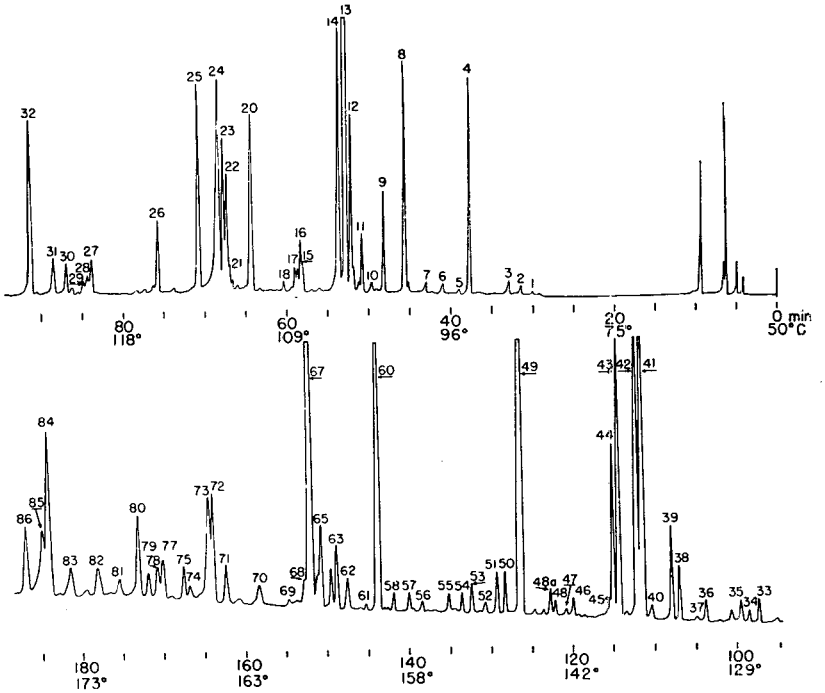

Fig. 1. Temperature programmed capillary column GLC analysis of the oxygenated fraction of hop oil (Brewers Gold hops). Column 192 ft. long × 0.01 in. I.D., coated with silicone SF 96 (50) plus tail reducers; helium carrier gas velocity 24.5 cm/sec; flame ionization detection.

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Dünnschicht- und säulenchromatographische Trennung einiger diastereomerer Dipeptide

Werden zwei optisch aktive Aminosäuren zu einem Dipeptid verknüpft, so tritt sehr häufig eine Racemisierung auf. Dabei racemisiert in der Regel die Aminosäure, die mit ihrer Carboxylgruppe zur Reaktion gebracht wird. Es entsteht ein Gemisch zweier diastereomerer Dipeptide. Zur Erkennung einer solchen Racemisierung sind bisher eine Reihe von Analysenmethoden herangezogen worden.

Die Messung der Drehung ist ungenau, da die spezifische Drehung von Peptiden oft gering ist und daher ein kleiner Anteil des durch Racemisierung entstandenen Diastereomeren nicht erkannt werden kann. Ebenso ist die oft mögliche fraktionierte Kristallisation für analytische Zwecke wenig geeignet. Gute Ergebnisse wurden mit der Gaschromatographie von N-Trifluoracetyl-dipeptid-methylestern[1] und neuerdings mit der Papierchromatographie freier Dipeptide[2] erzielt. In beiden Arbeiten finden sich weitere Literaturhinweise.

In der vorliegenden Mitteilung wird über die dünnschichtchromatographische Trennung einer Reihe von diastereomeren Dipeptiden berichtet. Von 12 zur Verfügung stehenden diastereomeren Dipeptiden konnten 9 aufgetrennt werden (Tabelle I). Das LL- (bzw. DD-) Isomer besass stets den höheren R_F-Wert. Die Trennung von Alanyl-alanin, Alanyl-asparagin und Alanyl-methionin gelang nicht.

Die unter den angegebenen Bedingungen entstehenden Flecke sind scharf. Die untere Nachweisgrenze wurde für Alanyl-leucin, Leucyl-leucin und Leucyl-tyrosin bestimmt. Es lassen sich 0.2 γ der einzelnen Diastereomeren noch ohne Schwierigkeiten erkennen. Dabei ist die Anfärbbarkeit für LL- (bzw. DD-) Isomere in der Regel etwas besser als für DL- (LD-) Isomere. Die Flecke für LL- (DD-) Isomere erscheinen schneller und sind zunächst violett gefärbt, während DL- (LD-) Isomere später und als mehr bräunliche Flecke erscheinen.

Beim Alanyl-leucin, das sich durch seine gute Löslichkeit besonders für diese Bestimmung eignet, konnten 0.5 γ des DL-Isomeren neben 1000 γ des LL-Isomeren noch ohne Schwierigkeiten nachgewiesen werden.

Die dünnschichtchromatographischen Trennungen können auf die Säule übertragen werden. Fig. 1 zeigt die Trennung von DL-Alanyl-L-leucin unter Verwendung von Kieselgel zur Säulenchromatographie unter 0.08 mm Korngrösse (Merck,

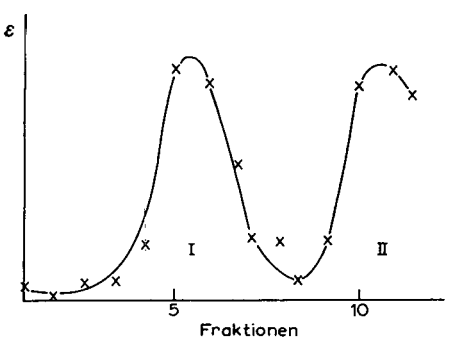

Fig. 1. Säulenchromatographische Trennung von DL-Alanyl-L-Leucin. (I) LL-Isomer; (II) DL-Isomer.

TABELLE I

DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG DIASTEREOMERER DIPEPTIDE

Als Kieselgel wurde Kieselgel G (Merck, Darmstadt), als Zellulose Zellulosepulver MN 300 (Macherey, Nagel & Co., Düren) verwendet.
Fliessmittel: I = *n*-Butanol–Wasser–Eisessig (8:2:2).
II = Essigester–Pyridin–Eisessig–Wasser (5:5:1:3).
III = *n*-Butanol–Wasser–Eisessig (8:2:4).
Die getrockneten Chromatogramme wurden mit Ninhydrin-Reagenz (Merck) besprüht und 10 Min. bei 80° aufbewahrt.

Dipeptid	*Konfiguration*	*Schicht*	*Fliessmittel*	R_F-*Wert*	
Ala-Leu	LL	Kieselgel	I (II)	0.56	(0.57)
	DL			0.30	(0.40)
	LL	Zellulose	I (II)	0.77	(0.60)
	DL			0.45	(0.25)
Ala-*n*-Leu	LL	Zellulose	II	0.64	
	DL			0.56	
Ala-Phe	LL	Zellulose	II	0.59	
	DL			0.54	
Ala-Ser	LL	Kieselgel	I (III)	0.19	(0.27)
	DL			0.13	(0.22)
	LL	Zellulose	I (II)	0.34	(0.23)
	DL			0.20	(0.16)
Ala-Val	LL	Kieselgel	I (III)	0.39	(0.47)
	DL			0.27	(0.42)
Leu-Leu	LL	Kieselgel	I (II)	0.60	(0.77)
	DL			0.48	(0.61)
	LL	Zellulose	I	0.87	
	DL			0.81	
Leu-Phe	LL	Kieselgel	I	0.38	
	DL			0.27	
	LL	Zellulose	I	0.89	
	DL			0.76	
Leu-Tyr	LL	Kieselgel	I	0.55	
	DL			0.44	
Phe-Leu	LL	Kieselgel	I	0.54	
	DL			0.43	

Darmstadt) und Fliessmittel I (vergl. Tabelle I). Die Länge der verwendeten Säule betrug 17 cm, ihr Durchmesser 2 cm. Die Auswertung erfolgte kolorimetrisch mit Ninhydrin[3].

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Use of silica gel G slurries in thin-layer chromatography

Silica gel G coatings for use in thin-layer chromatography have been prepared from individual batches of a silica gel suspension in an aqueous medium. Conventionally, a mixture of two parts of water or aqueous solution and one part of silica gel G is prepared by thorough shaking; then the plates are coated within a minute or two before the mixture sets to an unworkable gel[1].

The preparation of a large amount of suspension which could be used to coat a number of plates over a period of time would be considerably more convenient. It has been determined that a mixture of 2.5 parts of either water or of some aqueous solutions to one part of silica gel G will form a slurry* which can be used for at least three months to prepare satisfactory coatings. DAUVILLIER[2] prepared silica gel suspensions whose setting time was increased by an unstated amount. However, this procedure required modification of the commercially available silica gel G.

Experimental

(*a*) *Apparatus and reagents.* Silica gel G and silica gel H were Merck & Co. products. All other chemicals used were reagent grade. Brinkmann thin-layer chromatographic equipment was used for the preparation of the coatings.

(*b*) *Procedure.* 200 g of the desired silica gel was stirred into 500 ml of either distilled water, 0.1 *M* sodium hydroxide, or 0.1 *M* potassium bisulfate. The mixture was shaken mechanically for 0.5 h and then allowed to stand until needed. Just prior to the coating operation the slurry was shaken vigorously to resuspend the silica gel.

Fifty ml of the slurry was used to coat five 20 × 20 cm glass plates. The coated plates were allowed to stand until the slurry set. When water was used, a normal setting time was observed; both the acidic and basic solutions retarded setting for about 10 min. The coated plates were then oven-dried or air-dried as desired.

Discussion

Slurries of silica gel G in water, 0.1 *M* sodium hydroxide and 0.1 *M* potassium bisulfate have each been used to prepare coated plates at intervals over a period of three months. Their use over a longer period of time seems feasible. R_F values obtained on these coatings for a number of organic bases fell within the range of values found[3] using coatings prepared from the conventional mixture of two parts of water to one part of silica gel G. The coatings prepared from each of the slurries show as good or better adhesion to the plates and resistance to abrasion as do the conventional coatings. Formation of aggregates of silica gel has not been noted with any of the slurries prepared using the above solutions.

A mixture of one part of silica gel G and 2.5 parts of water sets within 10 min to a loose watery gel; when this gel is stirred it breaks up, giving two phases. Coatings prepared from a mixture which had gone through a "set" stage gave more variable R_F values than the slurries prepared as directed. Mixtures prepared from one part of silica gel G to 2 parts of each of the three solutions given above will set in less than 10 min despite vigorous shaking throughout this time. Shaking is only useful in preventing setting of these mixtures when the proper ratio of solution to silica gel is achieved.

* The word "slurry" is reserved for those mixtures prepared as described in the experimental section.

Shaking the slurry during its make-up probably prevents any structure formation during the hydration of the calcium sulfate. During the drying step this structure formation then takes place in such a manner as to produce a more water-resistant coating. Plates which have been coated using a slurry, and then thoroughly air-dried, lose only the surface layer of the coating when rinsed in a stream of cold water. Most of the silica gel adheres to the glass and must be removed by rubbing. Coatings prepared from the conventional 2:1 mixture are almost completely washed off the plate when treated in the same manner. The adherent layer on plates coated from slurries makes these coatings more resistant to flaking when they are developed in solvent systems containing a high percentage of water.

Not all solutions can be used to prepare satisfactory slurries by the procedure outlined. Slurries prepared with solutions 0.1 *M* in phosphate ion produced, within three days, crystalline appearing aggregates which presumably were calcium phosphate. A slurry prepared using McILVAINE's citrate–phosphate buffer, pH 3.2, was too thin to coat. A mixture prepared using the same buffer in a 2:1 ratio of it to silica gel G coated satisfactorily and did not set to a gel even if not shaken.

Silica gel H slurries are also usable over a period of time. Aside from the higher cost of silica gel H, only one disadvantage of this material has been noted. A silica gel H slurry in 0.1 *M* NaOH after one week's standing contained aggregates of silica gel which could not be dispersed by shaking. The coatings prepared using silica gel H slurries have the same mechanical properties as those prepared from silica gel G slurries. Silica gel H would have an advantage when coatings are prepared using materials, *e.g.* phosphate ion, which react with the calcium sulfate in silica gel G.

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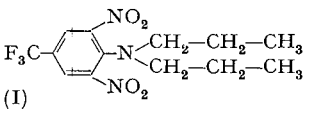
Separation of trifluralin and some related compounds by two-dimensional thin-layer chromatography

Trifluralin (I) (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine) is a selective pre-emergent herbicide for use on agronomic crops[1–3]. It is active against a great variety of broadleaf weeds and annual grasses.

Prior to starting metabolic studies of trifluralin in plant and soil systems, it was necessary to investigate the chromatographic behavior of this compound,

related compounds and possible metabolites. Two-dimensional thin-layer chromatography on Silica Gel GF separated trifluralin and 17 related substances. Optimum separations were obtained with benzene–ethylene dichloride (1:1) and *n*-hexane–methanol (98:2).



(I)

The compounds that are not colored are detected as blue absorbing spots when the Silica Gel GF plate is exposed to short wavelength U.V. radiation.

Experimental

Thin-layer plates (20 cm × 20 cm × 0.2 cm) were coated with Silica Gel GF 254 (Brinkmann Instruments, Inc., Long Neck, N.J.) as described by STAHL[4,5] using a suspension of 30 g of Silica Gel GF in 60 ml of distilled water in a 250 μ spreader. The plates were activated by drying at 110° for 60 min.

Five μg of trifluralin and each of the 17 compounds (Table I) in benzene solution were applied at a point of 3 cm from the left edge and 3 cm from the bottom of the plate.

After developing in solvent I (benzene–1,2-dichloroethylene (1:1)), the plates were dried at room temperature for 30 min, rotated 90° and developed in solvent II (*n*-hexane–methanol (98:2)). The chromatography jars were lined with filter paper to insure saturation.

Results and discussion

Fig. 1 shows the separation obtained with the compounds listed in Table I. The detection limit of the colored compounds and those that show as blue spots under short wavelength U.V. radiation is approximately 0.5 μg.

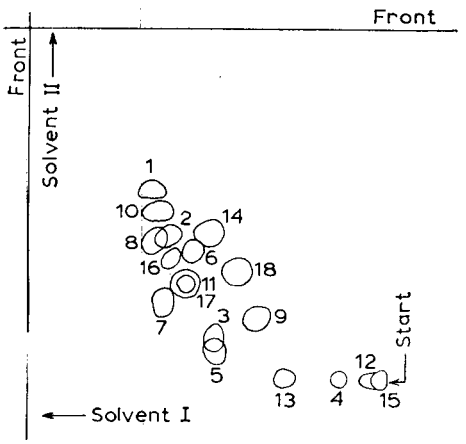


Fig. 1. Thin-layer chromatogram of trifluralin and 17 related compounds. Silica Gel GF. Solvent I = benzene–1,2-dichloroethylene (1:1); solvent II = *n*-hexane–methanol (98:2).

TABLE I

INVESTIGATION OF TRIFLURALIN AND RELATED SUBSTANCES

Number	*Name*	*Color on TLC*
1	Trifluralin	pale yellow
2	α,α,α-Trifluoro-2,6-dinitro-N-(*n*-propyl)-*p*-toluidine	intense yellow
3	2,6-Dinitro-4-trifluoromethylaniline	green yellow
4	α,α,α-Trifluoro-5-nitrotoluene-3,4-diamine	yellow
5	α,α,α-Trifluoro-5-nitro-N^4-(*n*-propyl)-toluene-3,4-diamine	yellow brown
6	2,6-Dinitro-N-(*n*-propyl)-*p*-toluidine	intense yellow
7	N^2,N^2-Di-(*n*-propyl)-3-nitro-5-trifluoromethyl-O-phenylenediamine	light yellow
8	N,N-Di-(*n*-propyl)-2,6-dinitro-*p*-toluidine	colorless
9	N^4,N^4-Di-(*n*-propyl)-α,α,α-trifluorotoluene-3,4,5-triamine	colorless
10	N,N-Di-(*n*-propyl)-2-nitro-α,α,α-trifluoro-*p*-toluidine	yellow brown
11	3,5-Dinitrobenzotrifluoride	colorless
12	2,6-Dinitro-α,α,α-trifluoro-*p*-cresol	intense yellow
13	2-Nitro-4-trifluoromethylaniline	pale yellow
14	N-(*n*-Propyl)-2-nitro-*p*-toluidine	deep brown-red
15	3,5-Dinitro-4-(di-*n*-propylamino)-benzoic acid	yellow
16	N-(*n*-Propyl)-2-nitro-α,α,α-trifluoro-*p*-toluidine	yellow
17	3,5-Dinitro-4-methoxybenzotrifluoride	colorless
18	3,5-Dinitro-4-(di-*n*-propylamino)-methylbenzoate	pale yellow

Since it is often difficult to obtain the 0.5 μg of metabolites in plant and soil samples, other methods of detection are needed. The separation of the compounds obtained with the thin-layer chromatographic system are adequate to permit dividing the chromatogram into zones which can be investigated by other techniques. In our laboratories, nanogram to picogram quantities of material have been isolated from thin-layer chromatographic plates and measured by gas–liquid chromatography. When radioactive labelled materials are used in metabolic studies, the separated zones can be eluted and counted by liquid scintillation counter.

Acknowledgements

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A simple technique in developing thin-layer bioautographs

The literature includes several reports on bioautographic techniques for antibiotic identifications. NICHOLAUS *et al.*[1] described a method utilizing tetrazolium chloride for zone definition and sensitivity. A combined procedure utilizing paper and silica gel was described by MEYERS AND SMITH[2].

It has been possible to greatly simplify these methods, as well as including other antibiotics not previously tested.

Materials and methods

This simplified method included the use of silica gel plates prepared by the standard procedure of STAHL[3] and developed in chromatographic chambers. For practical purposes drying periods extended for one to two hours at room temperature. This period of time was altered depending upon the solvents used. An agar medium was sprayed onto the dried plates with a Devilbiss paint spray gun attached to the laboratory air line. To standardize the pressure for more uniform results, a pressure reducing valve adjusted to 27 lb./sq.in. pressure was added to the line. Agar at 100° gave satisfactory results, in fact, the mixture of agar and air passing through the nozzle of the spray gun was sufficiently cool to allow solidification immediately on the upright plate.

An agar medium, cooled to 48° and inoculated with a suitable microbiological assay organism, was poured directly over the surface of the prepared plate. To insure even distribution of the medium, a box, 20 cm × 20 cm, was constructed of plexiglass to hold the plate. Volumes of agar ranging between 60–100 ml were sufficient to produce the desired effects over the surface of the described area. Lids, also prepared from plexiglass, were used to cover the plates during incubation.

Zones of inhibition were identified after incubation by directly viewing the opaque plates.

Results and discussion

The method of developing thin-layer plates described by NICHOLAUS *et al.*[1] includes a rather complicated procedure of adding tetrazolium chloride to the inoculated medium to protect the reagent from the atmosphere. They also used a pre-incubation period of 1 h at 0° to allow diffusion of the antibiotic into the inoculated agar while deterring growth of the organism. Variations in the percent inoculum and the agar depth were used to increase the sensitivity as well as zone definition.

Several attempts were made to apply the inoculated medium directly to the silica gel but the antibiotics were spread over the plate surface producing vast areas of inhibition. Since using the spraying device no malconfigurations of inhibitory zones were noticed.

Pressurized cans containing liquid propellants were also tried and worked satisfactorily but more uniform results were obtained with the present method.

Antibiotics tested in our laboratories include sodium penicillin G, tylosin base, erythromycin base, streptomycin sulfate, and several derivatives of tylosin.

A system using methanol–acetone (60:40) was satisfactory to differentiate penicillin, tylosin, streptomycin and erythromycin. Table I includes the R_F values for these antibiotics.

Only streptomycin failed to move from the original spot. Many different solvent systems were tried but all failed to produce the desired effect. This may be associated with the strong binding capacity of streptomycin and the solid substrate and the relative insolubility of the antibiotic in organic solvents. Although the literature contained many systems for separating streptomycin on paper, none were found for thin-layer methods.

TABLE I

R_F VALUES OF SOME ANTIBIOTICS

Antibiotic	*R_F value*
Sodium penicillin G	0.75
Erythromycin base	0.20
Streptomycin sulfate	Not mobile
Tylosin base	0.83

It was possible, however, to move streptomycin in an aqueous system. An R_F value of 0.27 was obtained under these conditions. Erythromycin and tylosin failed to move with water and an R_F value of 0.63 was determined for penicillin. With these two systems, it would be possible to identify each of the components. In fact, a two-dimensional system might be used.

It was also noted that higher concentrations of antibiotics applied to silica gel and located with a 1:1 solution of 2 % sodium carbonate and 1 % potassium permanganate could also be detected by microbiological methods. In this instance the plates were sprayed with a laboratory atomizer containing the reagent. After marking the yellow spots, the plates were dried at room temperature for 1 h and developed by microbiological methods.

It is assumed that the surface antibiotic is oxidized allowing for chemical detection and the sub-surface antibiotic diffused into the medium allowing for microbiological activity. This technique offers a unique advantage of qualitatively assaying a sample by both means showing biologically inactive degradation products as well as active components.

Thus far, three adsorbents have been tested: Silica Gel G, Silica Gel GF 254 and Kieselguhr G. All have proved satisfactory for our purposes. The added indicator in Silica Gel GF 254 allows detection of spots by U.V. light. But like the permanganate systems, larger quantities of antibiotics are required.

Two organisms, *Bacillus subtilis* A.T.C.C. 6633 and *Sarcina lutea* A.T.C.C. 9341, have been used for developing the bioautographs. *Bacillus subtilis* is routinely used for developing the paper chromatograms. Both of these organisms are used for standard microbiological assays and both were suitable for the detection of the antibiotics used in these studies. *Bacillus subtilis*, however, is more susceptible to streptomycin than *Sarcina lutea* and is recommended for this purpose.

Sensitivity, as defined for these procedures, is the smallest detectable quantity of antibiotic. This would vary according to the quantity of agar used, the concentration of inoculum and the antibiotic–organism relationship.

For the penicillin–*Sarcina lutea* combination on medium No. 1 (GROVE AND RANDALL[4]) the sensitivity was 0.005 units.

Bacillus subtilis' susceptibility to streptomycin on medium No. 5 (GROVE AND RANDALL[4]) approaches 0.1 μg. Tylosin against *Sarcina lutea* on penicillin seed agar was effective at a level of 0.025 μg. The smallest detectable level of activity for erythromycin against *Sarcina lutea* was 0.025 μg.

Two plates (Figs. 1 and 2) are included to illustrate the results of this technique. In certain instances, the poor zone definition is a result of the photographic equipment. Agar color and organism pigment create no problem in measuring mobilities directly on the plate surface.

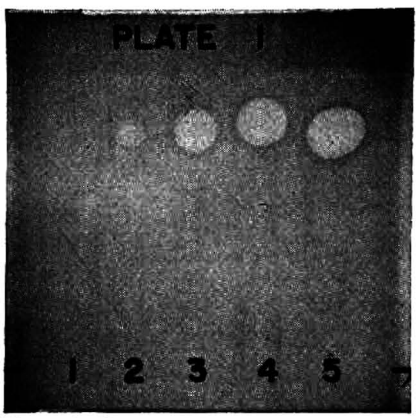

Fig. 1. Sodium penicillin G developed on Silica Gel G with *Sarcina lutea*. 1 = 0.005 units; 2 = 0.01 units; 3 = 0.025 units; 4 = 0.05 units; 5 = 0.1 units.

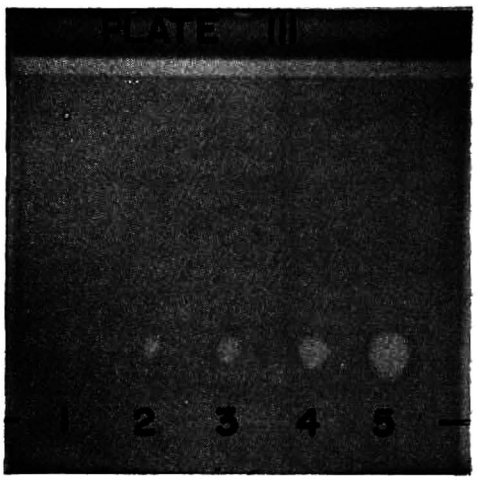

Fig. 2. Erythromycin base developed on Silica Gel G with *Sarcina lutea*. 1 = 0.025 μg; 2 = 0.05 μg; 3 = 0.1 μg; 4 = 0.2 μg; 5 = 0.25 μg.

For these reasons it appears that this method might be routinely used for the identification of minute quantities of antibiotic. Prior to this time, only one antibiotic could be unequivocally identified at these concentrations. The antibiotic, of course, is penicillin and the reagent penicillinase. Microbiological spectra could be used but it could not be recommended for routine use.

Additional systems are being screened as well as different absorbents and other antibiotics to find the ideal combinations for complete separation.

Gratitude is expressed to Mr. BARTH RAGATZ for technical assistance.

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Separation of 2,4-dinitrophenylhydrazones of oxo-terpenes by thin-layer chromatography

Several chromatographic methods have been developed for the separation of terpenes as such by MILLER and co-workers[1,2]. A thin-layer chromatographic method has been developed for the identification of carbonyls in the form of their 2,4-dinitrophenylhydrazones by ONOE[3]. Paper chromatography, adsorption chromatography, counter current distribution or liquid–liquid partition are time consuming and not so handy as thin-layer chromatography (TLC). Another thin-layer chromatographic method for the separation of aliphatic carbonyls (C_1 to C_3) as 2,4-dinitrophenylhydrazones has been developed by NANO AND SANCIN[4]. The present workers have developed a new method for the separation and identification of 2,4-dinitrophenylhydrazones of oxo-terpenes by thin-layer chromatography.

Application of this procedure in conjunction with either of the afore-mentioned procedures provides valuable clues to the identity of oxo-terpenes, which are generally present in small quantities in essential oils.

Experimental

Preparation of the thin-layer plates. Thin-layer plates (20 × 5 cm) were coated with a slurry of silica gel (chromatographic, E. Merck) mixed with 10% plaster of Paris and twice its weight of distilled water. The method of application was that described by LEES AND DE MURIA[5]. The coated plates were dried at 110° for 2–3 h in an oven. Four different solvent systems were used for the separation and identification of 2,4-dinitrophenylhydrazones. No spraying agent was used as the spots themselves were distinctly coloured.

TABLE 1

Synthetic mixture	*Components (as 2,4-dinitrophenylhydrazones)*
A	Carvone, menthone and pulegone
B	Menthone and pulegone
C	Salicylaldehyde, formaldehyde and phenylacetaldehyde
D	Salicylaldehyde, phenylacetaldehyde and pulegone
E	Carvone, dihydrocarvone and formaldehyde
F	Citral, α-thujone and citronellal
G	C_{10}-aldehyde, C_8-aldehyde and camphor
H	Menthone, citral and carvone
I	Acetaldehyde, camphor and acetone
J	Camphor and citronellal

Preparation of 2,4-dinitrophenylhydrazones. The method followed for the preparation of the 2,4-dinitrophenylhydrazones was as described by Guenther[6]. The derivatives were crystallized and recrystallized till they showed sharp melting points. Mixed melting points were taken to verify the authenticity of these compounds.

Solvent systems. The following solvent systems have been found to give clear separation of the synthetic mixtures of 2,4-dinitrophenylhydrazones:

S_1 = chloroform–carbon tetrachloride (1:19)
S_2 = chloroform–carbon tetrachloride (1:9)
S_3 = chloroform–carbon tetrachloride (3:17)
S_4 = petroleum ether–benzene (3:7).

The synthetic mixtures used were prepared by mixing 10 mg of each 2,4-dinitrophenylhydrazone, as shown in Table I.

A very clear separation of 2,4-dinitrophenylhydrazones in the case of the above synthetic mixtures was achieved. The results are tabulated in Table II.

TABLE II

R_F × 100 VALUES OF SOME 2,4-DINITROPHENYLHYDRAZONES OF OXO-TERPENES IN VARIOUS SOLVENT SYSTEMS

S. No.	*2,4-Dinitrophenylhydrazone of*	S_1	S_2	S_3	S_4
1	Formaldehyde	9	12	13	47
2	Acetaldehyde	10	11	15	51
3	Acetone	11	13	16	60
4	C_8-aldehyde	14	19	26	69
5	C_{10}-aldehyde	16	22	30	71
6	Citral	17	20	24	63
7	Citronellal	23	37	29	30
8	α-Thujone	17	31	23	82
9	Menthone	42	38	41	84
10	Pulegone	34	37	46	75
11	Carvone	29	32	36	79
12	Dihydrocarvone	22	29	33	80
13	Camphor	25	35	39	76
14	Salicylaldehyde	5	7	8	44
15	Phenylacetaldehyde	11	16	17	58

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Ein dünnschichtchromatographisches Verfahren zur quantitativen Bestimmung von Aminosäuren und Aminozuckern im Mikromasstab

Die Aminosäuren Alanin, Glutaminsäure und α-ε-Diaminopimelinsäure (DAP), sowie die Aminozucker Glucosamin und Muraminsäure sind als konstitutive Bestandteile der Bakterienzellwand bekannt[1]. Um diese Substanzen in den enzymatischen Spaltprodukten bakterieller Zellwände nebeneinander, quantitativ und im Mikromassstab analysieren zu können, wurde in Abwandlung bekannter Methoden[2] das folgende Verfahren ausgearbeitet. Es besteht in einer dünnschichtchromatographischen Trennung der Substanzen, Anfärbung mit Ninhydrin, Isolierung und Extraktion der Stoffe, Überführung des instabilen Ninhydrinkomplex in den stabilen Ninhydrin–Cadmium-Komplex und einer photometrischen Messung der einzelnen Stoffkomplexe.

Material

Es wurde mit einem künstlichen Gemisch gearbeitet, welches in 1 ml Wasser je 1 mg Alanin, Glutaminsäure, DAP, Glucosamin und Muraminsäure enthielt. Die Aminosäuren und Glucosamin wurden von der Fa. Calbiochem bezogen, die Muraminsäure wurde aus den Zellwänden von *Escherichia coli* B rein dargestellt[3]. Als Trägersubstanz bei der Dünnschichtchromatographie diente Cellulosepulver (300 MN, Macherey, Nagel & Co., Düren). Zum Beschichten der Dünnschichtplatten wurde ein Streichgerät der Fa. Desaga, Heidelberg benutzt.

Methoden

Dünnschichtchromatographie. Als Trägerplatten wurden 4 mm dicke Glasplatten 20 × 20 cm verwendet. Die Platten sind vor jedem Gebrauch durch Einlegen und sorgfältiges Spülen in heisser, konzentrierter Sodalösung fettfrei zu waschen. Die sauberen Platten werden mit einer Cellulosepulversuspension beschichtet; die Schichtdicke beträgt 250 μ. Die Aufschwemmung (15 g Pulver auf 90 ml Wasser) wird vor dem Einfüllen in das Streichgerät 2 Min. in einem Starmix homogenisiert. Die beschichteten Platten werden 15 Std. bei Zimmertemperatur oder in einem Thermostat (Luftumwälzer) nicht über 40° getrocknet und sind dann gebrauchsfertig. (Sollen die Platten aufbewahrt werden, so stellt man sie in einen Exsikkator.) Auf die trockene Platte wird das Substanzgemisch in Konzentrationen zwischen 2.5 und 0.25 μg pro Substanz punktförmig mit einer 1 μl Pipette aufgetragen. Durchmesser der Startflecken einheitlich 4 mm (grössere oder kleinere Flecken können bei der Trennung schwänzen), Abstand der Startflecken zum unteren Plattenrand 2 cm, Abstand der Flecken untereinander mindestens 1.5 cm. (Es empfiehlt sich, für eine Versuchsserie stets dieselbe Mikropipette zu benutzen, um Fehler durch Ungenauigkeiten der Pipettenkalibrierung zu vermeiden.)

Die Platten werden durch einen zweimaligen Trennungslauf in Glaströgen mit aufgeschliffenem Deckel (21 × 21 × 5 cm, Fa. Desaga, Heidelberg) bei 25° entwickelt. Trennungsgemisch ist Butanol–Pyridin–Eisessig–Wasser (60:45:4:30, v/v) (monophasisches Gemisch). Die Wände der Trennkammer werden mit lösungsmittelbefeuchtetem Filtrierpapier ausgekleidet. Man beschickt die Kammer 1 Std. vor dem ersten Lauf (Äquilibrierung der Kammeratmosphäre) mit 139 ml Lösungsmittel. Die Trennung wird abgebrochen, wenn die Lösungsmittelfront 1 cm vor dem oberen

Plattenrand steht, und man trocknet die Platte für 30 Min. bei 60° im Luftumwälzer. Dann wird bei unveränderten Bedingungen der zweite Trennungslauf durchgeführt. Danach trocknet man die Platte 5 Min. an der Luft vor und bringt sie anschliessend für 15 Std. zur völligen Trocknung in einen auf 11 mm Hg (Wasserstrahlpumpe) evakuierten Exsikkator über konzentrierte H_2SO_4 und frisches NaOH (in rotulis). (Für einen Exsikkator von 25 cm Durchmesser genügen 100 ml H_2SO_4 und 100 g NaOH.)

Anfärbung, Isolierung und Extraktion. Die getrockneten Platten werden kurz in eine 0.5 %ige Lösung (G/V) von Ninhydrin in Aceton getaucht und dann 105 Min. bei 70° in einem zuverlässig temperaturkonstanten Thermostat entwickelt. Die folgenden Arbeitsschritte sollen stets ohne Verzögerungen und in möglichst gleicher Zeit durchgeführt werden. Um die einzelnen Substanzflecke werden gleich grosse Areale markiert (z.B. mit einem sauberen Korkbohrer), die etwas grösser als die Farbflecke sein sollen.

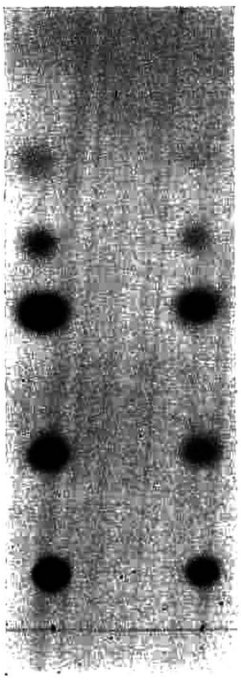
Das Material innerhalb der Markierungen wird mit einem kleinen Spatel von der Platte gekratzt, auf einen glatten, gefalteten Papierbogen geklopft und von dort über einen kleinen Trichter in Glasröhrchen (7 cm lang, 4 mm Innendurchmesser) gefüllt. Bei sorgfältiger Arbeitsweise (Abklopfen einzelner, hängenbleibender Substanzpartikel) lässt sich die Substanz jedes Fleckens ohne Verlust in den Boden eines Röhrchens übertragen. In gleicher Weise werden in Höhe der einzelnen Substanzflecke 5 Blindproben aus der Platte entnommen. Anschliessend wird jedes Röhrchen mit 0.4 ml einer 0.5 %igen Lösung (G/V) von Cadmiumacetat in Methanol[4] gefüllt und durch Einblasen von Luft mittels einer Glaskapillare die Substanz suspendiert (Vorsicht vor Überlaufen der Röhrchen). Man verschliesst die Röhrchen mit einem Plastikfilm (z.B. Para-film), suspendiert nach 2 Std. nochmals und zentrifugiert dann 10 Min. bei 3000 *g*. Bei vorsichtiger Handhabung kann man die überstehende Farblösung ohne Celluloseverunreinigungen abpipettieren und direkt in die Photometerküvetten einfüllen.

Photometrische Messung. Zur photometrischen Auswertung der Substanzlösungen wurde mit einem Eppendorf-Photometer bei 494 mμ, Photozelle 90 b, mittlerer Blende, Halbmikroküvetten von 0.5 cm Schichtdicke gegen 0.5 %ige Methanol–Cd-Acetatlösung gemessen. Die einzelnen Messzahlen wurden gegen die jeweiligen Blindwerte korrigiert.

Ergebnisse und Diskussion

Fig. 1 zeigt die chromatographische Auftrennung des Substanzgemischs bei 0.5 bzw. 0.25 μg pro Substanz, Fig. 2 die Extinktionskurven der fünf Substanzen für 2.5–0.25 μg. Innerhalb dieses Bereichs verlaufen die Kurven streng linear. Bei höheren Substanzmengen liegen die Extinktionswerte im Vergleich zu den Standardmessungen relativ zu niedrig. Bei geringeren Mengen streuen besonders die Messwerte der Aminozucker beträchtlich. Die Messungen sind mit einem durchschnittlichen Fehler von $\pm$ 5 % der Werte reproduzierbar. Gelegentlich treten auch grössere Abweichungen auf, die wohl vor allem auf unregelmässige Temperaturschwankungen während der langen Entwicklungszeit der Ninhydrinfärbung zurückzuführen sind. (Kürzere Entwicklungszeiten sind zwar für die Aminosäurebestimmungen möglich, führen aber bei den Aminozuckern zu falschen Ergebnissen.) Es empfiehlt sich, eine Substanzbestimmung nicht einfach durch Vergleich mit einmal aufgestellten Standardkurven

vorzunehmen, sondern auch einige Werte der Eichsubstanzen nachzumessen, so dass eventuelle Abweichungen berücksichtigt werden können.



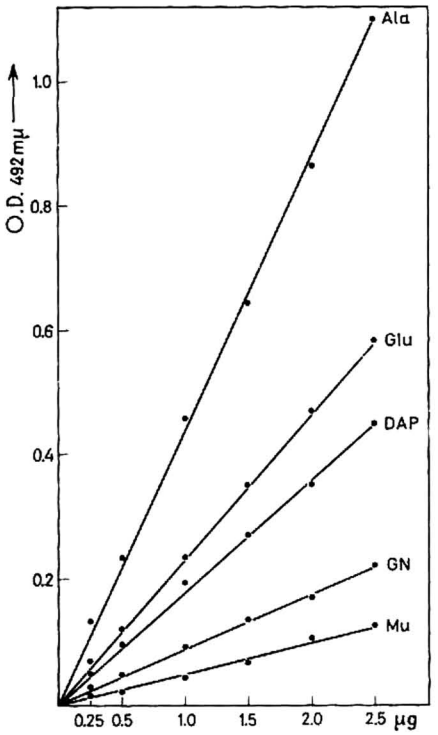


Fig. 1. Trennung von je 0.5 μg (links) und 0.25 μg (rechts) Substanz. Von oben nach unten: Muraminsäure, Glucosamin, Alanin, Glutaminsäure und DAP.

Fig. 2. Extinktionskurven für 2.5–0.25 μg Substanz. Mu = Muraminsäure; GN = Glucosamin; DAP = α–ε-Diaminopimelinsäure; Glu = Glutaminsäure; Ala = Alanin.

Dank

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Dünnschicht-Chromatographie einiger Zucker und Zuckeralkohole

Mit der Dünnschicht-Chromatographie von Zuckern und Zuckerderivaten auf Kieselgur G* haben sich erstmals STAHL UND KALTENBACH[1,2] befasst. RINK UND HERRMANN[3] entwickelten die Phenylosazone der Zucker, die sie aus Urin isoliert hatten, ebenfalls auf Kieselgur G, während WASSERMANN UND HANUS[4] die reinen Zucker auf einer Mischung von Kieselgur und Kieselgel G (60:40) chromatographierten. GRASSHOF[5,6] verwendete Magnesiumsilikat als Sorptionsmittel und RAGAZZI UND VERONESE[7] Kieselgel G.

Vor einigen Jahren haben JAYME UND KNOLLE[8] auf phosphatimprägnierten Glasfaserpapieren eine gute Auftrennung verschiedener Zucker erreicht. Die vorliegende Arbeit zeigt, dass in analoger Weise eine Verteilung der Zucker und Zuckeralkohole auf inerten Sorptionsmitteln wie z.B. Kieselgur G möglich ist. Auf phosphatimprägnierten Kieselgel G-Schichten (besser noch auf einer hochgereinigten Sorte) erfolgt ebenfalls eine Trennung, doch sind die R_F-Werte sehr viel kleiner. Selbst bei Mehrfachentwicklungen bleiben die R_F-Werte insgesamt auf Kieselgel G-Schichten kleiner, während auf den Kieselgur G-Schichten bei einmaliger Entwicklung eine Verteilung über die gesamte Trennstrecke erfolgt (siehe Tabelle I).

TABELLE I

Zucker	*R_F-Werte*	*R_G-Werte*	*Zucker*	*R_F-Werte*	*R_G-Werte*
Rhamnose	0.93	1.69	Dulcit	0.45	0.82
Glycerin	0.90	1.65	Saccharose	0.40	0.73
Ribose	0.75	1.36	Sorbit	0.39	0.71
Xylose	0.73	1.33	Galaktose	0.36	0.65
Sorbose	0.68	1.24	Maltose	0.30	0.55
Arabinose	0.65	1.18	Trehalose	0.23	0.42
Mannose	0.63	1.15	2-Ketogulonsäure	0.19	0.35
Fruktose	0.60	1.09	Laktose	0.17	0.31
Glukose	0.55	1.00	2-Ketoglukonsäure	0.10	0.18
Mannit	0.52	0.95	Raffinose	0.05	0.09

Nach Angaben von STAHL UND KALTENBACH[1,2] sind auf Natriumacetat gepufferten Kieselgur G-Schichten von 250 μ Dicke maximal nur 5 μg Zuckergemisch gut zu chromatographieren. Auf den phosphatimprägnierten Schichten lassen sich immerhin bis 25 μg Zucker aufbringen. Die runde Fleckenform bleibt erhalten und die Trennung der einzelnen Zucker ist gut. Die Verwendung eines aktiveren Sorptionsmittels könnte für die Durchlaufchromatographie[1,9] oder präparative Schicht-Chromatographie[10] von Interesse sein, zumal die maximal trennbaren Zuckermengen auf aktivem Kieselgel ein Vielfaches betragen.

Auf Kieselgur G-Schichten erhält man mit dem Fliessmittel: *n*-Butanol–Aceton–Phosphatpufferlösung** (40:50:10) bei einmaliger Entwicklung und 10 cm Laufstrecke, die in der Tabelle I angegebenen R_F- und R_G-Werte***.

* Zu Beziehen bei der Firma E. Merck A.G., Darmstadt, Deutschland.

** Anstelle von Phosphatpufferlösung kann auch Wasser verwendet werden, ohne dass sich die R_F-Werte wesentlich verändern.

*** Es handelt sich um Mittelwerte aus mehreren Bestimmungen. Die R_G-Werte (Bezugswerte auf Glukose) sind besser reproduzierbar aufzufinden.

Beschreibung der Versuche

Durch Mischen etwa gleicher Teile 0.1 *M* Phosphorsäurelösung und 0.1 *M* Dinatriumhydrogenphosphatlösung wurde eine Phosphatpufferlösung von pH 5.0 (pH-Papier) hergestellt.* Zur Bereitung von 5 Dünnschichtplatten der Grösse 20 × 20 cm von 250 μ Dicke wurden 20 g Kieselgur G mit 40 ml obiger Phosphatpufferlösung angerührt und nach STAHL[1] auf die Glasplatten aufgebracht. Nachdem die Schichten über Nacht an der Luft getrocknet waren, wurden 5–25 μg der Zucker in Form 0.5 %iger wässriger Lösungen auf die Startpunkte mit Mikropipette dosiert. Die Entwicklung erfolgte mit dem oben angegebenen Fliessmittel mit "Kammerübersättigung"[1] innerhalb von 30–35 Min.

Zur Detektion sprühte man mit Natriummetaperjodatlösung (a) vor, dann mit Benzidinlösung (b). Die Dünnschichtplatte wurde hierauf 5 Min. in ein Gefäss das Ammoniakdampf enthält eingestellt und schliesslich mit Silbernitratlösung (c) nachgesprüht.

(a) Natriummetaperjodatlösung ist eine 0.1 %ige wässrige Lösung von Natriummetaperjodat. Zu beachten ist, dass die Schicht erst etwas angetrocknet sein muss (lufttrocken), bevor mit Benzidinlösung weitergesprüht wird!

(b) Benzidinlösung: 2.8 g Benzidin werden in 80 ml Äthylalkohol (96 %) gelöst und zu dieser Lösung 70 ml Wasser, 30 ml Aceton und 1.5 ml *N* Salzsäure zugefügt.

(c) Silbernitratlösung: 1 ml gesättigte Silbernitratlösung wird unter Rühren zu 20 ml Aceton gegeben und dann so lange tropfenweise Wasser zugefügt, bis sich der Niederschlag eben wieder löst.

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* Bei Kieselgur, die eine Restaktivität aufweist, muss der pH-Wert stärker sauer eingestellt werden. Meist genügt es, in diesem Fall anstelle der beiden 0.1 *M* Lösungen lediglich eine 0.1 *M* Natriumdihydrogenphosphatlösung (pH ~ 4.8) zu verwenden.

Separation of chlorinated cresols and chlorinated xylenols by thin-layer chromatography

The separation of tar acids by thin-layer chromatography (TLC) has been described recently[1–3]. This paper describes the separation of chlorocresols and chloroxylenols by TLC and its usefulness in following the progress of chlorination of these tar acids. The paper chromatographic separation of these compounds has been reported earlier[4].

Materials and methods

The chlorocresols and chloroxylenols used for the separations were obtained from commercial sources[5].

Using a thin-layer applicator (Desaga, Heidelberg), glass plates (20 × 20 cm) were coated as usual with a well stirred suspension of silica gel G (E. Merck, Darmstadt; 30 g in 60 ml water) to give a layer approximately 270 μ in thickness. The plates were dried at 105–110° for 30 min and preserved in a desiccator. The compounds were dissolved in acetone and about 10 μg was applied with a glass capillary on starting points 2–3 cm from the edge of the plate. The plate was then placed inside the chamber for development. Three solvent systems, A, B and C, were used:

(A) Petroleum ether (80–100°) saturated with formic acid.

(B) Xylene saturated with formamide.

(C) The organic layer from a mixture of benzene–acetic acid–water (2:2:1, v/v).

About 40–60 min were taken for the solvent front to cover a distance of 15 cm. The plates were then sprayed with phosphotungstomolybdic acid (Folin-Denis reagent) and exposed to ammonia, which revealed the compounds as blue spots on a white background.

Results and discussion

Table I lists the R_F values of the chlorocresols and chloroxylenols in the three solvent systems, and gives the composition of the mixtures spotted. Fig. 1 shows the separation of these mixtures of chlorocresols and chloroxylenols using solvent B, which gave the best separations. Resolution of *p*-chlorocresols from parent cresols and of 6-chloro-2-methylphenol from 4,6-dichloro-2-methylphenol could not be achieved with any of the solvent systems tried.

Generally the R_F values increased with increasing substitution in the nucleus, an exception being 4,6-dichloro-3-methylphenol in solvent system B. The R_F values for *o*-substituted compounds were higher than for the corresponding *m*-isomer, probably due to the *ortho* effect[6]. Thus $R_F \times 100$ values in solvent B were: *m*-cresol = 26, *o*-cresol = 32, 4-chloro-3-methylphenol = 25, 6-chloro-2-methylphenol = 72, 3,4-dimethylphenol = 21, and 6-chloro-3,4-dimethylphenol = 44. The effect was more pronounced in the 2,6-disubstituted products, as seen in the $R_F \times 100$ values for: 4-chloro-2-methylphenol = 31, 6-chloro-2-methylphenol = 72, 6-chloro-3,4-dimethylphenol = 44, and 2,6-dichloro-3,4-dimethyl-phenol = 58. In disubstituted products this relative order was followed in all the solvent systems.

The technique was tried in following the progress of chlorination of 2,5-dimethylphenol in carbon tetrachloride with gaseous chlorine at 20 ± 2°. Samples withdrawn every 30 min were spotted. Fig. 2 reveals that 4-chloro-2,5-dimethylphenol is the

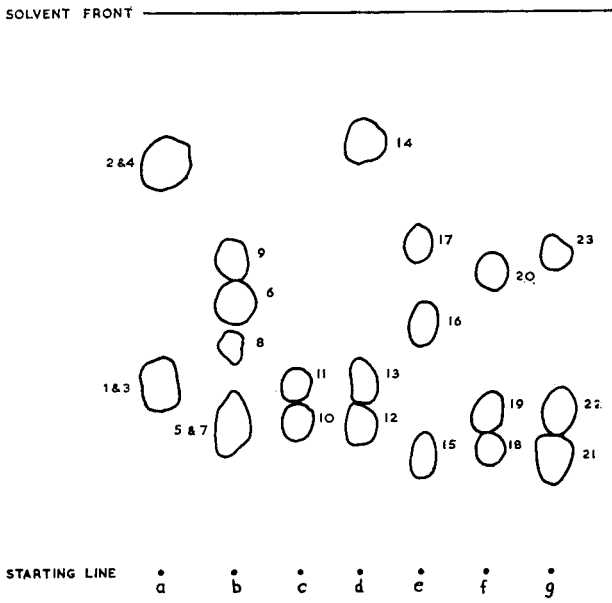

Fig. 1. Separation of chlorocresol and chloroxylenol mixtures. For the constituents of the mixtures a–g see Table I.

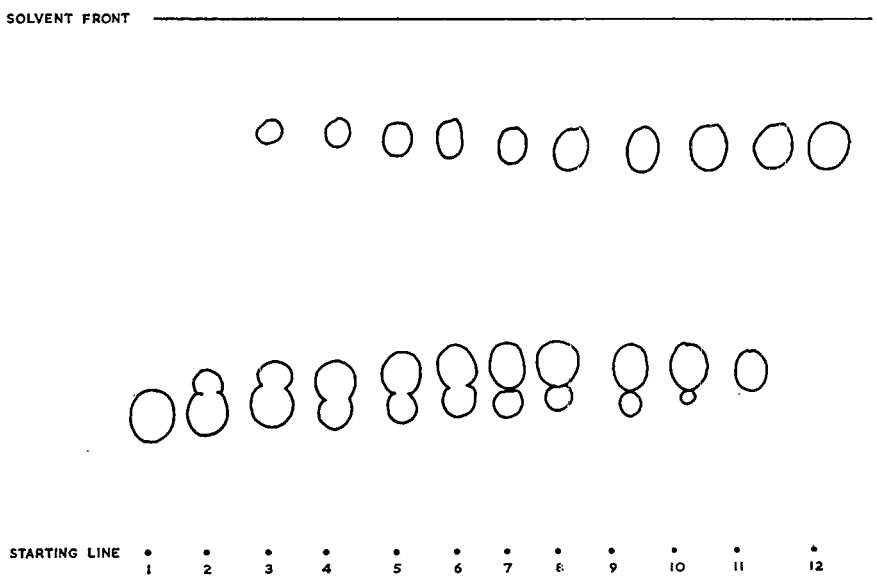

Fig. 2. Separation of chlorinated 2,5-xylenol samples withdrawn every 30 min.

TABLE I

R_F VALUES OF DIFFERENT CHLORINATED CRESOLS AND CHLORINATED XYLENOLS

Temperature of chromatography: 25 ± 3°.
Time of run for all solvent systems: 40–60 min.

Sl. No.	*Compounds*	*R_F values × 100 in various solvent systems*			*Phenol mixtures*	
		A	*B*	*C*	*Sl. Nos.*	*Code in Fig. 1*
1	2-Methylphenol	11	32	67		
2	6-Chloro-2-methylphenol	58	72	89	1 to 4	a
3	4-Chloro-2-methylphenol	06	31	65		
4	4,6-Dichloro-2-methylphenol	39	74	89		
5	3-Methylphenol	09	26	63		
6	6-Chloro-3-methylphenol	55	47	89		
7	4-Chloro-3-methylphenol	12	25	63	5 to 9	b
8	4,6-Dichloro-3-methylphenol	55	40	83		
9	2,4,6-Trichloro-3-methylphenol	66	56	89		
10	2,3-Dimethylphenol	12	26	71		
11	4-Chloro-2,3-dimethylphenol	12	31	71	10 and 11	c
12	2,5-Dimethylphenol	10	25	71		
13	4-Chloro-2,5-dimethylphenol	10	31	71	12 to 14	d
14	4,6-Dichloro-2,5-dimethylphenol	59	76	89		
15	3,4-Dimethylphenol	09	21	71		
16	6-Chloro-3,4-dimethylphenol	43	44	88	15 to 17	e
17	2,6-Dichloro-3,4-dimethylphenol	49	58	91		
18	3,5-Dimethylphenol	05	22	65		
19	4-Chloro-3,5-dimethylphenol	22	28	65	18 to 20	f
20	2,4-Dichloro-3,5-dimethylphenol	35	53	85		
21	5-Ethyl-3-methylphenol	04	22	66		
22	4-Chloro-5-ethyl-3-methylphenol	04	27	66	21 to 23	g
23	2,4-Dichloro-5-ethyl-3-methylphenol	35	56	84		

primary product of reaction. The dichloro (4,6) product appears only after one hour of chlorination. After 5 h, 2,5-dimethylphenol was totally converted to the mono- and dichloro-derivative. Half an hour later the monochloro-2,5-dimethylphenol was completely eliminated and only the dichloro-derivative was present.

Conclusions

In this study on the separation of chlorocresols and chloroxylenols by thin-layer

chromatography xylene saturated with formamide appeared to be the best solvent system. Resolution of *p*-chlorocresols from the parent cresols and of 6-chloro-2-methylphenol from 4,6-dichloro-2-methylphenol could not be achieved.

The application of TLC technique as an analytical tool in following the extent of chlorination is suggested.

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Separation of sterols and corresponding stanols on thin layers of silica impregnated with silver nitrate

In natural materials, such sterols as cholesterol and β-sitosterol very often occur as inseparable mixtures with the corresponding stanols. IKAN AND KASHMAN[1] have found a mixture of β-sitosterol and β-sitostanol in Israeli peat. Similar observations have been made by MCLEAN, RETTIE AND SPRING[2] with Scottish peat and by IVES AND O'NEILL[3] with Canadian peat moss.

In a previous communication[4] we have shown that by bromination of such mixtures, the unchanged stanols were easily separated on thin layers from the brominated sterols. In the present study the method applied by AVIGAN, DE GOODMAN AND STEINBERG[5] and MORRIS[6] for the fractionation of sterols, and by IKAN[7] for the separation of tetracyclic triterpenes on thin layers of silica impregnated with silver nitrate has been extended to include sterol–stanol mixtures. The sterols and the corresponding stanols had been shown to have practically the same R_F values on thin layers of silica gel G. However, on silica gel G impregnated with silver nitrate, the R_F values of the sterols were sufficiently different from the stanols. The R_F values and the colours obtained by spraying with 50 % sulfuric acid are summarized in Table I.

The following mixtures were separated: campesterol–campestanol, cholesterol–cholestanol, cholesterol–desmosterol, allocholesterol–cholestanol, lanosterol–dihydrolanosterol, agnosterol–dihydroagnosterol, β-sitosterol–β-sitostanol, stigmasterol–stigmastanol.

Experimental

Preparation of plates. The suspension for five plates (20 × 20 cm) was prepared by shaking 30 g of silica gel and 60 ml of water for 30 sec and applied uniformly to a thickness of 0.25 mm with a Desaga applicator. After 30 min at room temperature, the plates were heated in an oven at 125–130° for 45 min. After cooling they were

TABLE I

SEPARATION AND DETECTION OF STEROLS AND STANOLS BY THIN-LAYER CHROMATOGRAPHY

No.	*Sterol*	*R_F on silica gel G*		*Colours with 50 % H_2SO_4 (after charring)*	
		Treated with $AgNO_3$	*Untreated*	*Silica gel treated with $AgNO_3$*	*Silica gel untreated*
1	Campesterol	0.23	0.25	black	violet
2	Campestanol	0.26	0.25	brown	brown
3	Cholesterol	0.23	0.25	black	violet
4	Cholestanol	0.26	0.25	brown	brown
5	Allocholesterol	0.28	0.29	black	violet
6	Coprostanol	0.42	0.35	brown	brown
7	Demosterol	0.14	0.25	brown	brown
8	β-Sitosterol	0.23	0.25	black	violet
9	β-Sitostanol	0.32	0.25	brown	brown
10	Stigmasterol	0.23	0.25	black	violet
11	Lanosterol	0.41	0.42	brown	brown
12	Dihydrolanosterol	0.45	0.42	brown	violet
13	Agnosterol	0.89	0.92	brown	brown
14	Dihydroagnosterol	0.95	0.92	brown	violet

sprayed with concentrated aqueous-methanolic silver nitrate solution, 5 % relative to silica gel, and then activated at 120° for 30 min.

This method permits impregnation of only part of the plate, which can thus be used for comparative chromatography.

Development. The samples were dissolved in chloroform and applied with micropipettes along a line 2 cm above the rim of the plate. The experiments were performed at room temperature (25–27°). Chloroform was used as mobile phase. It was allowed to rise a distance of 15 cm. The plates were removed and the solvent was evaporated in air.

Detection. The sterols were detected by spraying with 50 % sulfuric acid, followed by heating in an oven at 150° for 10–15 min.

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Separation of positional and geometrical isomers of monoenoic aldehydes via the dinitrophenylhydrazones

Silica gel impregnated with silver nitrate used as an adsorbent for chromatography[1] possesses highly selective properties for separating substances with differences in number, position and geometrical configuration of the double bonds. This was demonstrated by application of this adsorbent in column[2,3] and especially in thin-layer chromatography[4,5].

DE JONG *et al.*[6] (*cf.* HAVERKAMP BEGEMANN AND KOSTER[7]) and URBACH[8] studied the separation of dinitrophenylhydrazones (DNPHs) of aldehydes using thin-layer chromatography on aluminium oxide impregnated with silver nitrate; BADINGS AND WASSINK[9] used silver nitrate on Kieselguhr for the same purpose. These authors described the separation of DNPHs of saturated, 2-monoenoic and 2,4-dienoic aldehydes. Moreover they separated the DNPHs of monoenoic aldehydes containing isolated *trans*- or *cis*- double bonds from DNPHs of 2-monoenoic aldehydes.

DE VRIES AND JURRIENS[5] demonstrated that the position of the double bond also exercises a certain influence on the R_F values by separating methyl esters of *cis*-6-, *cis*-9- and *cis*-12-octadecenoic acids. It could therefore be expected that DNPHs of the positional and geometrical isomers of straight-chain monoenoic C_6- and C_7-aldehydes can also be separated using silica gel G impregnated with silver nitrate.

Methods and results

The plates (20 × 40 cm), with an adsorbent layer of 0.5 mm thickness, were prepared as described by DE VRIES AND JURRIENS[5,10].

Two micrograms of each DNPH of the isomeric hexenal and heptenal series were dissolved in 10 μl carbon tetrachloride. These solutions were placed on the plate by means of a micropipet of 10 μl at a distance of 5 cm from and parallel to a short edge. In the same way the mixture of all DNPHs of the hexenal respectively heptenal series (containing 2 μg of each DNPH) was placed on the same plate.

Development took place as customary using the ascending technique with benzene as eluant. During and shortly after development the DNPHs were visible as yellow spots. After storing in air all the unsaturated DNPHs turned grey. This may be helpful in distinguishing the saturated from the 2-monoenoic components, which have about the same R_F values. As can be seen from Fig. 1 for the hexenals and from Fig. 2, showing the heptenals, all the geometrical and positional isomers of both these series were clearly separated.

The *cis*-3-enals show some tailing, probably because these compounds degrade during development owing to the presence of a reactive α-methylene group in the molecule.

Discussion

The results clearly indicate that silica gel G impregnated with silver nitrate possesses highly selective adsorption properties for the separation of positional and geometrical isomers of alkenal DNPHs.

cis-4- and *cis*-5-heptenal DNPHs and *trans*-3- and *trans*-4-hexenal DNPHs have long elution times. It is therefore necessary to use longer plates.

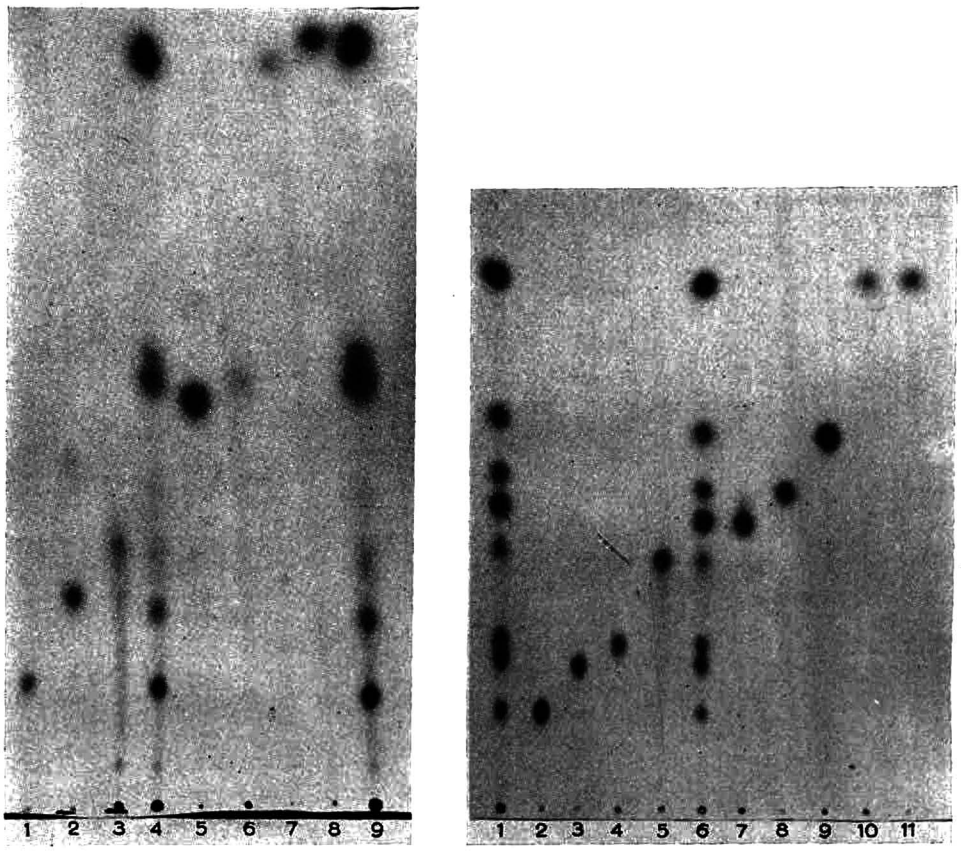

Fig. 1. Separation of a mixture of DNPHs of isomeric hexenals by TLC. Adsorbent: 30% silver nitrate–silica gel G; solvent: 100% benzene. 1 = 5-hexenal; 2 = *cis*-4-hexenal; 3 = *cis*-3-hexenal; 4 = mixture of hexanal and all isomeric hexenals; 5 = *trans*-4-hexenal; 6 = *trans*-3-hexenal; 7 = *trans*-2-hexenal; 8 = hexanal; 9 = mixture of hexanal and all hexenals.

Fig. 2. Separation of a mixture of DNPHs of isomeric heptenals by TLC. Adsorbent: 30% silver nitrate–silica gel G; solvent: 100% benzene. 1 = mixture of heptanal and all isomeric heptenals; 2 = 6-heptenal; 3 = *cis*-5-heptenal; 4 = *cis*-4-heptenal; 5 = *cis*-3-heptenal; 6 = mixture of heptanal and all isomeric heptenals; 7 = *trans*-5-heptenal; 8 = *trans*-4-heptenal; 9 = *trans*-3-heptenal; 10 = *trans*-2-heptenal; 11 = heptanal.

Only the *trans*-5- and *trans*-6-DNPHs of the nonenals were available. These nonenals could be separated using the same technique.

The technique described above may be used in the identification of unknown monoenoic aldehydes, which occur in minute amounts in oils and fats and which play an important role in imparting flavours[11] to these products. Identification and/or determination of these and other aldehydes has always been difficult in view of the extremely small amounts involved. As far as is known, attempts to separate the isomers described, by means of gas–liquid chromatography, which in itself is most suitable for analysing trace amounts, have been unsuccessful.

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Thin-layer chromatography of long-chain tertiary amines and related compounds*

Long-chain tertiary amines, *e.g.* trilaurylamine, tri-*n*-octylamine etc. are currently being examined as extraction agents in reprocessing systems for nuclear fuels. Certain commercially available amines sold as "tricaprylamine" are mixtures of straight-chain (normal) saturated tertiary amines where the alkyl groups comprise a C_8–C_{10} mixture with the C_8 chain predominating. Gas chromatographic analysis has established[1] that the main tertiary amines present are members of a homologous series: tridecyl-, didecyloctyl-, dioctyldecyl-, trioctyl-, and dioctylhexyl-amines. Secondary and primary amines with these alkyl groups are present as impurities, usually not amounting to more than 5 % by weight of the mixture. Many commercial samples of the higher *n*-alkyl tertiary amines containing only one alkyl group, *e.g.* tri-*n*-octylamine or tri-*n*-dodecylamine contain similar quantities of the corresponding secondary and primary amine impurities.

The extraction of uranium and plutonium by these long-chain amines varies according to the nature of the amino nitrogen present so that it is necessary in partition studies to be able to determine the relative amounts of primary, secondary and tertiary amine groups present in a particular sample of amine. Furthermore, if the amine has been exposed during use to conditions causing chemical degradation, *e.g.*, radiation in the presence of mineral acids, the type and amount of such degradation must be determined to define the limits of effective performance.

* Presented at a meeting of the Scottish Section of the Society for Analytical Chemistry, held in Dublin on 5th/6th September, 1963.

Methods are available for the analysis of long-chain amine mixtures based on gas chromatography[1], and on non-aqueous titrimetry[2], but in view of the speed and simplicity of TLC techniques it was of interest to devise suitable separation processes for the determination of the main amine components of such mixtures. Whilst it is not possible to separate the homologous series of tertiary amines present in "tricaprylamine" or to separate tri-*n*-octylamine from tri-*n*-dodecylamine, a facile separation into primary, secondary and tertiary long-chain amines has been made.

Experimental

Two sizes of glass plates were used: (i) 20 × 20 cm were coated with an aqueous slurry of Aluminium Oxide G using a Desaga type spreader and were dried by heating in an oven at 100° for 1 h; (ii) 3¼ × 3¼ in. lantern slide cover glasses were coated by the technique due to PEIFER[3] using a slurry of 60 g Aluminium Oxide G in 100 ml of a 70:30 mixture of chloroform and methanol. This type of plate was prepared immediately before use, dried in a stream of air for 5 min and used without further activation. Substrates prepared by these methods barely resolved the standard Desaga dye-mixture with the developing solvent (see below and Fig. 2) but were suitable for the separation of long-chain amines according to their functionality.

Spotting, development and detection

0.25 μl of amine (or an equivalent amount of a diluted sample) was a suitable sample size and isobutyl acetate was the most satisfactory solvent for separating long-chain amines from the associated secondary and primary amine impurities at room temperature. With the larger size plate where the solvent front moved through 10 cm it was sometimes preferred to use a 98:2 mixture of isobutyl acetate and glacial acetic acid, which produced a much more coherent secondary amine spot although at the expense of a slightly more diffuse tertiary amine spot. After evaporation of the developing solvent the plates were sprayed with an aqueous solution of cobalt thiocyanate [NH_4CNS, 10 g; $Co(NO_3)_2 \cdot 6H_2O$, 10 g; H_2O, 100 ml]. Primary, secondary and tertiary amines give blue colorations with this reagent against a pink background of the substrate. The colour of the sprayed plate fades to a uniform blue after exposure to air for approx. 2 h but the contrast may be restored by respraying with water or by steaming. Long-chain quaternary salts and long-chain amine oxides (but not long-chain alcohols or hydrocarbons) also give blue colours with cobalt thiocyanate and in some cases the colour produced differs slightly from that of the parent tertiary amine. Permanent records were preserved by photography.

Results

Fig. 1 is a typical chromatogram obtained by the above procedure using a 20 × 20 cm plate. R_F values are not quoted; the only significant value, the R_F value of secondary amine, varied widely with the activity of the substrate, which as stated above was low and difficult to reproduce from different plates. The tertiary amine spot was invariably found in or slightly below the solvent front whilst the primary amine spot only spread slowly from the point of application.

Fig. 2 shows the effect of omitting the acetic acid from the developing solvent. The secondary amine spots are more diffuse than in Fig. 1 but the tertiary and primary amine spots are more coherent.

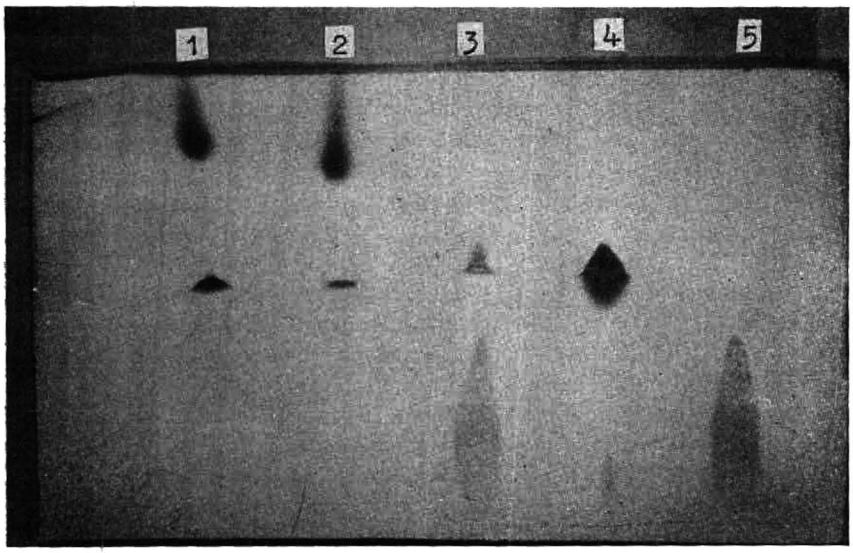

Fig. 1. Chromatograms (20 × 20 cm) on alumina, developed with isobutyl acetate–acetic acid (98:2). (1) Tri-*n*-octylamine containing 5% v/v di-*n*-octylamine. (2) Tri-*n*-octylamine containing 2% v/v di-*n*-octylamine. (3) Mono-*n*-octylamine showing di-*n*-octylamine as impurity. (4) Di-*n*-octylamine showing *n*-octylamine as impurity. (5) Mono-*n*-octylamine.

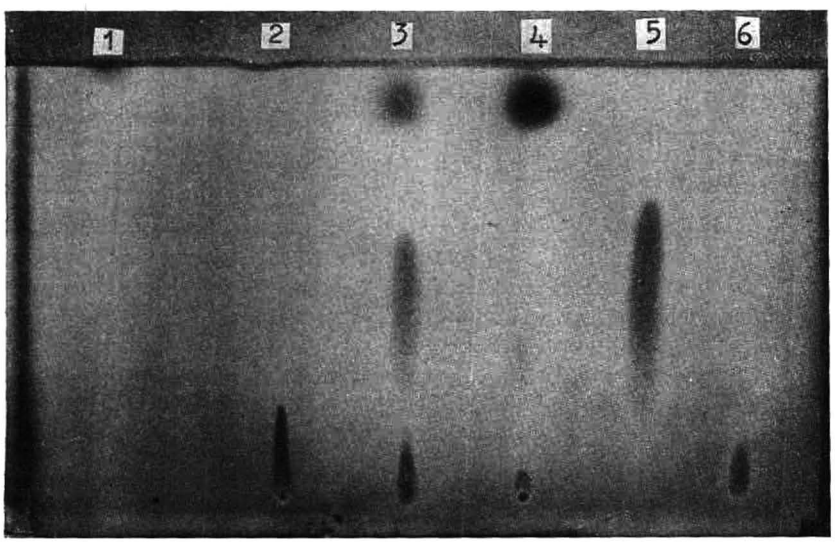

Fig. 2. Chromatograms (20 × 20 cm) on alumina, developed with isobutyl acetate. (1) Dyemarker. (2) "Tricaprylamine oxide". (3) Mixture of equal parts by vol. of mono-, di- and tri-*n*-octylamine. (4) Tri-*n*-octylamine. (5) Di-*n*-octylamine. (6) Mono-*n*-octylamine.

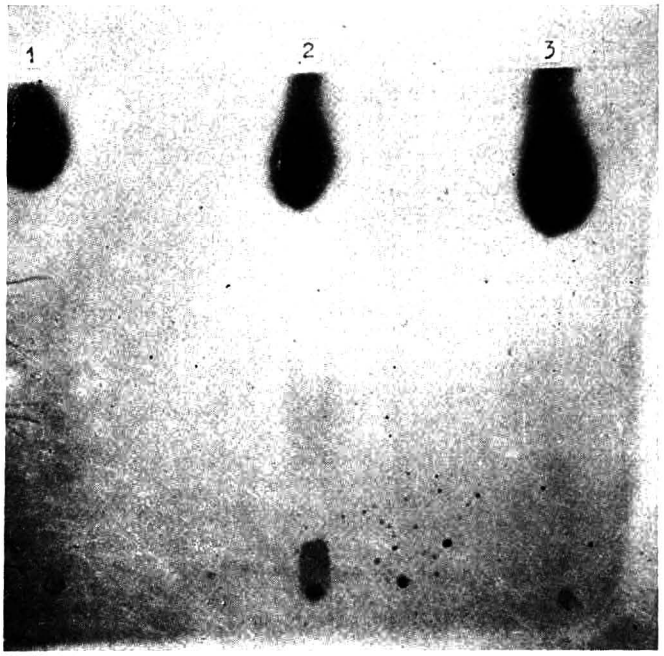

Fig. 3. 3¼ × 3¼ in. alumina coated chromatoplate developed with isobutyl acetate showing three different samples of "tricaprylamine".

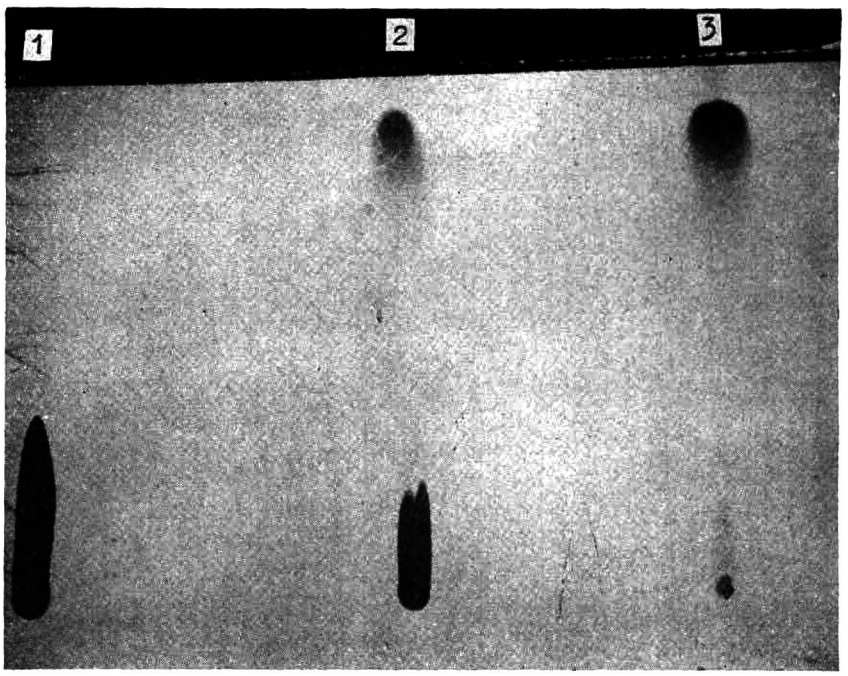

Fig. 4. 3¼ × 3¼ in. alumina coated chromatoplate developed with benzene. (1) "Tricaprylamine methosulphate." (2) "Tricaprylamine methosulphate" containing 25% unreacted "tricaprylamine". (3) "Tricaprylamine" with primary and secondary amine impurities.

The use of the smaller plate to compare the primary and secondary amine impurities in three commercial samples of "tricaprylamine" is shown in Fig. 3. The solvent front moves through 5 cm and the development requires only 30 min. Sample (2) clearly shows an increase in primary and secondary amine content over samples (1) and (3). Comparison with samples of known composition was preferred for quantitative work. Whilst lacking the precision of the gas chromatographic (GC) method the TLC method is faster and more convenient because the size of the primary and secondary amine spots is a measure of the total quantities of these components present whereas the GC method requires the measurement of the peaks of the individual components, which, in the case of "tricaprylamine" with its homologous series of amine components to identify and measure, becomes rather involved.

The conversion of long-chain tertiary amines to quaternary salts or to amine oxides by reaction with appropriate reagents (*e.g.* dimethyl sulphate or hydrogen peroxide, resp.) may also be followed by this TLC technique. Quaternary salts and amine oxides are more polar than the parent tertiary amine and are more strongly adsorbed on the substrate. Progress of a reaction where tertiary amine is being converted may be followed by spotting on to the smaller chromatoplate, developing with benzene and spraying with cobalt thiocyanate. Unreacted tertiary amine is carried in the solvent front whilst the reaction product moves only a short distance from the point of application (see Fig. 4). In this way, it is possible to detect as little as 0.2 % amine oxide in "tricaprylamine" using a 1 μl sample and 0.5 % of "tricaprylamine" in a "tricaprylamine oxide" preparation using the same size sample.

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Separation of proline and hydroxyproline derivatives by thin-layer chromatography

The recent publication of MYHILL AND JACKSON[1] concerning the separation of proline and 4-hydroxyproline N-nitroso derivatives prompted us to report similar results obtained with other derivatives. This note will summarize the thin-layer chromatographic separation of N-nitroso derivatives and of carboxy-*n*-butyl esters of the following imino acids: (a) proline, (b) 3,4-dehydroproline, (c) 3-hydroxyproline (*cis*-form), (d) 3-hydroxyproline (*trans*-form), (e) 4-hydroxyproline, (f) 4-allo-hydroxyproline*.

Procedure

n-Butyl esters. The hydrochlorides of the imino acids (0.2–0.5 mg each) were esterified with 10 ml of *n*-butanol in the presence of a strong cationic resin (Amberlyst 15, kindly obtained from Rohm and Haas Co.). The mixture was gently rotated at 120° for one hour. The ratio between imino acids and resin was 1:20 (w/w). After esterification *n*-butanol was decanted and the resin was filtered on glass under slight vacuum. To the dry resin was then added 10 ml of benzene, containing 0.2 ml of *n*-butylamine; the mixture was refluxed at 70–80° for 15 min.

The resin was discarded and the benzenic solution containing the *n*-butyl esters of the imino acids was concentrated under vacuum (22 mm Hg) in an ice bath.

N-Nitroso derivatives. Nitrous acid (1 ml/mg of imino acid) was added to the mixture of imino acids (0.2–0.5 mg each) at 100° until change of the colour. In order to avoid the destruction of 3,4-dehydroproline the nitrous acid must be prepared from sodium nitrite and acetic acid instead of the usual mixture of sodium nitrite and hydrochloric acid. The N-nitroso derivatives of imino acids were then concentrated under vacuum.

Thin-layer chromatography. Kieselgel G (Merck Co.) was mixed with distilled water (1:2, w/v), stratified with an automatic apparatus (previously described[2]) on glass plates (20 × 20 cm) and heated for one hour at 100–105°.

The amounts of imino acid derivatives deposited were from 10 to 50 μg in volumes of 10–50 μl.

The solvent for *n*-butyl esters was benzene-*n*-butanol (75:25, v/v), and for *N*-nitroso derivatives *n*-butanol–acetic acid–water (120:30:50, v/v).

The substances were visualized with ninhydrin in *n*-butanol saturated with water (0.2 %) or with isatin (0.2 %) in *n*-butanol containing 5 % acetic acid followed by *p*-dimethylaminobenzaldehyde (1 %).

Results

The R_F values obtained for the two series of imino acids with the two staining procedures are reported in Tables I and II.

Good separations of the *n*-butyl esters of imino acids were achieved. This last procedure has been applied with satisfactory results to samples of bovine serum albu-

* Compounds (a), (e) and (f) were obtained from Mann Co., (b) was kindly given by Dr. B. WITKOP, N.I.H., Bethesda, Md., (c) and (d) were kindly given by Dr. D. OGLE, University of Cincinnati.

TABLE I

R_F VALUES OF *n*-BUTYL ESTERS OF IMINO ACIDS

Compound	R_F	*Colour developed with ninhydrin*
n-Butylproline	0.14	yellow-brown
n-Butyl-3,4-dehydroproline	0.55	violet
n-Butyl-3-hydroxyproline (*cis*-)	0.24	yellow
n-Butyl-3-hydroxyproline (*trans*-)	0.10	yellow
n-Butyl-4-hydroxyproline	0.59	yellow
n-Butyl-allo-4-hydroxyproline	0.47	yellow

The solvent used was benzene–*n*-butanol (75:25). No colour appeared with isatin or dimethyl-aminobenzaldehyde spray.

TABLE II

R_F VALUES OF IMINO ACIDS AND THEIR NITROSO DERIVATIVES

Compound	R_F	*Colour developed with*		
		Ninhydrin	*Isatin*	*p-Dimethyl-aminobenzaldehyde*
Proline	0.28	orange	blue	yellow
NO_2-Proline	0.27	yellow	blue	blue
3,4-Dehydroproline	0.29	yellow-green	—	violet
NO_2-3,4-Dehydroproline	0.48	yellow	—	violet
3-Hydroxyproline (*cis*-)	0.29	pink	—	pink
NO_2-3-Hydroxyproline (*cis*-)	0.28	yellow	—	pink
3-Hydroxyproline (*trans*-)	0.23	pink	—	pink
NO_2-3-Hydroxyproline (*trans*-)	0.23	yellow	—	pink
4-Hydroxyproline	0.29	orange	—	pink
NO_2-4-Hydroxyproline	0.28	yellow-pink	—	pink
4-Allo-hydroxyproline	0.23	orange	—	yellow
NO_2-4-Allo-hydroxyproline	0.23	yellow	—	pink

The solvent used was *n*-butanol–acetic acid–water (120:30:50).

min and gelatin after hydrolysis. The nitroso derivatives of the various imino acids on the contrary cannot be easily separated under the experimental conditions of the present work.

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Scales for measurement of R_F values in paper and thin-layer chromatography

The R_F value of a component located on a paper chromatogram can be determined rapidly, without direct measurement of the distances travelled by solvent and component, using one of several simple aids devised for the purpose. In effect, each of these devices lays down a scale of one hundred arbitrary units between starting line and solvent front, and provides for the rapid and precise determination of the number of such units separating a component from its point of origin on the starting line of the chromatogram. This number of units is converted into the R_F value of the component by suitable location of the decimal point. R_F values of components on thin-layer chromatograms can be determined with these same devices, though the considerable patience and care required during their manipulation over the fragile surface of the adsorbent may preclude the use of several of them in this field, now one of major importance in analytical chemistry. The following brief review classifies the devices available for the measurement of R_F values, and their description offers a perspective from the point of view of their applicability to the thin-layer method. A new "R_F measure", which has proved suitable for use with both paper and thin-layer chromatograms, is also described.

The first R_F scale[1], consisting simply of a rubber strip inscribed with a scale dividing its length into ten equal units, has been developed further by SEGEL[2] (expansible scales are the subject of a patent[3]).

BERBALK[4] has described the use of a sliding wire potentiometer, part of a conventional circuit in which the millivoltmeter can be set by means of a variable resistance to read 100 mV for the length of wire corresponding to the distance travelled by the solvent front.

The other R_F scales which have been devised depend on the proportionality properties of similar triangles; the scales are either mechanically adjustable devices or inscribed transparent sheets. The former group includes the apparatus (Fig. 1) of JERCHEL, JACOBS AND MÖHLE[5], which can accommodate long distances of solvent flow apparently only at the expense of accuracy in the higher R_F values; other representatives of this group are the devices of SAVOIA[6] (Fig. 2), CLEMENTS[7] (Fig. 3; GLAZKO AND DILL[8] describe a similar device in which an arm moves across a transparent isosceles triangle, the arm being pivoted at the apex of the triangle, whose base is divided into ten equal units) and KOUDELA[9] (Fig. 4). Mechanical devices of these

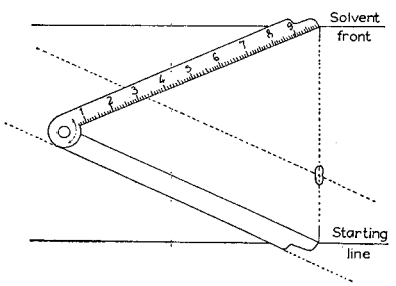

Fig. 1

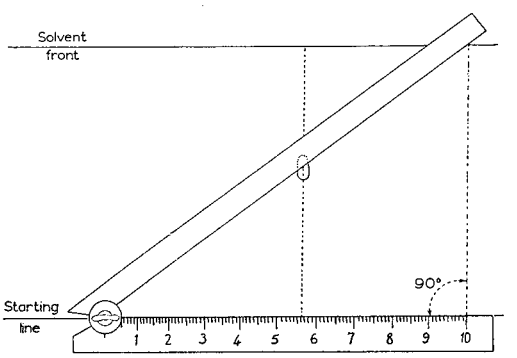

Fig. 2

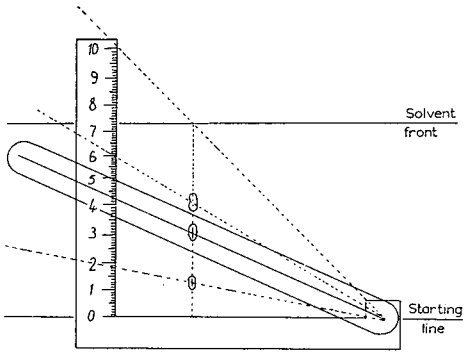

Fig. 3.

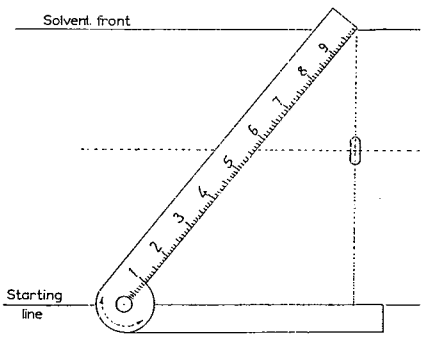

Fig. 4.

types are capable of adequate accuracy (though KOUDELA's scale is less satisfactory from this point of view), but are likely to be more or less destructive on thin-layer surfaces and cumbersome if constructed for use with relatively large chromatograms. Transparent scales embody considerable practical advantages, in the simplicity of their construction, in the ease of their alignment on a chromatogram, and in their applicability, without undue caution, to measurements on thin-layer chromatograms. They yield accurate R_F values, although ROCKLAND AND DUNN's "Partogrid"[10] (Fig. 5; HOTTA AND MORISHITA[11] describe a similar device) sacrifices this accuracy if constructed also to accommodate relatively short distances of solvent flow. A new device[12], the "R_F measure" (Fig. 6), does not suffer this disadvantage. NETTLETON AND MEFFERD[13] have described a modification of the "Partogrid" which enlarges the range of distances of solvent flow which can be accommodated conveniently by this device; these authors also propose[13] an R_F scale consisting of a transparent sheet inscribed with ten curves of common point origin, and state the mathematical derivation of this family of curves.

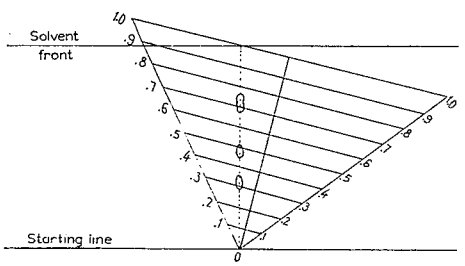

Fig. 5.

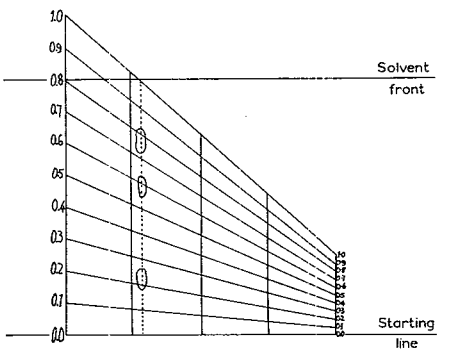

Fig. 6.

R_F values in both paper and thin-layer chromatography would be measured most satisfactorily with a development of BERBALK's potentiometer. An arm fixed to the sliding contact would be brought close to, but supported so as not to touch, the surface of the chromatogram; and accurate R_F values should thus be obtainable for long and short distances of solvent flow. Clearly, the nature of the application would

not normally warrant the cost of such a device, and a realistic selection of the best aid from this small field would be based on an assessment of other contenders for simplicity in construction and manipulation, for accuracy, and for compatibility with the thin-layer technique.

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Centrifugal chromatography

XIII. Centrifugal chromatography of cations*

A complete qualitative analysis of cations is usually a time-consuming operation. Nevertheless one very often needs extensive information about cations present in a given sample. In a laboratory that is arranged for inorganic analysis this is not a difficult task, because besides classical separation procedures with H_2S, other methods like flame photometry may be used. But in laboratories of a more biochemical character one always meets with difficulties in inorganic analysis. Recently POLLARD *et al.*[1,2] have published a systematic analysis of cations via paper chromatography that is very suitable for this purpose, but it has the disadvantage common to chromatographic separations that it takes a long time. The present paper shows how the chromatographic analysis of cations can be shortened by means of a centrifugal acceleration technique.

Experimental

For centrifugal paper chromatography the apparatus with central-spot development described by PAVLÍČEK *et al.*[3] was used.

Chromatographic separation was performed on Whatman No. 3 paper, the solvent system being *n*-butanol with 0.5 % benzoyl-acetone saturated with 0.1 *N* nitric acid.

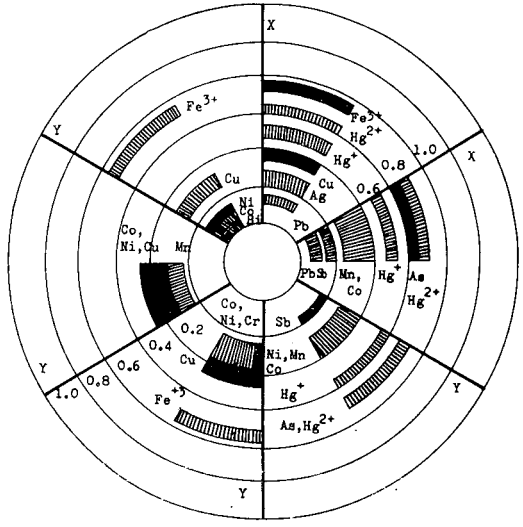
The sample for analysis (if in the solid state) was dissolved or mixed (if it is in

* For Part XII, see J. ROSMUS, M. PAVLÍČEK AND Z. DEYL, *Proc. Symp. Thin-Layer Chromatography, Rome, May, 1963.*

1. KCNS sat. soln. in H_2O + acetone (1:1): Bi, Co, Cu.
2. Ammonia + 1% soln. of dimethylglyoxime in ethanol: Ni.

1. 2 *N* NaOH saturated with Br_2 + 2 *N* NaOH (2:1), then SO_2, then 0.05% soln. of benzidine in acetic acid and ammonia vapours: Mn.
2. Cu salt of thio-oxamide, 0.1% soln. in methanol, then ammonia vapour: Co, Ni, Cu.

1. 2 *N* NaOH sat. with Br_2, diluted with 2 *N* NaOH (2:1): Mn, Co, Ni, Cr, Cu, Fe^{3+}.
2. 2 *N* H_2SO_4 + 20% H_2O_2 (1:1): Cu, Cr.



(Fig. 1a)

1. K_2CrO_4, 1% soln. in water: Pb, Ag, Hg^+, Hg^{2+}.
2. Concentrated ammonia: Pb, Ag, Cu, Hg^+, Fe^{3+}.

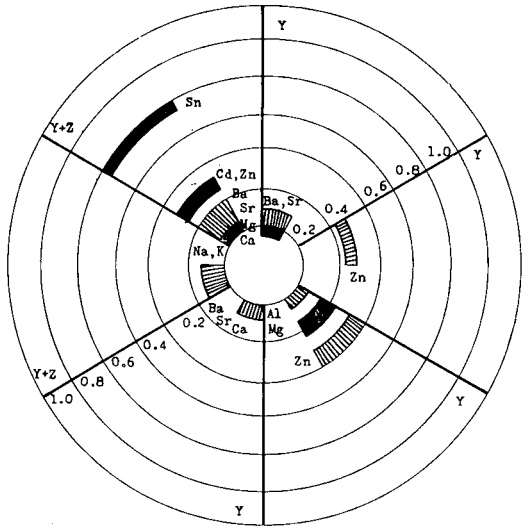
1. 2 *N* HCl + $(NH_4)_2S$, dil. (1:1): Pb, Mn, Co, Ni, Ag, Cd, Hg^+, As, Hg^{2+}.
2. 2 *N* NaOH + 20% H_2O_2, then SO_2: Ni, Hg^+, Hg^{2+}.

1. $(NH_4)_2S$ + concentrated HCl, then 5% soln. of Na_3PO_4 in water: Ni, Mn, Co, Hg^+, As, Hg^{2+}.
2. 5% soln. of phosphotungstic acid: Sb.

1. 8-Hydroxyquinoline, 0.5 % soln. in 60 % aq. ethanol, ammonia vapours: Al, Ca, Sr, Ba, Mg, Cd, Zn, Sn.
2. Acetic acid: Al, Zn, Sn.

1. Uranylacetate + Zn-acetate, saturated soln. in 1 *N* acetic acid: Na.
2. Pb–Co nitrate: K.

1. Gallacetophenone, 1 % soln. in NH_4OH: Ca, Sr, Ba.



(Fig. 1b)

1. NH_3 vapours + sodium rhodizonate, 0.1 % soln. in water: Sr, Ba, Zn, Sn.
2. 2 *N* HCl + 2 *N* CH_3COOH (1:1): Ba.

1. $CuSO_4 \cdot 5\ H_2O$, 1 % soln. in water, soln. contains 10 % of H_2SO_4; then: 2 g $HgCl_2$ + 3 g NH_4SCN in 100 ml H_2O: Zn.

1. Quinalizarin, 0.02 % soln. in methanol: Al, Zn.
2. 2 *N* NaOH + 20 % H_2O_2 (1:1): Mg.

Fig. 1. Scheme of cation separation. X, Y and Z designate different modes of preparing the sample on the start-line (see text); texts at individual sections describe the composition of the detection agent and the detected cations respectively.

If two detecting agents are presented then the section is cut into two sub-sections and each is detected separately. Only when the second reagent is introduced by the word "then", is the whole section detected by successive spraying with both reagents.

solution) either with 2 *N* nitric acid (spotted as X), or with 2 *N* hydrochloric acid (spotted as Y).

Spots on the startline, designated as Z, were prepared by spotting the solution Y and spraying with sodium hypobromite, and after drying they were sprayed twice with 2 *N* acetic acid.

The samples were spotted on two discs of Whatman No. 3 chromatographic paper. On the first, two samples of X and four samples of Y were spotted. On the second chromatogram there were two samples designated as Z and four samples designated as Y. The system of detection and the nature of detection agents are given in Fig. 1.

One run took about 40 min in the solvent system used, and a complete analysis was finished within 1.5 h (mostly passive working time). After development the chromatograms were dried and cut into six sections, each for detection purposes.

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Cation-exchange separation of protactinium-233 from irradiated thorium

A knowledge of the analytical separation of protactinium is of technical importance in connection with the chemical processing of irradiated thorium. Several ion-exchange methods have been proposed for separating protactinium from thorium[1–6]. Because of strong hydrolytic and irreversible behavior of protactinium in contact with glass and metal surfaces, most of the ion-exchange systems suffer from an appreciable loss of protactinium during the ion-exchange chromatographic process except for the system involving hydrofluoric acid as a component of eluent. We have been exploring the effective ion-exchange procedures for several elements in thiocyanate–hydrochloric acid media[7–10]. The present paper describes a new ion-exchange chromatographic procedure for separating carrier-free protactinium-233 from neutron-irradiated thorium with a strong acid cation exchanger in thiocyanate–hydrochloric acid solution. Because of the tendency of protactinium to form a stable complex with thiocyanate ions, the cumbersome hydrolytic property of protactinium can almost be excluded so that the rapid separation of protactinium-233 from irradiated thorium was achieved with a shorter column.

Experimental

Dowex 50 W, X-8, of "analytical grade", was used (particle size 100 to 200-mesh). Before use the resin was further purified as described before[7]. Two grams of the

dried resin in the hydrogen form was slurried with water and passed into a small ion-exchange column, internal diameter 1 cm, pulled to a tip, and plugged with glass wool at the outlet of the column. The resulting bed was 4 cm long. Eluent was fed on to the top of the column through a 100 ml separatory funnel attached to the inlet of the column.

Protactinium-233 was produced by irradiating approximately 25 mg of thorium nitrate in a TRIGA MARK II reactor of St. Paul's University, Yokosuka, for 6 h at a neutron flux of 5×10^{11} n/cm^2/sec. The activity of the protactinium-233 was measured in a standard well-type scintillation counter. Thorium was determined by titration with 0.001 *M* EDTA solution using Xylenol Orange as indicator.

Procedure

Before use the resin column is treated with a thiocyanate solution (1 *M* NH_4SCN–0.5 *M* HCl). The irradiated thorium nitrate is dissolved in 5 ml of the same thiocyanate solution as is used for the column treatment. The sample solution is loaded onto the top of the column and allowed to pass down the column at a flow rate of ~ 0.6 ml per min. Elution of the protactinium-233 is commenced with 1 *M* NH_4SCN–0.5 *M* HCl solution, at a flow rate of ~ 0.8 ml per min, when the sample solution almost reaches the top of the column. The protactinium-233 is recovered in the first 100 ml of the effluent. Removal of the thorium is completed by elution with approximately 55 ml of 2 *M* NH_4SCN–0.5 *M* HCl solution. The effluent is collected and titrated for thorium with EDTA after diluting with water.

Results and discussion

Protactinium does not adsorb to any great extent from the 1 *M* NH_4SCN–0.5 *M* HCl solution, while the thorium shows an equilibrium distribution coefficient of about 150 on a Dowex 50-like cation exchanger, Diaion SK 1, in the same solution[7]. Thus, the difference in the equilibrium distribution coefficients of protactinium and thorium is large enough for a good separation with this cation exchanger. Typical elution profile curves are shown in Fig. 1 for the separation of protactinium-233 and thorium. The elution band which appears with maximum counting rate near 15 ml

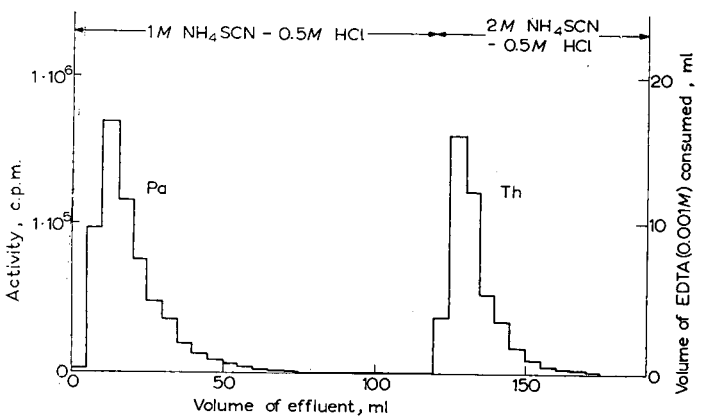

Fig. 1. Separation of carrier-free protactinium-233 from irradiated thorium.

was found by γ-spectrometry to consist of protactinium-233. No other peaks due to foreign activities were found. Recovery of thorium is quantitative. No color reaction of thorium–Xylenol Orange complex was found in the eluted fractions of protactinium. Almost 90 % of the protactinium-233 is effectively recovered in the first 50 ml of effluent. Cross contamination of protactinium in the eluted thorium fraction comes to about 1 % of the total activity of protactinium concerned. Only 6 % of the protactinium-233 was found to remain on the ion-exchange column, mostly on the glass wool used for the plug at the outlet of the column.

The retention of the protactinium is considered to be due to the protactinium fraction which is not thiocyanato-complexed. Even in a protactinium solution in 0.01 *M* HF–6 *M* HNO_3, a few per cent of the protactinium was found not to be fluoro-complexed[4].

The elution behavior of protactinium does not seem to be affected seriously by the flow rate of the eluant. Slowing the flow rate down to a half still produces much the same elution curve as illustrated in Fig. 1. Uranium behaves like protactinium, but is removed much more easily in a sharp band, so that the fissile uranium-233, daughter of protactinium-233 and the desired product of thorium bombardment, can effectively be enriched in the eluted protactinium fraction.

The present method of isolation of protactinium-233 is rapid and gives a reasonably high recovery of carrier-free protactinium-233 without loss of thorium. If desired, the procedure can readily be adapted to handling substantially larger amounts of irradiated thorium. The column can also be operated without attention during the operation step.

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Book Reviews

Proceedings of the Conference on Methods of Preparing and Storing Marked Molecules, Brussels, November 13–16, 1963, published by Euratom (report EUR.1625.e.), 1359 pp., price 1,000 Belgian Francs.

These proceedings consist of about 70 review and original papers dealing with radiochemical preparations, radiation chemistry in addition to the main topic of the conference. It contains a wealth of chemical information and the reviewer was impressed by the almost universal application of chromatographic methods in the work described.

Half the volume presents the papers in their original languages and the second half are the English translations of those which were not originally in English.

It is a pity that proof reading and translation did not get the care the volume deserves.

This report (EUR 1625) is available for purchase at the following addresses: Central Sales Office for Publications of the European Communities, 2, place de Metz, Luxemburg (Grand Duchy); and Presses Académiques Européennes, 98, Chaussée de Charleroi, Brussels (Belgium).

Annual Reports on the Progress of Chemistry, for 1963, published by the Chemical Society, London, Vol. LX, 1964, 681 pages.

This book contains a survey of the papers which appeared most relevant in the chemical literature in 1963, and the chapters are devoted to selected topics in physical chemistry and to extensive reviews of inorganic, organic, biological and analytical chemistry, and of chemical crystallography. It is clearly almost impossible to critically evaluate papers after so short a time has elapsed since their publication, and therefore the selection is often arbitrary, and the choice of articles reviewed is acceptable only thanks to the personal skill of the reviewers. In addition, since to a great extent, the first results of chemical research find their expression in proceedings of conferences, internal reports of research institutions and dissertations, which cannot be adequately reviewed, here the choice is made increasingly difficult. For this reason, these *Annual Reports* cannot be considered as a substitute for a systematic bibliography of research, such as is needed as an aid to original research work, but this book can be praised as the continuation of an established tradition in the field of chemical review publications; its actual utility is confined, therefore, to supplying preliminary information on the topics reviewed.

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2. FUNDAMENTALS, THEORY AND GENERAL

2c. Thermodynamics and theoretical relationships

GIDDINGS, J. C.: Plate height in coiled columns. *J. Chromatog.*, 16 (1964) 444–447 — a criticism of MATSEN AND HARDING'S paper (*J. Chromatog.*, 12 (1963) 145); true plate-height equation is $H = 7\,vr_0^4/12R_0^2\gamma D_g$, where v is flow velocity, r_0 and R_0 are tube and coil radius resp., D_g is gaseous diffusion coefficient and γ obstruction factor for molecular diffusion in the gas.

LUCKHURST, G. R.: Non-ideality of the gas phase in frontal analysis. *J. Chromatog.*, 16 (1964) 543–545 — pressure coefficient $\bar{P}$ is in the example given twice as high as that for infinite dilution; the measurement of the pressure dependence of the k' factor may be used to measure the second virial coefficient of the solute.

3. TECHNIQUES I

3b. Column performance and filling studies

FÜRST, H. AND KÖHLER, H.: Imprägnieren von Trägermaterial für die Gas-Chromatographie mit Hilfe der Wirbelschicht. *Chem. Tech. (Berlin)*, 16 (1964) 669 — the solid stationary phase is inserted in fluid support material (tube I.D. 50 mm; gas flow-rate 20 l/min) at the temperature of the melting point of the stationary phase (*e.g.*, mannitol at 190° and inositol at 250°).

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ROONEY, T. B. AND AZNAVOURIAN, W.: Minimizing time for gas chromatographic analysis of complex mixtures. *Anal. Chem.*, 36 (1964) 2112–2115 — a digital computer search technique was used in the study on minimum analysis time with respect to liquid loading and exit gas velocity; other variables were studied indirectly.

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3c. Apparatus, accessories and materials for GC

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DERGE, K.: Doppelkolonnen-Gas-Chromatograph. *Chemiker-Ztg.*, 88 (1964) 390–393 — description of F & M type chromatograph.

DUTTON, H. J. AND MOUNTS, T. L.: Micro vapor-phase hydrogenation monitored with tandem chromatography-radio-activity. *J. Catalysis*, 3 (1964) 363–367 — design is given.

HOPE, D. A. AND NEWMAN, D. E.: A simple temperature programmer for a furnace to give an accurately repeatable top temperature value. *J. Sci. Instr.*, 41 (1964) 519 — circuit gives a temperature repetition better than 0.2 % after controlled cooling: useful for GC.

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NEYMARK, M. E. AND BEZRODNYĬ, V. I.: (Injection device for sampling in the chromatograph). *Zavodsk. Lab.*, 30 (1964) 1519–1520 — description of UdSSR authorized Pat., No. 159689 (1963); errors for 1–2 μl 8 %, for 10–20 μl 5 %.

SKAUG, O. E.: A new principle for heating of gas chromatographs. *Scand. J. Clin. Lab. Invest.*, 15, Suppl. 76 (1963) 75 — direct heating of column walls by means of 24 V, 100 A power supply.

4. TECHNIQUES II

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4d. Special microtechniques

HUBER, J. F. K. AND KEULEMANS, A. I. M.: Possibility and limitation of the identification of materials from their behaviour in isothermal-extraction gas-chromatography. *Z. Anal. Chem.*, 205 (1964) 263–274.

HUYTEN, F. H. AND RIJNDERS, G. W. A.: Some rapid identification methods in gas chromatography. *Z. Anal. Chem.*, 205 (1964) 244–262.

JANÁK, J.: Multi-dimensional chromatography using different developing methods. II. Microscopic and colorimetric investigation of compounds separated by gas chromatography in submicro-quantities. *J. Chromatog.*, 16 (1964) 494–501 — classical colorimetric and crystallographic methods can be applied to the identification of nano- to picogram quantities of compounds from GC effluents, if they are examined under the microscope; tar compounds are used in the investigation.

KARR, JR. C., CHILDERS, E. E., WARNER, W. C. AND ESTEP, P. E.: Analysis of aromatic hydrocarbons from pitch oils by liquid chromatography on gas chromatography analog. *Anal. Chem.*, 36 (1964) 2105–2108 — retention data of 57 aromatics are given.

4f. Measurement of physico-chemical and related values

BEREZKIN, V. G., KRUGLIKOVA, V. S. AND BELIKOVA, N. A.: (Kinetics of bimolecular chemical reactions, as studied by a pulse chromatographic technique). *Dokl. Akad. Nauk SSSR.* 158 (1964) 182–185.

IKELS, K. G.: Determination of the solubility of nitrogen in water and extracted human fat. *J. Gas Chromatog.*, 2 (1964) 374–379 — experimental dara are given.

PURNELL, J. H.: Physical measurement by gas chromatography. *Endeavour*, 23 (1964) 142–147 — a critical review for educational purposes.

TIVIN, F.: Thermodynamics of an inorganic system via gas–liquid chromatography. *Dissertation Abstr.*, 25 (1965) 794 — heats of solution of some metal chlorides in molten-salt phases were determined.

WOLF, F. AND LOSSE, A.: Gas-Flüssig-Verteilungskoeffizienten für dünne Flüssigkeitsschichten auf polaren Trägern. 2. Mitteilung. *Kolloid-Z.*, 196 (1964) 147–152 — thermal treatment of silica gel surfaces increases and organic modification decreases the partition coefficient in coated phases.

ZHENG, GUO-KANG, SUO, YU-GUANG, GIN, YI AND PENG, ZHE-CHUN: (Activity coefficients from gas–liquid chromatography). *Acta Chim. Sinica*, 30 (1964) 506–508 — C_4 and C_5 hydrocarbons in several solvents.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

JONES, K. AND GREEN, R.: Safety in air separation plants: Determination of atmospheric pollutants by gas chromatography. *Nature*, 205 (1964) 67–68 — concentration 0.001–0.22 v.p.m.; bis(2-methoxyethyl) adipate–di-2-ethylhexyl sebacate (2.1:1) on Chromosorb P at 50° for C_4; di-2-ethylhexyl sebacate on silica gel at 50° for C_1–C_3, including C_2H_2; retention data are given.

KONTOROVICH, L. M. AND BOBROVA, V. P.: (Determination of impurities in acetylene). In A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 150–152 — up to 100–200 p.p.m.: H_2, CH_4, divinyl, diacetylene, dimethylacetylene; up to 0.1–0.25%: CO, CO_2, propadiene, methylacetylene, vinylacetylene; in 1–4%: O_2 and N_2.

5b. Other hydrocarbons

ASINGER, F., FELL, B. AND STEFFAN, G.: Über die relativen Reaktivitäten der Doppelbindung in stellungs- und konfigurationsisomeren höhermolekularen *n*-Olefinen. I. Synthese und physikalische Eigenschaften der stellungs- und konfigurationsisomeren *n*-Undecane. *Chem. Ber.*, 97 (1964) 1555–1561 — on DC silicone grease at 160°, on β,β'-iminodipropionitrile at 70° and 100°, and on β,β'-oxydipropionitrile at 70°.

EĬZEN, O. G. AND ARUMEEL, E. KH.: (Application of gas chromatography for the determination of the chemical composition of Estonian shale gasoline). In A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 179–185 — retention data of 22 C_3–C_5 hydrocarbons on diacetonylacetone at 15° and 18 C_5–C_8 on 4 nitrile phases at 100°, 150° and 200°.

FERRAND, R., ISELIN, P., MOREL, P. AND AUBRY, S.: Étude de la coupe 350–450°C d'un goudron de houille. I. Méthodes de fractionnement et premiers résultats. *Chim. Ind. (Paris)*, 91 (1964) 370–379 — on SE-30 and SE-52 at 250–270°.

HARTWIG, M.: Isomerisierendes und hydrierendes Spalten von *n*-Paraffinen an Palladiumkatalysatoren. *Brennstoff-Chem.*, 45 (1964) 234–239 — C_1–C_6 paraffins on isoquinoline.

JAWORSKI, M. AND BOBIŃSKI, J.: (Gas–liquid chromatographic analysis of crude benzene). *Chem. Anal. (Warsaw)*, 9 (1964) 1003–1009 — retention data of 19 hydrocarbons (b.p. 36–182° on PPGA and PEG phthalate at 135°.

KRICHMAR, S. I. AND STEPANENKO, V. E.: (Chromatographic method for purity determination of raw ethyl benzene and isopropyl benzene). In A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 153–156 — mean deviation 6–7% for concentrations of 0.01–0.06%.

LULOVA, N. I., TARASOV, A. I., KUZ'MINA, A. V., FEDOSOVA, A. K. AND LEONT'EVA, S. A.: (Use of gas chromatography for the investigation of complex hydrocarbon mixtures including non-hydrocarbon components). In A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 162–172 — retention data of 17 different C_4–C_8 hydrocarbons on 3 stationary phases at 40° and 60°.

PANKOV, A. G., DOLGOVA, N. A., MOSKVIN, A. F., KNYAZEVA, M. F., FEL'DBLYUM, V. S. AND ROMANOV, I. B.: (Identification of compounds by means of gas–liquid chromatography in connection with other physico-chemical methods). In A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 173–178 — application to C_6 olefin analysis.

STREIBL, M.: (Chemical composition of carbonization gasoline. I. Isolation and identification of substances from the 27–93°C boiling point range fraction). *Chem. Průmysl*, 14 (1964) 630–633 — the following stationary phases are used: Apiezon L, paraffin oil, PEGA and tris(2-cyanoethoxy) propane.

VIGDERGAUZ, M. S. AND CHABROVA, O. G.: (Investigation of various stationary liquid phases for the chromatographic separation of isomeric aromatic hydrocarbons). *Neftekhimiya*, 4 (1964) 932–937 — Bentone-34–dinonyl phthalate (60:40) at 75° was found to be the best phase for C_8 aromatics; retention data of 33 C_6–C_{11} aromatic hydrocarbons are given.

YAMAMOTO, H., O'HARA, M. AND KWAN, T.: Hydrogenation and dehydrogenation of cyclohexene and cyclohexa-1,3-diene on transition metals, by gas-chromatographic pulse technique. *Chem. Pharm. Bull. (Tokyo)*, 12 (1964) 959–967.

5c. Halogen derivatives of hydrocarbons

VIEHE, H. G. AND FRANCHIMONT, E.: Geometrische Isomerenpaare mit bevorzugter *cis*-Struktur. VI. 1,4-Difluor- und 1,4-Dichlor-butadien-(1,3). *Chem. Ber.*, 97 (1964) 602–609 — on 4 stationairy phases at 40–120°.

6. ALCOHOLS

HÜCKEL, W. AND WÄCHTER, J.: Beiträge zur Konstellationsanalyse. XI. (Solvolyse von Toluolsulfonsäureestern. XIV). Cycloheptanol, *cis*- und *trans*-1-Methylcycloheptanol-(2). *Ann.* 672 (1964) 62–77 — on Hyprose and Carbowax 1500 at 160° and 80°.

IKEDA, R. M., SIMMONS, D. E. AND GROSSMAN, J. D.: Removal of alcohols from complex mixtures during gas chromatography. *Anal. Chem.*, 36 (1964) 2188–2189 — small outlet section of column is coated with 1% H_3BO_3 for the removal of alcohols.

KAGAN, YU. B., BASHKIROV, A. N., MOROZOV, L. A. AND KOLESNIKOVA, L. P.: (Analysis of a mixture of C_4–C_7 alcohols by the gas–liquid chromatographic method). *Neftekhimiya*, 4 (1964) 924–931 — retention data of 29 alcohols on triethanolamine, diglycerol and two mixed phases at 105–115°; sodium caproate is used as tailing reducer.

7. PHENOLS

BARBER, E. D., SAWICKI, E. AND MCPHERSON, S. P.: Separation and identification of phenols in automobile exhaust by paper and gas liquid chromatography. *Anal. Chem.*, 36 (1964) 2442–2445 — on tricresyl phosphate + orthophosphoric acid at 135°.

KAISER, R.: Direkte und automatische Kopplung der Dünnschicht-Chromatographie an Gas-Chromatographen. *Z. Anal. Chem.*, 205 (1964) 284–298 — applied to phenol analysis.

NAUCKE, W. AND TARKMANN, F.: Gaschromatographische Bestimmung der Abwasser-Phenole, besonders in Torf-Schwelwässern. *Brennstoff-Chem.*, 45 (1964) 263–269 — retention data are given for 25 phenols (up to C_9) on Apiezon L at 180°.

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Erratum

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Table II, last column, top line: for PLL^{**} read PPL^{**}.

A QUANTITATIVE THEORY OF THE INTERRELATIONSHIP BETWEEN SOLUTE RETENTION VOLUMES IN GAS–SOLID AND LIQUID–SOLID CHROMATOGRAPHY. STUDIES ON WATER DEACTIVATED ALUMINA

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INTRODUCTION

In contrast to gas–liquid chromatography (GLC), which has been used in the analysis of an extremely large and varied number of sample types, gas–solid chromatography (GSC) has found only limited application as an analytical separation procedure, and its use has been confined largely to the determination of mixtures of the fixed gases and lower hydrocarbons. A number of reasons for the relative unpopularity of GSC separation are apparent. First, adsorption isotherms in GSC systems are frequently non-linear, even at low column loadings, and occasionally irreversible; this leads to several severely detrimental effects: component retention volumes which vary with sample size, band tailing, and incomplete recovery of sample from the column. Second, retention volumes in GSC are generally excessively large, particularly for large polar molecules, leading to impossibly long separation times for all but the simplest organic mixtures. Third, adsorbents are in general more difficult to standardize and reproduce than comparable liquid phases used in GLC; not only do adsorbent properties change markedly with subtle differences in the preparation of the solid phase, but the presence of small amounts of adsorbed material (particularly water) has a critical effect on adsorbent performance. Fourth, active adsorbents at elevated temperatures frequently induce catalytic alteration of the sample. Finally, the number of different, commercially available adsorbents which appear useful in GSC is relatively small, compared with the large number of different liquids available for GLC, and this has created the impression of greater flexibility or versatility for GLC *versus* GSC analysis.

GIDDINGS[1], in a recent communication, has pointed out that despite the above limitations on GSC, this technique may actually possess a greater future analytical potential than does GLC. The column efficiencies theoretically attainable in GSC appear to be several orders of magnitude higher than are possible in GLC, and GIDDINGS has also called attention to the unique selectivity provided by adsorption separation in some cases. Furthermore, many of the foregoing limitations on GSC are today more apparent than real. The highly sensitive detectors that have been developed for GLC permit GSC separations with extremely small sample sizes, which in turn means linear adsorption isotherms and constant sample retention volumes[1]. Similarly, it has long been known in the case of liquid–solid chromatography that addition to the adsorbent of small quantities (less than a monolayer) of

strongly adsorbed substances suppresses the more active adsorption sites, hence reducing band tailing and increasing the sample sizes that can be used in linear isotherm operation. This same technique has been successfully applied to GSC by a number of different workers: EGGERTSEN *et al.*[2] who used squalane to deactivate carbon, SCOTT[3] who used silicone oil to deactivate alumina, and PETITJEAN AND LEFTAULT[4] who used various other heavy oils as well as pyrolysis products (coke?), to deactivate the oxide surface of aluminum capillaries. Adsorbent deactivation in this fashion can also reduce the excessive retention volumes characteristic of GSC to more convenient levels, as shown by SCOTT's work[3,5] with water and NaOH deactivated alumina. With 40 % NaOH on alumina SCOTT was able to separate *n*-alkanes boiling to C_{36}. Chemical modification of the surface can similarly be used in GSC to reduce retention volume[6] and improve isotherm linearity[7]; thus, after reacting a silica surface with trimethylchlorosilane, VASIL'EVA *et al.*[6] found much *lower* retention volumes for benzene and *n*-hexane in GSC separations with this adsorbent than in comparable GLC systems. Variation of the geometrical structure of the adsorbent, particularly the elimination of small pores, can also improve isotherm linearity as discussed by KISELEV *et al.*[7] for silica and HALÁSZ AND HORVÁTH[8] for carbon. The use of an adsorbing carrier gas or vapor has been shown by GREENE AND ROY[9] to give much the same advantages in GSC as adsorbent deactivation: improved band shape (and presumably greater isotherm linearity) and shorter retention volumes. Another expedient for reducing the retention volumes of sample components in GSC is by effectively reducing the amount of adsorbent in the column. HALÁSZ AND HORVÁTH[8] accomplished this by using glass beads coated with carbon; they were able to separate the *n*-alkanes through C_{13} in a short time. Similarly, KISELEV *et al.*[7] used low surface area silica gels to accomplish the separation of the *n*-alkanes through C_{29} in a reasonable time. The reduction of adsorbent surface area is precisely equivalent in this respect to dilution of the adsorbent by glass beads or other means. SCHWARTZ *et al.*[10] have also described the use of adsorbent coated capillaries (similar to those of PETITJEAN AND LEFTAULT[4], which appear to have much the same advantages in this respect as large pore adsorbents and coated glass beads. SCHWARTZ *et al.*[10] also emphasize the advantage of these solid coated capillaries over liquid coated capillaries in their freedom from column bleeding.

Suppression of adsorbent activity by deactivation or by the use of an adsorbing carrier gas might also be expected to reduce the catalytic activity of the adsorbent, and hence minimize the likelihood of sample reaction in GSC separation. Adsorbent standardization has come to be less of a practical problem as more is learned about the variables which influence adsorbent behavior (*e.g.* see discussion in refs. 7, 11 and 12). Similarly, for maintaining the water content (and hence activity) of the adsorbent in GSC constant, SCOTT[3] used water saturation of the incoming carrier gas. Finally, the limited number of adsorbents that have been used in previous GSC and LSC separations (principally alumina, carbon, silica, and molecular sieves) does not really imply a significant limitation in the separation possibilities of GSC, relative to GLC with its vast number of potential stationary phases. The separations provided by adsorbents are in many cases fundamentally different than those achievable with *any* liquid phase, while many structurally dissimilar liquids possess little uniqueness in their separation capabilities as GLC substrates. In addition, recent years have seen the creation of specific adsorbents[13] for given separations, as well as the

synthesis of many new, unique adsorbents (for example molecular sieves of varying geometry).

The foregoing paragraphs suggest a potentially promising future for the GSC technique, and there are currently a number of groups actively working on the realization of this potential. It is therefore important to clarify the basic principles of GSC separation as quickly as possible. The usefulness of any elution chromatographic procedure can be evaluated in terms of two essentially independent considerations: column efficiency or band width, and separation factors or relative retention volumes. The theoretical treatment of GIDDINGS[1], which is concerned almost solely with column efficiency in GSC, seems to provide an adequate initial basis for guiding present experimental work aimed at optimizing column efficiency in GSC. A satisfactory overall theoretical framework requires a corresponding treatment of the variation of GSC retention volumes with solute molecular structure and experimental conditions. Previous theoretical and experimental investigations in this area are essentially non-existent. For the related technique of liquid–solid chromatography (LSC), a vast amount of experimental data have been acquired, and reasonably satisfactory theories of the dependence of solute retention volumes on molecular structure and experimental conditions have been set forth (*e.g.* see ref. 12). In general, some parallelism is expected between the relative separation of different compounds in GSC and LSC when the same adsorbent is used. Thus, for LSC separations on carbon, it has long been known that adsorption of the solute increases with molecular size or number of carbon atoms[14], and is relatively unaffected by the presence of polar solute groups or double bonds, or even decreases with their addition to the solute[15]. Similarly, several GSC studies on carbon[2,8,16–18] have confirmed a separation order according to carbon number or molecular weight, with little effect of solute double bonds or ring closures. GRIFFITHS *et al.*[19] studied the displacement of a number of polar and hydrocarbon solutes from carbon, and HALÁSZ AND HORVÁTH[8] carried out similar elution GSC studies on carbon; both groups again confirmed the preferential adsorption of the heavier hydrocarbons, particularly with respect to polar molecules of similar boiling points.

By contrast with adsorption on carbon, LSC separation on alumina or other metal oxides shows little dependence of solute adsorption on solute size or number of aliphatic carbon atoms, but a pronounced increase in adsorption with addition of polar groups or double bonds to the solute molecule. Similarly, in GSC separations over alumina, SCOTT[3] has shown the preferential adsorption of ethylene relative to the larger propane on sufficiently active columns; PETITJEAN AND LEFTAULT[4] have reported similar data for GSC separation over alumina. KLEMM *et al.*[20,21] have studied both the LSC and GSC separation of several of the substituted hydrocarbons and pyridines on alumina, and found the GSC data[21] "...consistent with data on adsorbabilities obtained earlier from (LSC)".

These past qualitative comparisons of separation in GSC and LSC systems have been useful in summarizing the differences in GSC between such adsorbents as carbon and the metal oxides. However, a more detailed theoretical analysis is required in order to take full advantage of our considerable knowledge and understanding of separation in LSC systems for application to corresponding GSC systems. Previous investigations carried out in this laboratory[12,22–24] have resulted in a detailed, quantitative treatment for correlating and predicting retention volume data in

LSC separation over alumina and, to a lesser extent, other adsorbents. Certain theoretical considerations have been advanced[12] which suggest that this treatment might easily be generalized to include the similar treatment of retention volume in GSC systems. If so, this would permit the prediction of separation in GSC systems on the basis of comparable LSC data, and provide considerable insight into the factors controlling retention volume in various GSC systems. Finally, some measurements most conveniently carried out in GSC systems might shed additional light on the theory of separation in LSC, assuming the existence of a satisfactory unified theory of LSC and GSC separation.

With the foregoing considerations in mind, GSC separations were performed with a variety of organic solutes, using an alumina comparable to previously studied (*e.g.* ref. 12) samples. The interpretation, correlation, and generalization of these data was then carried out in terms of previous theories of LSC separation[12,22-24].

EXPERIMENTAL

All of the experimental GSC data presently reported were obtained in conventional gas chromatography units equipped either with thermal conductivity or flame ionization detectors. Inlet He carrier gas could be water saturated at ambient temperature (21°) if desired by passing the gas through two large galvanized steel bubblers. The lines between the bubblers and the gas chromatography unit were preheated to approximately 83° by means of heating tape. The column was thermostated at 83° ± 1°, and exit flow rates were measured with a soap bubble flow meter. For the runs with water saturated He, the water content of the equilibrated adsorbent was chromatographically checked[12] following the GSC runs.

In one set of experiments the water content of the adsorbent was intentionally reduced to 1.5 % H_2O–Al_2O_3 by bypassing the bubbler and passing dry He through the column for 24 h prior to running GSC separations (for a subsequent 4 h). The resulting water content of the adsorbent (1.5 % H_2O) in this case was measured by the breakthrough time for water equilibrated He in a subsequent re-equilibration of the column with water (to 2.7 % H_2O–Al_2O_3). All experiments were carried out with the precalcined (400°, 16 h) water deactivated Alcoa F-20 alumina used previously[12,22-24].

Retention volumes in nominal ml as measured by the exit He flow rate and elution times were converted to specific corrected retention volumes U_g': the actual volume of He (at column conditions of pressure and temperature) per gram of adsorbent required to elute a peak maximum, corrected for the dead volume of the column. U_g' is similar to the specific retention volume U_g defined by JANAK[25], and is defined in the present way for theoretical reasons (so as to be equivalent to retention volume in LSC systems). Actually, since the pressure and temperature corrections involved operate in opposition, there is little difference between values of U_g, U_g', and the uncorrected (measured under ambient conditions) retention volume per gram of adsorbent. Duplicate determinations of U_g' for a single solute in different adsorbent columns showed good repeatability, averaging ±3 %.

All U_g' values reported are for linear isotherm column loadings. Linear column capacities (the amount of sample per unit weight of adsorbent sufficient to reduce the linear isotherm value of U_g' by 10 %[12]) were measured for several solutes, and

all values were found to vary between $2 \cdot 10^{-4}$ to $5 \cdot 10^{-4}$ g/g for 2.7 % H_2O–Al_2O_3. These linear column capacities are similar to those involved in comparable LSC systems[26]. All U_g' values for 2.7 % H_2O–Al_2O_3 were measured within the linear isotherm region, or extrapolated from values obtained at loadings below $2 \cdot 10^{-4}$ g/g. Column linear capacities for 1.5 % H_2O–Al_2O_3 were observed to be considerably lower, as expected[26].

Band shape and column efficiencies were not of major interest in the present studies. It was observed that most bands were symmetrical for elution from 2.7 % H_2O–Al_2O_3, while slight tailing was observed for the bands eluted from 1.5 % H_2O–Al_2O_3. All studies were carried out in the region of minimum HETP, and HETP was observed to be approximately constant for most solutes: 0.5 mm for the $^1/_8$ in. columns, and 2.0 mm for the $^1/_4$ in. columns.

For a number of solutes having retention volumes U_g' greater than 300 ml/g on 2.7 % H_2O–Al_2O_3, it was established by dual measurement with flame and thermal conductivity detection that two elution peaks are obtained; one at 320 to 350 ml/g, and a second one at some larger retention volume. The first peak was found to be a water peak (U_g' for water approximately 370 ml/g), which appears to be the result of displacement of adsorbed water by initial adsorption of the solute. This phenomenon (double peaking) is even more common when the carrier gas is saturated with materials more weakly adsorbing than water, as will be discussed in a following paper.

A UNIFIED THEORY OF SOLUTE RETENTION VOLUME IN GSC AND LSC SEPARATION, AND ITS EXPERIMENTAL VERIFICATION

Development of the theory

In linear isotherm LSC or linear elution adsorption chromatography (LEAC), the equivalent retention volume of a solute $\underline{R}^\circ$ (ml/g) can be related[12] to adsorbent activity α and surface volume V_a, eluent strength ε°, the area A_s required by the solute for adsorption on the adsorbent surface, and the dimensionless free energy of adsorption of the solute from pentane onto calcined adsorbent S°:

$$\log \underline{R}^\circ = \log V_a + \alpha(S^\circ - A_s\varepsilon^\circ). \qquad (1)$$

Values of the parameters V_a and α have been tabulated[12] for the adsorbents alumina, silica, Florisil, and "X" sieve; ε° values have been listed for a number of eluents[12,22] and can be estimated for other eluents[22] when certain solute parameters are available. Solute A_s values can be calculated in a straightforward fashion[12], and the solute adsorption energies S° can be quantitatively related[12,23] to solute group adsorption energies, solute intramolecular steric and electronic interactions, and the orientation of solute groups with strong adsorbents sites. Presumably, these solute S° values (which vary with both solute and adsorbent type) determine the unique separation characteristics of both LSC and GSC systems.

Equation (1) has been derived on the assumption that a competition exists between a solute molecule and some number m eluent molecules for a given place on the adsorbent surface; it is further assumed that the net solute adsorption energy is given as the inherent solute adsorption energy minus m times the eluent adsorption energy. All solution energy terms are hence ignored in the derivation of equation (1),

and the empirical success of equation (1) in a variety of different eluent systems makes this omission appear justifiable. There are additional reasons for believing this latter approximation is valid in LSC systems. Thus, such exceptions to eqn. (1) as have been noted[22] in the case of "anomalous" eluents have been shown to arise from peculiar interactions of the solute or eluent with the adsorbent, rather than corresponding solution interactions. Similarly, the eluent strength ε^c of binary mixtures can be accurately predicted from the eluent properties of the constituent pure solvents[12] by assuming that only the adsorption energy terms are important. Finally, that this should be so (adsorption energy terms much more important than solution energy terms) is not really too surprising, considering that the solution energy terms appear to be generally much smaller than corresponding adsorption energy terms[12], and that only a fraction of the solution bonds are normally broken when a molecule adsorbs.

If the solution energy terms in LSC separation are in fact negligible, there is then little difference in the theoretical treatment of retention volume in LSC and GSC systems. In LSC systems, eluent strength values ε° are calculated relative to the standard weak eluent pentane ($\varepsilon^\circ = 0.00$). In GSC, when negligible adsorption of the carrier gas (or eluent) occurs, the effective ε° value of the carrier gas ε°_g will necessarily be negative. This is equivalent to a positive adsorption energy (per unit of area or A_s) for pentane, equal $-\varepsilon^\circ_g$. This value would be expected to vary between adsorbents in the same manner that the adsorption energy of pentane varies. An effect in GSC which is necessarily overlooked by any relationship (such as equation (1)) for retention volume dependence in LSC separation is the large increase in translational freedom which accompanies the transfer of a molecule from a fixed (adsorbed) phase to the gas phase. If we assume negligible translational freedom in the adsorbed or solution phases, this effect cancels out in LSC (as evidenced by the absence of any term reflecting this effect in eqn. (1)), while for GSC the net decrease in translational energy upon adsorption of a molecule will be equal to $1.5\ R\ T$ (R is the gas constant, T the absolute temperature), which is equivalent to an added, constant term (—0.65) in eqn. (1). Finally, U_g' as defined in the EXPERIMENTAL section is thermodynamically equivalent to the quantity $\underline{R}^\circ$ in LSC. Thus, our final, revised equation for solute retention volume in linear isotherm GSC (corresponding to eqn. (1) in LSC) is simply:

$$\log U_g' = \log V_a - 0.65 + \alpha(S^\circ - A_s\varepsilon^\circ_g). \qquad (2)$$

The experimental U_g' values acquired in the present study can be used to evaluate the validity of eqn. (2) for GSC separation. Before attempting this, however, it should be noted that $-\varepsilon^\circ_g$ will be large, and it is therefore of great importance that the A_s values selected for the various solutes studied be as accurate as possible. In previous LSC studies, using eqn. (1) to correlate retention volume data between different eluents, the range in eluent strength values for a given solute has generally been fairly small, and small errors in A_s have not been critical. Furthermore, most of the solutes studied in these previous LSC investigations have been relatively large, planar aromatics, where the calculation of A_s is simpler than for most of the solutes that could be conveniently studied in the present investigation. For these reasons, we will first turn to the re-examination of the calculation of solute A_s values, before attempting the experimental verification of eqn. (2) for GSC separation.

Calculation of solute A_s values

A_s values (molecular areas) for a number of organic molecules have been measured directly by the BET method, although these values are not always constant between different adsorbents[27], and even show marked variation for the same adsorbent type as pore diameter[28] or water content[29] is varied. A number of such data[27,30,31] have been summarized in Table I. If we seek to generalize these data by comparison with calculated values (using covalent bond and Van der Waals radii as summarized by PAULING[32]), the calculated values are invariable small because of inefficient packing on the adsorbent surface. Following a suggestion by PIERCE[32],

TABLE I

CORRELATION OF EXPERIMENTAL MOLECULAR AREAS A_s FROM BET DATA

Adsorbate	A_s (Å^2)			
	Exptl.			*Calc.*[b]
	Ref. 26[a]	*Ref. 29*	*Ref. 30*	
Hydrogen		8.7		11.1
Methane		16.8	18	19.7
Acetylene		22.2		22.8
Ethane		23.7		27.3
Propane			40	34.9
1-Butene		42.7		41.5
n-Butane		46.9	47	42.3
Isobutane			33	42.9
Neopentane			38	42.9
n-Hexane	57.7			57.3
n-Heptane		62.5		64.8
Benzene	48.3	32.3		51.1
Ammonia		15.4		18.7
Oxygen		15.4		16.2
Nitrogen	(16.2)[c]	(16.2)[c]		17.0
Carbon monoxide		17.1		17.7
Carbon dioxide		20.5		21.5
Carbon disulfide		39.9		32.3
Fluorodichloromethane		40.2		36.1
Ethyl chloride	32.6	26.1		33.8

[a] Data for carbon only.
[b] From covalent and Van der Waals radii, assuming the latter is 0.5 Å greater for adsorbed molecules.
[c] Standard.

we have attempted the calculation of these solute molecular areas assuming a Van der Waals radius larger than those of PAULING by 0.4–0.7 Å. The use of Van der Waals radii increased by 0.5 Å give the best fit to the data of Table I, and molecular areas calculated by this procedure are also included in Table I. The resulting agreement between calculated and experimental molecular areas is reasonably satisfactory ($\pm$ 4.3 Å^2 standard deviation, omitting the questionable value of LIVINGSTON for benzene). Presumably, the same method of calculating molecular areas can be used for the solutes presently studied to obtain A_s values with a similar precision.

Verification of equation (2) for GSC separation

Table II presents experimental U_g' values for 38 solutes in the system 2.7 %

TABLE II

SOLUTE RETENTION VOLUMES FOR GSC SEPARATION OVER 2.7% H_2O–Al_2O_3 AT 83°[a]

Solute	*Log U_g' (exptl.)*		(A_s)[d]	$(S°)$[e]	*Log U_g'*	
	Column 1[b]	*Column 2*[c]			*Best exptl.*	*Calc.*[f]
Hydrocarbons						
Propane		0.48	4.1	—0.04	0.48	0.14
n-Butane		0.85	5.0	—0.02	0.85	0.70
n-Pentane	1.23	1.23	5.9	0.00	1.23	1.21
n-Hexane	1.65	1.66	6.8	0.02	1.65	1.74
n-Heptane	2.09	2.10	7.6	0.04	2.10	2.22
n-Octane	2.51	2.55	8.5	0.06	2.53	2.75
n-Nonane		3.00	9.4	0.08	3.00	3.29
n-Decane		3.48	10.3	0.10	3.48	3.82
1-Hexene	1.82		6.7	0.64	1.82	2.03
Cyclopentane		1.14	5.2	0.08	1.14	0.85
Cyclopentene		1.31	5.1	0.70	1.31	1.14
1,4-Pentadiene		1.50	5.7	0.62	1.50	1.44
Benzene	2.06	2.07	6.0	1.86	2.06	2.33
Toluene	(2.51)[g]	2.70	6.8	1.92	2.70	2.82
o-Xylene		3.15	7.5	2.07	3.15	3.32
m-Xylene		3.08	7.6	1.98	3.08	3.33
p-Xylene		3.08	7.6	1.98	3.08	3.33
Non-hydrocarbons						
Dimethyl sulfide	1.85		4.2	2.65	1.85	1.73
Thiacyclobutane		2.30	4.7	2.65	2.30	2.02
Thiacyclopentane		2.88	5.0	2.92	2.88	2.35
Dimethyl ether	1.82		3.7	3.5	1.82	1.93
Ethyl bromide	1.55		3.9	2.0	1.55	1.19
Methyl iodide	1.29		3.7	2.0	1.29	1.07
1-Chlorobutane		2.21	5.8	2.0	2.21	2.29
Chlorobenzene	(2.55)[g]	2.73	6.8	2.06	2.73	2.90
Fluorobenzene	2.17		6.3	1.97	2.17	2.56
Acetone	(2.52)[g]	3.05	4.2	5.0	3.05	3.07
Acetonitrile	(2.55)[g]	2.72	3.1	5.0	2.72	2.43
Methanol	(2.54)[g]	3.54	2.9	6.5	3.54	3.16
Methyl acetate	(2.50)[g]	3.55	4.8	5.0	3.55	3.42
Nitromethane	(2.51)[g]	3.01	3.8	5.4	3.01	3.07
Water	2.57					
Solutes for which U_g' could not be calculated						
Cyclohexane	1.57		?	0.10		
Cyclohexene	1.82		?	0.72		
Carbon tetrachloride	1.69		5.0	?		
Dichloromethane	1.63		4.1	?		
Dibromomethane	2.18		4.6	?		
Chloroform	2.02		5.0	?		

[a] Carrier gas: He saturated with H_2O at 21°.
[b] Thermal conductivity detection, 5.5 g adsorbent in 11 in. × $^1/_4$ in. column, 60 ml/min carrier gas flow rate.
[c] Flame ionization detection, 1.25 g adsorbent in 11 in. × $^1/_8$ in. column, 14 ml/min carrier gas flow rate.
[d] In units of $^1/_6$ the area of a benzene molecule[12].
[e] Directly measured experimental values where possible[21,34], otherwise calculated according to ref. 12.
[f] From eqn. (2), assuming $\varepsilon°_g$ equal —1.02, and using values of log V_a (—1.57) and α (0.57) from ref. 12.
[g] Value for water displacement peak.

H_2O–Al_2O_3 at 83°. For 32 of these solutes it is possible to calculate A_s and $S°$ values from previous LSC data. By rearrangement of eqn. (2) to express $\varepsilon°_g$ as a function of the remaining variables, a best average value of $\varepsilon°_g$ can be calculated from these data, equal —1.02. Use of this value and the known values of α and log V_a from previous LSC studies[12] then permits the calculation of U_g' values for each of these same 32 solutes. The resulting calculated values are compared in Table II with experimental values; the correlation is also shown graphically in Fig. 1. The overall agreement between experimental and calculated U_g' values is impressive. The standard deviation between calculated and experimental values is ± 0.25 log units.

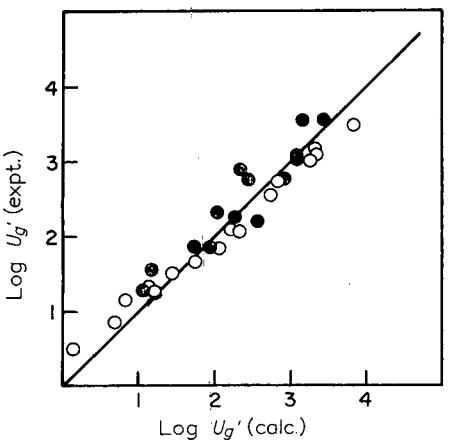

Fig. 1. Comparison of experimental U_g' values with values calculated from eqn. (2): 2.7 % H_2O–Al_2O_3 at 83°. O = hydrocarbon values; ● = non-hydrocarbon values.

If we assume the 4.3 Å² uncertainty in the A_s values of Table II which the correlation of Table I implies, this is equivalent to an uncertainty in the calculated values of U_g' from Table II of ±0.32 log units (4.3 Å² is equal to 0.51 A_s units, and the uncertainty in U_g' due to our assumed A_s values is then $\pm 0.51\ \alpha\varepsilon°_g$). Similarly, several of the $S°$ values of Table II have been determined indirectly, and must be uncertain to the extent of at least ± 0.1–0.3 log units, while the temperature extrapolation from room temperature[12] introduces an uncertainty of ±0.1 log units in comparable LSC studies. Finally, similar correlations of eqn. (1) in LSC systems for data involving different eluents are never more precise than ±0.1 log units, and the standard deviation tends to rise as the difference in eluent strengths increases; in the system of Table II we are essentially comparing two eluents of greatly differing strengths, pentane and He. On balance we can conclude that the correlation of Table II is certainly as good as could have been expected, and adequately verifies eqn. (2) as a sound relationship for correlating GSC and LSC data.

Additional data on a dryer adsorbent (1.5 % H_2O–Al_2O_3) are shown in Table III and Fig. 2. Here, the $\varepsilon°_g$ value calculated from the data of Table II was used, so that these values of U_g' could be calculated directly. For the five solutes with the exception of methyl iodide, the standard deviation between experimental and calculated U_g' values is ±0.26 log units, providing additional proof of the general validity of eqn. (2). The methyl iodide never cleared the column of 1.5 % H_2O–Al_2O_3

TABLE III

SOLUTE RETENTION VOLUMES FOR GSC SEPARATION OVER 1.5% H_2O–Al_2O_3 AT 83°[a]

Solute	*log* U_g'		(A_s)[b]	(S°)[b]
	Exptl.	*Calc.*[c]		
n-Butane	1.15	1.20	5.0	0.02
n-Pentane	1.67	1.80	5.9	0.00
n-Hexane	2.19	2.40	6.8	0.02
1-Hexene	2.41	2.73	6.7	0.64
Carbon tetrachloride	2.24	2.55	5.0	2.09[d]
Methyl iodide	> 3.0	1.64	3.7	2.0

[a] Thermal conductivity detection, dry He carrier gas, 5.5 g adsorbent in 11 in. × $^1/_4$ in. column, 60 ml/min carrier gas flow rate.
[b] Same values as in Table II.
[c] From eqn. (2); α equal 0.64, log V_a equal —1.40 (ref. 12); ε_g equal —1.02.
[d] Calculated from U_g' value of Table I.

and is believed to have been catalytically decomposed by this more active alumina (probably by reaction with adsorbed water to give strongly held HI and methanol).

If solution interactions were significant in determining retention volume in LSC systems, these effects would be expected to be largest for the polar non-hydrocarbons of Table II. This in turn would result in apparently larger values of $-\varepsilon^\circ_g$ for these solutes, since preferential solution interactions of pentane with the polar solutes would yield an effectively larger eluent strength for pentane. Similarly, the non-hydrocarbon data (solid circles of Fig. 1) would tend to lie above the average (solid line), while the hydrocarbon data (open circles) should lie below. If the two sets of compounds, hydrocarbons and non-hydrocarbons, of Table II are handled separately in determining ε°_g, values of ε°_g are obtained equal —1.00 and —1.06, respectively. While the value of $-\varepsilon^\circ_g$ for non-hydrocarbons is thus slightly higher, the actual difference appears well within the experimental variability of the data. This is better illustrated by the extensive overlap of both open and closed circles in Fig. 1 about the solid line.

Some additional GSC retention volume data for separation on alumina have been reported in the literature, but in no case have sufficient data on the adsorbent

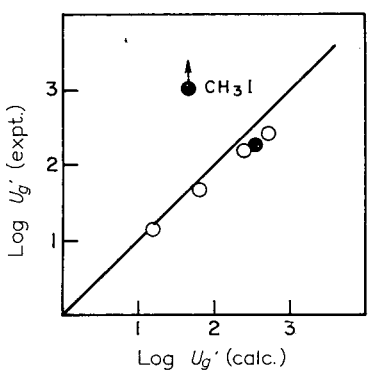

Fig. 2. Comparison of experimental U_g' values with values calculated from eqn. (2): 1.5% H_2O–Al_2O_3 at 83°. O = hydrocarbon values; ● = non-hydrocarbon values.

been reported to unambiguously determine the values of log V_a and α required by eqn. (2). If we assume that the "activity C" alumina reported by SCOTT[3] is equivalent to fully deactivated Alcoa F-20 alumina (both adsorbents contain a monolayer of adsorbed water), the hydrocarbon retention volume data for this adsorbent (Appendix 2, ref. 3), can be correlated with eqn. (2) as shown in Fig. 3. The calculated values of U_g' appear to be uniformly low, implying that the surface volume V_a for SCOTT's adsorbent is higher than for Alcoa F-20 of similar deactivation; this seems not unreasonable. If log V_a is assumed 0.4 log units greater for SCOTT's alumina, a reasonable correlation of his data with eqn. (2) results (dashed line of Fig. 3). In any event,

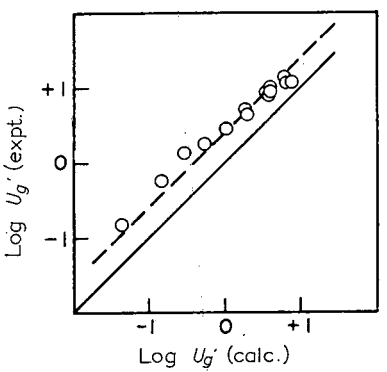

Fig. 3. Comparison of experimental U_g' values with values calculated from eqn. (2): Data of SCOTT[3] (Appendix 2) for elution from alumina plus a monolayer of water at 15°; log V_a assumed equal —2.00, α equal 0.57 in calculation. – – – = calculated curve for log V_a equal —1.60.

eqn. (2) predicts the correct relative retention volumes in Fig. 3 regardless of the value of log V_a assumed. We conclude that eqn. (2) is generally valid for predicting retention volume data in most GSC systems.

The *n*-alkane data of Figs. 1–3 show a definite tendency to fit a line of slightly lower slope, the calculated values for the small alkanes (C_1–C_3) being low, and the values for the large alkanes (C_6–C_{10}) high. This can be rationalized in terms of a small but consistent error in the calculation of the A_s values, as discussed in a later section.

DISCUSSION

Assuming the general validity of eqn. (2), there are a number of direct implications possible with respect to the potential of GSC in the separation of complex organic mixtures, particularly with alumina as adsorbent. First, with respect to the separation of isomers, eqn. (2) predicts that as long as the A_s values are equal (which is generally at least approximately true in the case of isomers), separation factors will be identical in LSC and GSC separation, assuming the same adsorbent, identical adsorbent activity, and equal column temperatures. This means that in principle many of the separation possibilities recognized in LSC can be transferred to GSC as well. However, the apparent advantage of GSC separation with respect to corresponding GLC systems tends to be reduced by two other considerations: GSC separations must always be carried out at higher temperatures and/or lower adsorbent

activities, which tends to reduce the separation factors for isomers, and many GLC systems show separations of isomers similar to those afforded by LSC (although frequently in reduced degree). Thus, while GIDDINGS[1] has referred to the good separation of *meta*- from *para*-xylene in a modified GSC system as an example of the unusual separations potentially available by GSC, many GLC systems are known which provide some separation of these two isomers. Similarly, while a number of similar separations of "positional" isomers in LSC over alumina have recently been cataloged[24], it is noted that these LSC separations become much poorer at low adsorbent activities. In general, we should conclude that reported isomer separations in LSC can provide useful guides for similar separations by GSC, that corresponding isomer separations will generally be less sharp by GSC than by LSC, and that we should not lose sight of the possibility of similar separations by GLC.

Another application of GSC which might appear potentially attractive arises from the marked selectivity of the metal oxides for polar molecules, permitting their ready separation by LSC from mixtures with less polar substances. It is interesting in this connection to compare the relative compound type selectivity of GSC over alumina with GLC separation over several common substrates. Table IV summarizes a number of experimental GLC data in this respect, along with calculated values for GSC over alumina. Compound type selectivity is here measured by the effect on solute retention volume of various polar groups, in terms of the equivalent number of methylene groups required to produce the same retention volume. It is seen that the selective retardation of the aliphatic hydroxyl, acetyl, and acetate groups on alumina ranges from 9–10 equivalent methylene groups, while the less polar

TABLE IV

COMPARISON OF COMPOUND TYPE SELECTIVITY IN GLC SEPARATION OVER VARIOUS LIQUID STATIONARY PHASES WITH GSC SEPARATION OVER ALUMINA

Solute group	*Equivalent number of methylene groups*[a]								
	GSC[b]	*GLC*[c]							
		P[d]	*K*[e]	*R*[f]	*A*[g]	*B*[h]	*C*[i]	*O*[j]	*Q*[k]
–OH[m]	9.2	8.5	7.6	4.6	3.7	1.9	2.5	3.3	3.0
$-CO-CH_3$[m]	9.0	8.5	7.2	4.8	4.7	4.1	3.7	4.0	4.0
$-CO_2CH_3$[m]	9.8	7.7	6.7	4.9	4.5	4.3	4.3	4.2	3.9
–O–[m]	5.2	3.2	2.4	1.4	1.0	1.1	1.0	1.1	0.9
$C_6H_6/n-C_6H_{14}$[n]	1.4	3.7	3.4	1.6	1.2	1.1	0.5	0.7	1.6

[a] Number of methylene groups required to replace group at right and give same solute retention volume.
[b] Separation over alumina, any temperature; calculated values, eqn. (2).
[c] Separation over indicated stationary phases at 100°; data of ref. 35.
[d] Polydiethylene glycol succinate.
[e] Polyethylene glycol.
[f] Polyethylene glycol.
[g] Diisodecyl phthalate.
[h] Di-2-ethylhexyl sebacate.
[i] Silicone oil.
[j] Silicone grease.
[k] Apiezon "L".
[m] Substituents on an alkyl chain.
[n] Increase in retention volume between benzene and *n*-hexane.

ether linkage increases retention volume by an equivalent of about 5 methylene groups. These calculated values would appear accurate to about ± 1 methylene group, on the basis of the correlation of Table II. The most polar liquid stationary phase, polydiethylene glycol succinate (P), is seen to be about equivalent to GSC over alumina in this regard, while the selectivity of the remaining stationary phases steadily declines as these liquids become less "polar". Similarly, the selectivity of GSC over alumina for unsaturated hydrocarbons can be measured by the increased retention volume of benzene relative to *n*-hexane; in this respect the data of Table IV show much *higher* selectivity in the case of the more polar GLC substrates than for GSC over alumina. While the data of SCOTT[3] suggest that highly active aluminas may show somewhat increased selectivity for unsaturated hydrocarbons than indicated by LSC studies on less active adsorbents (Tables II–IV), the possibility of using GSC in separating the unsaturated hydrocarbons over highly active aluminas is ruled out by the experience of PETITJEAN AND LEFTAULT[4], who noticed extensive reaction and loss of olefins under such conditions. Furthermore, separating even moderately complex (*i.e.* high molecular weight) samples by GSC appears to require highly deactivated adsorbents for convenient retention times, as well as for suppressing adsorbent catalyzed sample reactions. On balance, it appears that GSC over alumina offers little advantage over GLC with polar substrates in the selective separation of polar from non-polar sample components. Because of the similarity of the various metal oxide adsorbents in this respect (*e.g.* see comparison of alumina, silica, and Florisil[36]), the same generalization can doubtless be applied to other metal oxide adsorbents as well.

A most important problem in GSC separation is the extension of the technique to as high boiling samples as possible, and some workers (*e.g.* ref. 5) have regarded the upper sample boiling point limitation in GSC to be higher than for GLC, because of the volatility and decomposition of liquid substrates at sufficiently high temperatures. The preceding study of alumina, in conjunction with the development of eqn. (2), permits us to examine the question of sample boiling point limitation in GSC in some detail. A major necessity for high temperature GSC operation with metal oxide absorbents is heavy deactivation of the adsorbent, since a combination of high temperature and active adsorbent invariably results in reaction of some sample components during separation; furthermore, it has been noted in our laboratory[37] that GSC separations over undeactivated alumina at the highest temperatures practically lead to a much lower sample boiling point limit than in conventional GLC separation, because of the very strong adsorption of most molecules on such an adsorbent. This observation can be confirmed by simple calculation from eqn. (2): at 400° it is estimated that the U_g' value of n-$C_{30}H_{62}$ is about $5 \cdot 10^6$ (ml/g) on undeactivated alumina; under the same flow rates and column loadings of Table II, an elution time of about one year would be required!

Adsorbent deactivation by water as in the present studies is obviously restricted to lower temperature operation, since maintaining a deactivated adsorbent at high temperatures involves prohibitive water partial pressures in the carrier gas. For the present alumina, for example, it was found that the equilibrium water vapor pressure over 2.7 % H_2O–Al_2O_3 is 1.4 mm at 24° (static measurement) and 18 mm at 83° (data of Table II). If a constant heat of water adsorption is assured, this means that use of 2.7 % H_2O–Al_2O_3 at 150 and 250° would require water partial pressures

in the carrier gas of 140 and 1100 mm, respectively. Since it is unlikely that a more active alumina than this would be desirable in most GSC separations of heavy samples, the upper column temperature limit with water deactivation would seem to be about 250°. Under these conditions it can be calculated that n-$C_{20}H_{42}$ would have a U_g' value of about 10^4 ml/g, and would require about 16 h to elute, for a column size and eluent flow rate similar to that used in Table II.

A number of procedures other than elution with water vapor have been suggested for adsorbent deactivation in GSC, which seem suited for higher temperature GSC operation. Addition of up to a monolayer of nonvolatile liquid (or solid) to the adsorbent would seem to accomplish the same function as water addition, and would not require addition of excessive amounts of vapor to the carrier gas in order to maintain the deactivation of the adsorbent. PETITJEAN AND LEFTAULT[4] used Apiezon L and polyester succinate to deactivate alumina coated capillaries, and claimed no change in the selectivity of the adsorbent for olefins and paraffins. SCOTT[3], however, found that addition of silicone oil to alumina gave a complex combination of adsorption and partition effects. Experiments to be described in a following paper on GSC with an adsorbing carrier gas also show considerable adsorption of solutes on the surface of adsorbed vapor, and it is likely that similar adsorption on the deactivating liquid would occur in GSC with adsorbents deactivated by heavy liquids. Possibly the proper application of a deactivating non-volatile liquid to the adsorbent, so as to cover 50–90 % of the surface in a monolayer and remove all active sites, would show a minimum of partition and adsorption on the deactivating liquid, but this remains to be investigated experimentally. PETITJEAN AND LEFTAULT[4] have also suggested deactivating the surface of metal oxides with a layer of decomposition products (presumably carbon) formed by the *in situ* pyrolysis of an organic compound. This technique may eventually show some promise, although the latter workers were unsuccessful in applying it to their GSC system. A major problem in such a deactivation procedure is the production of large amounts of finely divided carbon on which adsorption can occur, leading effectively to GSC on carbon rather than the original metal oxide adsorbent. Finally, SCOTT[5] has used NaOH to deactivate alumina to the point where n-$C_{36}H_{74}$ was eluted below 400°. The data of SCOTT[5] can be used to calculate a surface volume and activity function for his adsorbent at 250°, 40 % NaOH–Al_2O_3: log V_a, —1.8; α, 0.22, compared with values calculated for fully deactivated (monolayer adsorbed water) Alcoa F-20 at 250° of log V_a, —2.0; α, 0.27. The major question with respect to SCOTT's adsorbent is what relationship it has to alumina, since sufficient NaOH has been added to produce several monolayers of adsorbed NaOH. The above adsorbent parameters suggest that 40 % NaOH–Al_2O_3 is not greatly different from alumina deactivated with just less than a monolayer of adsorbed water, but this comparison may not be a critical one.

Other techniques for deactivating the adsorbent during GSC separation include elimination of small pores, which are believed to correspond to active adsorption sites[6], and the use of adsorbing vapors in the carrier gas[9] (other than water for surface deactivation). The former technique may well prove one of the most promising in this regard, while the latter technique can be further broken down into use of adsorbing carrier gas components plus water, and higher boiling substances instead of water. Both of these latter techniques are examined in a following paper.

The present studies contain certain implications for the theory of LSC as well as

GSC separation. Beyond verifying the assumption of negligible net solution interaction energies contained in our previous theory of LSC separation, the general validity of this LSC theory has been further confirmed by the extreme extrapolation involved in going from LSC to GSC systems. The present investigation has also been responsible for a further improvement in the calculation of solute A_s values for LSC as well as GSC systems (Table I). In this connection it should be pointed out that the calculation procedure of Table I gives the A_s values of the n-alkanes as $1.4 + 0.9\,n$, where n is the number of carbon atoms in the alkane. The data of Tables II to IV and Figs. 1 to 3, however, strongly suggest that a more accurate value is $A_s = 2.1 + 0.75\,n$. This change provides a small improvement in the fit of the latter data to eqn. (2), but makes quite a considerable change in calculation of U_g' for the larger n-alkanes as presented earlier in this section. The latter relationship for A_s was assumed in these calculations.

Another addition to the theory of LSC provided by the present study has been the measurement of $S°$ values on alumina for the compounds CCl_4, $CHCl_3$, and CH_2Cl_2 (2.1, 2.7, 2.9, respectively), which in turn permits the calculation of eluent strength values $\varepsilon°$ for these solvents as in a previous treatment[21] (see Table V).

TABLE V

ELUENT STRENGTH VALUES FOR DIFFERENT SOLVENTS

Eluent	$\varepsilon°$	
	Exptl.	*Calc.*
CCl_4	0.18	0.33
$CHCl_3$	0.40	0.37
CH_2Cl_2	0.42	0.43

Agreement between calculated and experimental $\varepsilon°$ values is satisfactory for the last two eluents, but the calculated value is considerably too high for CCl_4. This suggests some peculiarity in the elution behavior of CCl_4 which deserves further study.

Finally, if we recognize that the adsorption energy of the He carrier gas used in the present study is actually zero, or close to it, then we can set up an absolute scale of $\varepsilon°$ values (adsorption energies per unit area) to replace the previous relative scale based on pentane as reference eluent (for alumina as adsorbent). The revised absolute $\varepsilon°$ values then range from 0.00 for He, to 1.02 for n-pentane, and 1.97 for methanol. The range 1.02 to 1.97 corresponds to normal LSC separation (no known liquid eluents are significantly weaker than pentane), and absolute values of $\varepsilon°$ close to 0.00 correspond to normal GSC. It is interesting to speculate on what sort of separations would involve absolute $\varepsilon°$ values intermediate between 0.00 and 1.00. The use of a strongly adsorbing carrier gas (other than the water required to maintain adsorbent activity) is a possibility examined in a following paper. Another possibility is use of eluents maintained at the critical state by means of high temperatures and pressures, where the eluent properties of the solvent should be intermediate between those of the liquid and gas phase; related separations have been described by KLESPER AND CORWIN[38]. Separations carried out in this area intermediate between LSC and GSC are virtually unknown, and it seems worthwhile to investigate their potential.

SUMMARY

A simple theory is proposed which relates solute retention volume in gas–solid and liquid–solid chromatography, and a quantitative equation has been derived which permits retention volume in gas–solid chromatography to be predicted upon the basis of previously measured liquid–solid chromatographic data. A number of experimental retention volume data have been measured for the gas–solid chromatographic separation of several solutes on alumina of varying water content; these data are accurately correlated by the above equation, verifying the theory. The future potential of gas–solid chromatography for the separation of complex organic mixture has been briefly examined in terms of the theory, and some of the problems of gas–solid chromatography are discussed.

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AN IMPROVED TECHNIQUE FOR BACK-FLUSHING GAS CHROMATOGRAPHIC COLUMNS

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INTRODUCTION

Back-flushing of gas chromatographic columns is a procedure that is widely practised when the sample to be analysed contains components that are eluted after the compounds of interest. Its virtue is that it can cut down analysis time and it may be used to protect the detector or a second column from contamination with undesirable compounds.

Back-flushing may be used to obtain a separation of compounds where a straight through system fails. Two columns with different stationary phases are used in series, the first column separating the compounds into two groups, A and B. The group with the longest retention time, B, is back-flushed off the first column. The separation of group A is completed on the second column. If back-flushing is not used, one or more of the compounds in group B may interfere with the final separation of group A on the second column.

THE TRADITIONAL METHOD OF BACK-FLUSHING

Fig. 1 shows a schematic flow diagram of a typical back-flushing system. With the taps in the position shown, the column operates normally. When both taps are turned through 90° the first column is back-flushed and the second column continues on forward flow. Both four-way taps have to be at the column temperature, the inlet tap, in order to avoid contamination of the inlet carrier gas with purge material. The needle valve is adjusted to maintain the same flow of carrier gas through the second column, when the first column is being back-flushed.

A number of variations of this back-flushing system are used but they are all likely to suffer from reduced separation efficiency or peak distortion compared with a straight through system. The source of the trouble is the lack of suitable multiway taps for use in the sample path. The taps available usually have a comparatively large dead volume, which if they are used with packed columns tends to broaden the peaks, and which if they are used with capillary columns destroys the separation efficiency. A technique of adding carrier gas to the flow through a four-way tap has been used to reduce the effect of the dead volume[1]. The technique, while useful, is limited in its applicability and it dilutes the sample to the detector.

The difficulties associated with multiway taps are not confined to the problem of dead volume; the tap has to withstand the column conditions of temperature and pressure without leaking, yet the use of grease is undesirable as absorption of components can occur. A constructional drawback is that the barrels of the taps have to be situated in the oven while they must be capable of being operated from outside the oven.

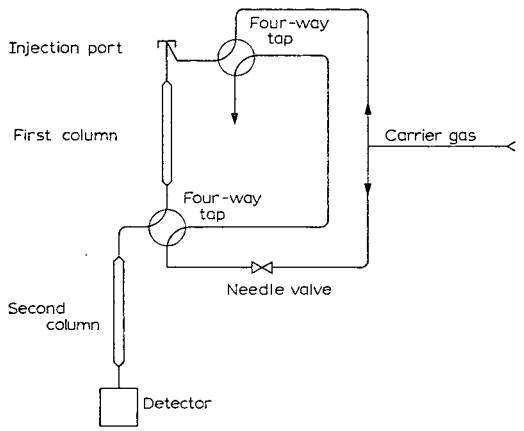

Fig. 1. Schematic flow diagram of a typical back-flushing system.

DESIGN AND CONSTRUCTION OF THE IMPROVED METHOD

A system of back-flushing has been developed which avoids the use of taps in the sample path or in the oven. Fig. 2 shows the scheme applied to two packed or two capillary columns and Fig. 3 that applied to one packed and one capillary column.

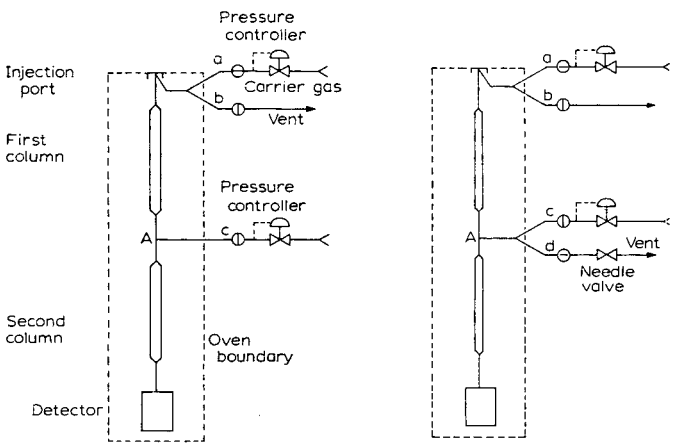

Fig. 2. Back-flushing system applied to two packed or two capillary columns.

Fig. 3. Back-flushing system applied to one packed and one capillary column.

Metal columns have been used in all cases but there is no obvious reason why glass should not be used. To keep the volume of the T-piece at A small, T-pieces made of brass or stainless steel $^3/_4$ in. long were used with a 42 thou hole to take a 20 thou I.D. capillary column, or $^1/_8$ in. I.D. holes to take $^1/_8$ in. O.D. connections to packed columns. Spring loaded glass taps have been used successfully up to an inlet pressure of 30 p.s.i.g. Edwards pressure controllers type V.P.C.1 have been found satisfactory for regulating the carrier gas inlet pressures.

METHOD OF OPERATION

When a sample is injected, tap a (Fig. 2) is open and taps b and c are shut. Carrier gas flowing through tap a carries the sample through the first column. When the components of interest have passed on to the second column taps b and c are opened and tap a is shut. Carrier gas now enters through tap c and divides into two streams at point A. One stream back-flushes the first column carrying unwanted components to atmosphere through tap b. The other stream flows through the second column, where separation of the components already on this column is completed.

Base line disturbance on the recorder trace on turning the taps is avoided by adjusting the two pressure controllers so that the pressure at point A is the same irrespective of the direction of flow in the first column. This is achieved by setting the pressure controller before tap a to give the optimum flow conditions for the particular separation being studied and then adjusting the pressure controller before tap c until the base line kick on changing from forward flow to back-flush is eliminated. To avoid pressure build up behind closed taps the pressure controllers used should incorporate a bleed system, enabling the pressure to be controlled when there is no flow along the line.

The method of operation and setting up is slightly different when the scheme is applied to a packed first column and a capillary second column.

On forward flow the taps are set as shown in Fig. 3. Carrier gas entering through tap a is split at the end of the first column, the major part of it going to atmosphere through tap d and the needle valve. This makes it possible for the optimum flow rates to be maintained in both the packed and capillary columns; in other words, the packed column and vent are acting as the conventional sample splitter. Back-flushing is achieved by closing taps a and d and opening taps b and c.

In setting up this system a pressure gauge connected by a capillary T-piece to point A is useful. The pressure at point A to give the optimum flow rate through the capillary column is first determined. The inlet pressure and the needle valve setting are then adjusted on forward flow to maintain the pressure at point A and to give the optimum flow rate through the packed column.

THE EFFECT OF COLUMNS WITH UNEQUAL PNEUMATIC RESISTANCE

Where both the first and second columns have the same pneumatic resistance, the pressure difference from the inlet to point A will be the same as the pressure difference from point A to atmosphere at the detector. When the first column is back-flushed the pressure at point A remains unchanged but the pressure at the injection point drops to atmospheric. The back-flush gas flow rate will be the same

as the forward flow rate because the pressure difference across the first column will be the same but in the reverse direction.

As a consequence the time required to back-flush off any unwanted compounds will be the same as the time elapsed from injection to starting to back-flush.

If the pneumatic resistance of the first column is less than that of the second column the rate of reverse flow will be greater than the rate of forward flow as the pressure difference from point A to atmosphere will be greater than the pressure difference from the inlet to point A. This will not affect the operation of the system in any way except that unwanted compounds will be back-flushed off the first column more quickly. A limitation may be reached, when the pneumatic resistance of the first column is much less than that of the second, where the back-flush flow becomes so large that the pressure controller before tap c is unable to maintain the required pressure. In this case the back-flush flow rate can easily be reduced by introducing a needle valve or other restriction into the purge line before tap b.

Where the pneumatic resistance of the first column is greater than that of the second the operation will not be affected in any way except that the time required to back-flush off unwanted components may become appreciable. In order to increase the back-flush flow rate a choice of methods is available; probably the easiest is to introduce a restriction between the second column exit and the detector; a short length of capillary restricted by flattening has been found satisfactory. Another method which gives excellent results is to connect the purge line to a controlled vacuum system; a vacuum pump and Edwards V.P.C.1 controller have been used. A third method is to operate the system with the detector above atmospheric pressure; an Edwards V.P.C.1 controller, on the exit of a katharometer or a flame detector, has been used. Any of these methods may be used where a second column is not required.

ADVANTAGES OF THE IMPROVED TECHNIQUE

The advantage of this method of back-flushing over the conventional use of multiway taps are:

1. The dead volume associated with the use of taps in the sample path is eliminated. Consequently capillary columns may be back-flushed without loss of efficiency.

2. The absence of taps in the sample path eliminates the possibility of absorption of sample components on tap lubricants or low friction plastics.

3. The taps used are outside the oven, at ambient temperature, consequently no special design of tap or oven is required and the system is easy to add to an existing chromatograph.

This method has been used successfully in many applications including the analysis of trace impurities in phenols and esters and for removal of heavy materials from reactor products during analysis.

SUMMARY

A technique of back-flushing gas chromatographic columns is described which avoids the use of taps in the sample path. The dead volume and the danger of ab-

sorption of compounds in the tap lubricant normally associated with back-flushing systems is eliminated. The taps used can be located outside the oven at ambient temperature, consequently the apparatus is easy to construct and existing chromatographs can be readily modified. Capillary and other high efficiency columns have been successfully back-flushed using this technique.

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BEITRAG ZUR GASCHROMATOGRAPHIE DER TETRACHLORIDE, $SiCl_4$, $SnCl_4$ UND $TiCl_4$

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EINLEITUNG

Im Hinblick auf umfangreiche physikalisch-chemische Untersuchungen an binären Systemen aus Tetrahalogeniden der IV. Gruppe des Periodensystems[1] waren systematische Reinheitsuntersuchungen der Halogenide $SnCl_4$, $TiCl_4$ und $SiCl_4$ auf gaschromatographischem Wege erforderlich. Es sollte dabei die Art der Verunreinigungen und ihre Mengen nachgewiesen, sowie ihre Entfernung durch geeignete Reinigungsverfahren überprüft werden.

Dabei zeigte es sich, dass für derartige Untersuchungen auch vom methodischen Standpunkt Interesse bestand.

Für die gaschromatographische Analyse des Siliziumtetrachlorids lagen bereits in der Silikonchemie erprobte Trennsäulen vor. So geht der Einsatz der Nitrobenzolsäule bei den hier beschriebenen Reinheitsbestimmungen auf FRIEDRICH[2] zurück, welcher an ihr Silangemische trennte.

Die Säulenfüllungen für die Gaschromatographie von Zinn- und Titantetrachlorid, welche in uns bekanntgewordenen Publikationen beschrieben wurden, schienen sich in keiner Weise für Reinheitsbestimmungen dieser Substanzen zu eignen. Diese liessen vielmehr erkennen, dass die gaschromatographische Bestimmung dieser Substanzen auf Schwierigkeiten stösst, was insbesondere ihre Trennung in gut aufgelösten Profilen anbetrifft. So berichtet FREISER[3] in einer kurzen Mitteilung über die Trennung von Zinn- und Titantetrachlorid. Er verwendete *n*-Hexadekan bei 102° als stationäre Phase, wobei die Arbeitstemperaturgrenze des *n*-Hexadekans weit überschritten ist. KELLER UND FREISER[4] erprobten Squalan und Oktadekan als stationäre Phase. Die Trennungen am Squalan waren, wie die Chromatogramme von KELLER[5] zeigen, mangelhaft. Untersuchungen von WACHI[6] (zitiert nach KELLER[5]) mit Silikonöl und Apiezonfett N und M blieben ohne Erfolg, wie auch der Einsatz eutektischer Schmelzen von anorganischen Salzen. Die Trennversuche von KELLER[5] an Apiezonfett und Silikonöl schlugen ebenfalls fehl; auch an Paraffinwachs konnten nur schlecht aufgelöste Profile erhalten werden.

Auf Grund dieser Ergebnisse mussten die chromatographischen Untersuchungen von Zinn- und Titantetrachlorid für diese analytischen Zwecke noch einmal aufgegriffen und darüber hinaus auch grundsätzlich auf die Trennung solcher Gemische ausgedehnt werden.

EXPERIMENTELLES

Es wurde für die Mehrzahl der Untersuchungen ein kommerzielles Gerät (GCHF 18/2 der Firma Giede, Berlin) mit Wärmeleitfähigkeitszelle verwendet. Die Trennsäulen sowie auch der Detektor bestehen aus V_2A-Stahl.

Besondere Aufmerksamkeit erfuhr die Überführung der Tetrachloride vom Vorratsgefäss in die Apparatur, die möglichst hydrolysefrei erfolgen musste (Fig. 1).

Die Dosierung erfolgte mit 1 ml Tuberculinspritzen. Da die Metallkolben den Glaswandungen der Spritzen nicht völlig gasdicht anliegen, wurde am oberen Teil derselben ein Silikongummiring als zusätzliche Dichtung angebracht. Dadurch wurde ein Hydrolysieren der Analysensubstanz zwischen Kolben und Glaswand verhindert. Die Dosierung wurde in folgender Weise vorgenommen: Die Spitze der Kanüle befindet sich in einem Silikongummistopfen (Gummischleuse) und ist daher vollständig abgeschlossen. Das Vorratsgefäss besteht aus einer Glasampulle, die mit einer Silikonkappe versehen ist. Die Injektionsspritze wird mit der Gummischleuse auf die Silikonkappe aufgedrückt und diese mit der Kanüle durchstossen. Nach der Füllung wird die Kanüle in die Schleuse zurückgezogen. Sodann kann die Injektionsspritze mit der Gummischleuse auf die Silikondichtung vor der Trennsäule aufgesetzt werden. Die Kanüle wird durch die Dichtung gestochen und so die Probe dem Trägergas beigemischt (Fig. 1). Nach beendeter Dosierung wird die Kanüle wieder in die Schleuse zurückgezogen.

Folgende Trennsäulen wurden verwendet:

Säule A: 30 Gew. % Nitrobenzol auf Diaphorit. Säulenlänge 1 m, Durchmesser 6 mm, 8 Stunden unter Arbeitsbedingungen stabilisiert.

Säule B: 45 Gew. % Silikonöl FF 7100 (Silikongummivorprodukt, Hersteller VEB Chemiewerk Nünchritz) auf Diaphorit. Länge 1 m, 6 mm Durchmesser (stabilisiert bei 250°, wobei etwa 10 % der Trennflüssigkeit abdampfen).

Säule C: 28.3 Gew. % Apiezonfett N auf Sterchamol. Säulenlänge 1 m, Durchmesser 6 mm, 8 Stunden unter Arbeitsbedingungen stabilisiert.

Die Trennsäulen wurden wie folgt präpariert:

Die Trägersubstanz wurde in einer Stielfritte G 4 mit der in absolutem Äther gelösten stationären Phase zusammengebracht. Durch die Fritte wurde Stickstoff geleitet, wodurch das Lösungsmittel langsam verdampft. Anschliessend wurden die Säulenfüllungen B und C bei 150° etwa 8 Stunden getrocknet und danach in die Säulen eingegeben.

ERGEBNISSE

Siliziumtetrachlorid

Die Leistung der Trennsäule A ergibt sich aus Fig. 2, dem Chromatogramm eines Gemisches von Silanen und Siliziumtetrachlorid. Diese Trennsäule schien daher für die Reinheitsuntersuchungen gut geeignet.

Es standen zwei $SiCl_4$-Proben verschiedener Herstellung zur Verfügung, die für die genannten allgemeinen physikalisch-chemischen Untersuchungen Verwendung finden: $SiCl_4$ I wurde durch Chlorieren von Siliziumdioxyd und Kohle bei 500° erhalten, während $SiCl_4$ II ein Chlorierungsprodukt von Rückständen der Silikon-

gummiproduktion darstellt. In $SiCl_4$ I konnte etwa 0.1 % Silikochloroform als Verunreinigung nachgewiesen werden. Im $SiCl_4$ II waren ausserdem noch etwa 1.5 % Methyltrichlorsilan enthalten. Silikochloroform lässt sich destillativ leicht entfernen, während die Trennung von $SiCl_4$–Methyltrichlorsilan grössere Schwierigkeiten bereitet.

Im Destillationsrückstand von $SiCl_4$ I fanden sich geringe Mengen Siliziumoxychlorid (Si_2OCl_6). Für den Nachweis wurde die Säule B verwendet, weil die Nitrobenzolsäule für hochsiedende Verbindungen nicht mehr verwendet werden kann.

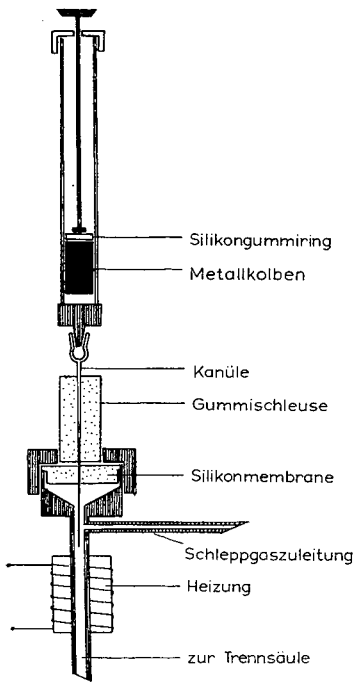

Fig. 1. Probendosierung. Einführung der Substanz in den Chromatographen.

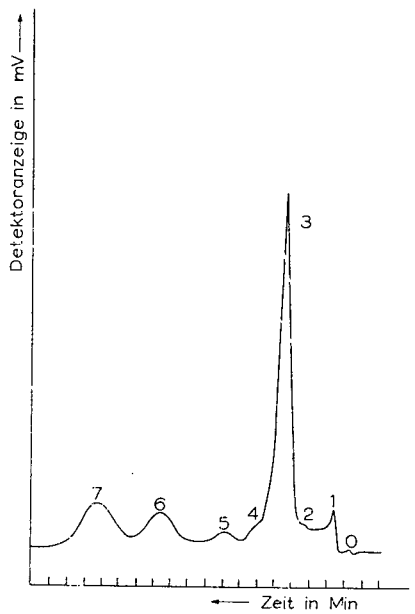

Fig. 2. Chromatogramm eines Silangemisches: Chlorwasserstoff (1), Silikochloroform (2), Siliziumtetrachlorid (3), Methyldichlorsilan (4), Trimethylchlorsilan (5), Methyltrichlorsilan (6), Dimethyldichlorsilan (7). Arbeitsbedingungen: Säule A; Säulentemperatur 100°; Schleppgasgeschwindigkeit: 1.9 l/St N_2.

Zinn- und Titantetrachlorid

Bei Verwendung von Säule B erscheinen Zinntetrachlorid bei 120° Säulentemperatur und Titantetrachlorid bei 175° als gut aufgelöste Maxima. Allerdings waren die Retentionszeiten so niedrig, dass das Erkennen von Verunreinigungen mit noch kleinerer Retentionszeit wenig erfolgversprechend war.

Die Säule C dagegen erlaubte nicht nur die Reinheitsuntersuchungen von $TiCl_4$ und $SnCl_4$, sondern gestattete deren einwandfreie Trennung zusammen mit der Erfassung von $SiCl_4$ und anderen niedriger siedenden Komponenten (Fig. 3). Die volle Trennwirkung dieser Säule wird erst erreicht, wenn eine etwa 8-malige Vordosierung

mit $TiCl_4$ erfolgt war. Die ersten Proben wurden nämlich in verzerrten Profilen aufgezeichnet. Offenbar wurden geringe, der Säule trotz der Vorbehandlung noch anhaftende Wassermengen hydrolytisch beseitigt. Erst danach ist die Säule stabil.

In dem untersuchten $TiCl_4$ (VEB Elektrochemisches Kombinat Bitterfeld) konnten als Verunreinigungen Chlor, Phosgen und Siliziumtetrachlorid mit insgesamt 0.6 % nachgewiesen werden (Fig. 4). Eine mehrmalige Destillation über Kupfer liefert ein gaschromatographisch reines Produkt.

Im Zinntetrachlorid (VEB Laborchemie Apolda) waren Chlor und $SiCl_4$ mit insgesamt 0.02 % als Verunreinigungen enthalten. Beide können durch eine einmalige Destillation abgetrennt werden.

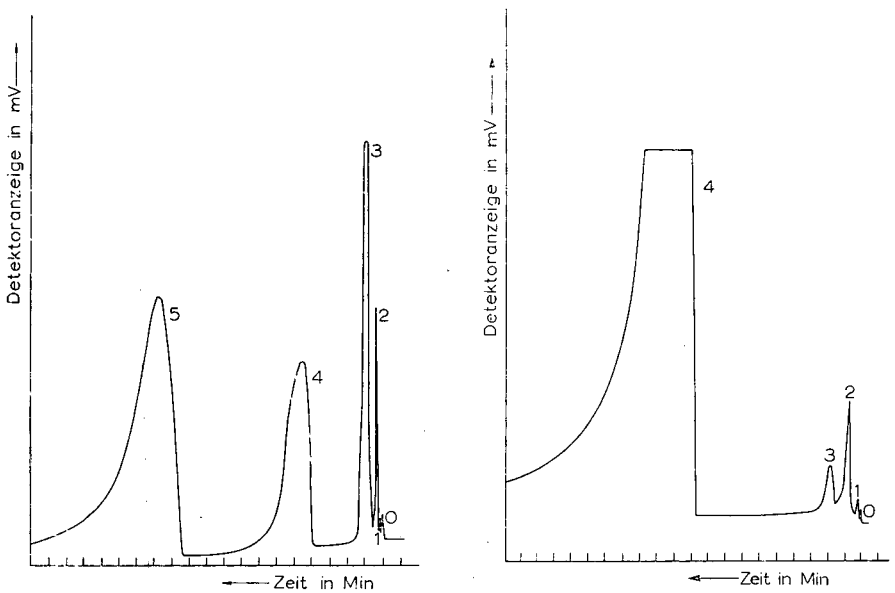

Fig. 3. Chromatogramm eines Gemisches von: Chlor (1), Phosgen (2), Siliziumtetrachlorid (3), Zinntetrachlorid (4), Titantetrachlorid (5). Arbeitsbedingungen: Säule C; 100°; 3.2 l/St H_2.

Fig. 4. Chromatogramm des verunreinigten $TiCl_4$. Die Verunreinigungen wurden durch Destillation angereichert. Chlor (1), Phosgen (2), Siliziumtetrachlorid (3), $TiCl_4$ (4). Arbeitsbedingungen: Säule C; 100°; 4.5 l/St H_2.

DISKUSSION

Die hier mitgeteilten Ergebnisse bezüglich der gaschromatographischen Trennung von $SnCl_4$ und $TiCl_4$ stimmen mit jenen von KELLER und WACHI (zitiert nach KELLER) nicht überein. Wie gezeigt, lassen sich insbesondere an Säulenfüllungen mit Apiezonfett gut aufgelöste Profile erreichen. Selbst wenn die von den Autoren verwendeten Apiezonfette und die Silikonöle in ihrer chemischen Zusammensetzung von den hier verwendeten mehr oder weniger verschieden sein sollten, so ist doch der Spielraum kaum gross genug, um die verschiedenen Ergebnisse zu erklären. Die hier dargestellten positiven Untersuchungen führen wir auf folgendes zurück:

(a) Auf die weitgehend hydrolysefreie Überführung der Substanzproben mittels einer Gummischleuse vom Vorratsgefäss in den Chromatographen.

(b) Auf die Stabilisierung der Apiezonsäule durch öfter wiederholte Vordosierung mit $TiCl_4$, wodurch Feuchtigkeitsreste in der Säule entfernt werden.

Hinsichtlich einer von KELLER vermuteten Reaktion zwischen Probe und Trennsystem haben bei keinem unserer Versuche Anhaltspunkte vorgelegen, wenn nur die Stabilisierung der Säulen vor Beginn der Trennung erreicht worden war.

ZUSAMMENFASSUNG

Es wird über die gaschromatographische Reinheitsbestimmung von $SiCl_4$, $SnCl_4$ und $TiCl_4$ berichtet. An einer Trennsäule aus Apiezonfett auf Sterchamol gelingt auch die Trennung von Gemischen dieser Stoffe, wenn auf hydrolysefreie Einführung der Substanzproben geachtet wird und Feuchtigkeitsreste in der Trennsäule durch Vordosierung von $TiCl_4$ entfernt werden.

SUMMARY

The determination of purity of $SiCl_4$, $SnCl_4$ and $TiCl_4$ by gas–liquid partition chromatography is described. The separation of these compounds, on columns with Apiezon on Sterchamol as stationary phase, is successful when the samples are unhydrolysed prior to injection and any moisture remaining in the column is eliminated by preinjection of $TiCl_4$.

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SEPARATION OF AMINO ACIDS AS THEIR N-TRIFLUOROACETYL-*n*-BUTYL ESTERS BY GAS CHROMATOGRAPHY

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Gas-liquid chromatography techniques may lead to rapid analysis of quantities of amino acids smaller than those detectable by classic ion-exchange column chromatography[1-3]. For example HUNTER *et al.*[4] and ZLATKIS *et al.*[5] used gas chromatography for the separation of aliphatic amino acids which had been converted to the corresponding aldehydes by means of ninhydrin. BIER AND TEITELBAUM[6] separated the amines obtained from decarboxylation of the corresponding amino acids. BAYER *et al.*[7,8] reported the separation of some aliphatic amino acids as methyl esters. By using nitrous acid LIBERTI[10] transformed the amino acids to α-hydroxy acids which were methylesterified and then separated by gas–liquid chromatography.

A gas-chromatographic separation has also been reported for methyl esters of α-chloro acids obtained from some aliphatic amino acids[11].

More recently several attempts of separation were made by combining the esterification of the carboxyl group with an acetylation of the amino group. Thus different authors have preferred one or other of the following transformations: N-acetyl-*n*-propyl[18], *n*-butyl[9], or *n*-amyl[14] esters; or N-trifluoroacetyl methyl[12,13], *n*-butyl[17], or *n*-amyl[22] esters.

Amino acids have also been separated by gas chromatography after their esterification with trimethyl-silane[15]. Finally HORNING *et al.*[16], using a different approach, separated the phenylthiohydantoin and dinitrophenyl derivatives of amino acids.

The present study concerns a method for the preparation of the N-trifluoroacetyl-*n*-butyl esters of eleven amino acids and the determination of the best conditions for their separation by gas chromatography.

MATERIALS

A Fractovap model C analytical unit P.AID/f (Carlo Erba, Milan) with hydrogen flame detector and automatic temperature programmer was used. Two meter stainless steel coiled columns (internal diameter 2 mm, external diameter 4 mm) were packed with 3 different stationary phases (obtained from Applied Science Laboratories Inc.): (a) neopentyl-glycol succinate, 1 %; (b) EGSS-X 1 %; (c) Carbowax 20 M, 1 %. The solid supports were Gas Chrom P (80–100 mesh) or Chromosorb (100–120 mesh).

The L-amino acids, chromatographically pure, were obtained from Mann Research Laboratories Inc., New York, N.Y., isoleucine was obtained from Hoffman

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La Roche, Basel. The *n*-butanol, methylene chloride and trifluoroacetic anhydride, gas-chromatographically pure, were obtained from Fluka, Switzerland.

A cationic resin, Dowex 50Wx4, from Down Co., was used after separation to a uniform size of about 200 mesh.

Thin-layer chromatography was made on glass plates (20 × 20 cm) by stratification of silica gel (Kieselgel G, Merck) with an automatic apparatus[23].

PREPARATION OF THE N-TRIFLUOROACETYLAMINO ACID *n*-BUTYL ESTERS

The hydrochlorides of the amino acids (total amount from 2.5 to 5 mg) were esterified in 10 ml of *n*-butanol together with 100 to 200 mg of dry Dowex 50Wx4, H^+ form* (200 mesh) as catalyst[19,20]. After 3 h at 130° the butanol was decanted and the resin washed twice with 10 ml butanol. Ten ml of a citrate buffer (semi-saturated solution) at pH 6.95 and 20 ml of methylene chloride were then added to the washed resin. The effect of pH on the percentage of butyl esters eluted from the resin is shown in Fig. 1.

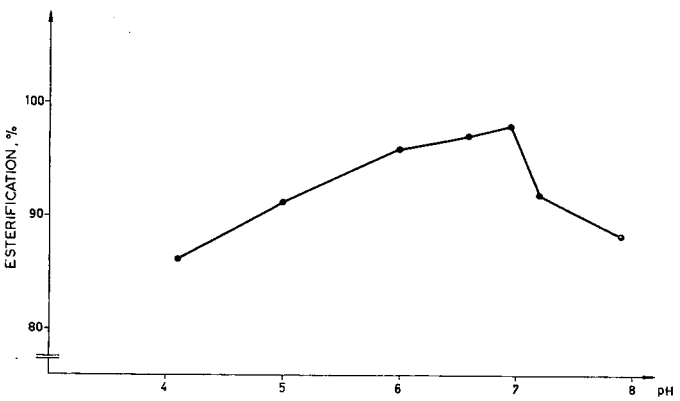

Fig. 1. Per cent esters eluted at different pH values.

After shaking for 5 min, the aqueous phase was discarded while the organic layer containing the esters of the amino acids was concentrated, under vacuum (15 mm Hg) in an ice bath, to a volume of 10 ml.

The butyl esters were then mixed, with continuous stirring, with 5 ml of a solution of trifluoroacetyl anhydride 1.5–2 % in methylene chloride. The excess of the reagent was removed under vacuum (15 mm Hg) whilst in an ice bath.

The residue was dissolved in 0.5 ml of methylene chloride and 3–5 μl of this were introduced into the chromatographic column.

With this procedure it was possible to prepare also the N-trifluoroacetyl, *n*-amyl or methyl esters. However, we selected the *n*-butyl ester because, being less volatile than the methyl esters, it could be manipulated with a minimal loss. Furthermore, under our experimental conditions, the *n*-butyl esters could be separated in a shorter time than the *n*-amyl esters.

* The use of resins with a higher degree of cross-linking (Dowex 50Wx8 or x12 or x20) or of carboxylic resins (Amberlite IRC50) does not improve the esterification.

QUANTITATIVE ESTIMATIONS

To determine whether the esterification of the amino acids with *n*-butanol was complete the reactivity to ninhydrin (0.2 % in *n*-butanol) after migration on a thin-layer chromatoplate was studied. With the solvent used (benzene–*n*-butanol (75:25, v/v)) the butyl esters showed various R_F's, after a 90-min run, while unmodified amino acids do not migrate from the point of application (see Fig. 2). Under these conditions methylene chloride extract did not show any trace of unmodified amino acids.

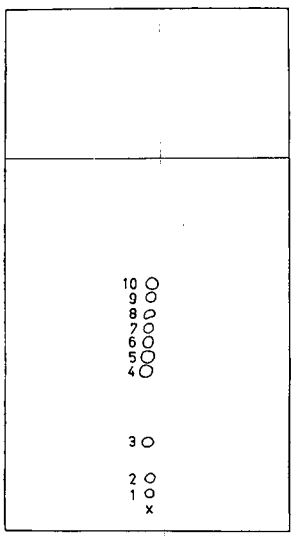

Fig. 2. Separation of a mixture of ten amino acid butyl esters by thin-layer chromatography. Spot identification by means of ninhydrin reagent (colours in parentheses).

Acidic hydrolysis of the amino acid *n*-butyl esters performed with 6 *N* HCl for 24 h and subsequent reaction with ninhydrin[21] confirmed that the esters represented up to 98 % of the amino acids passed through the process. After trifluoroacetylation the reactivity to ninhydrin was completely negative.

Further studies are in progress to obtain esterification of other amino acids and quantitative results after gas chromatography. The solution of these problems might permit the application of the procedure for the complete analysis of protein hydrolysates.

GAS-CHROMATOGRAPHIC SEPARATION

The column temperature was programmed from 90° to 200° with a linear increase of 2.5°/min. This temperature program was selected after various attempts as the most suitable for obtaining the separation of the 11 amino acid derivatives used (alanine, valine, isoleucine, leucine, glycine, proline, threonine, methionine, aspartic acid, phenylalanine, and glutamic acid). The flow rate of the carrier gas nitrogen was 14 c.c./min and the volume/min, as measured through a by-pass, was 5 c.c. Among

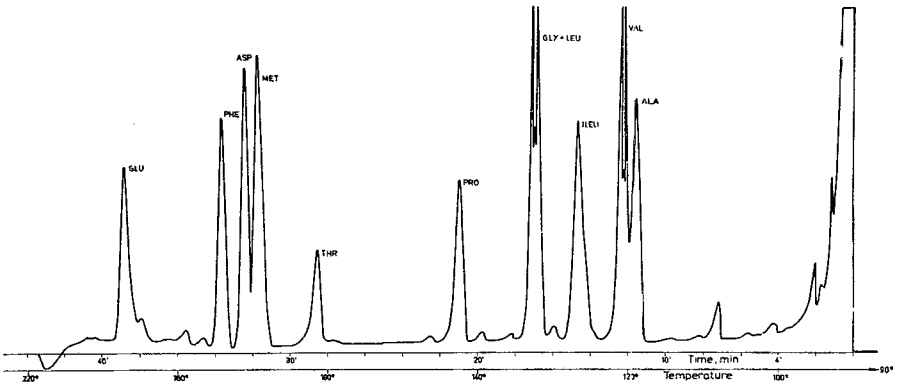

Fig. 3. Gas chromatogram of a mixture of 11 amino acids. NeoPGS 1 %. Carrier N_2 14 cc/min, H_2 23 cc/min, air 300 cc/min. Program 2.5°/min.

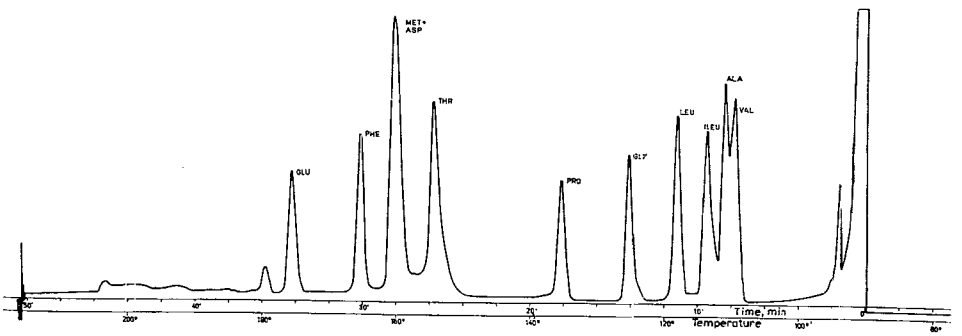

Fig. 4. Gas chromatogram of the same amino acids mixture. EGSS-X 1 %. Carrier N_2 14 cc/min H_2 23 cc/min, air 300 cc/min. Program 2.5°/min.

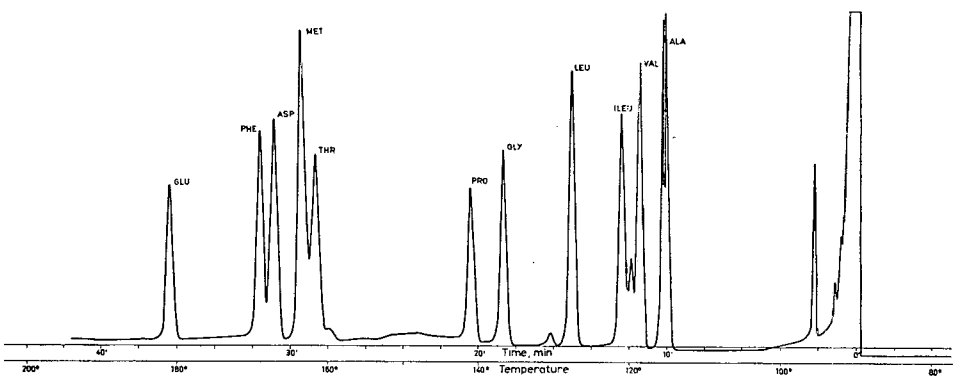

Fig. 5. Gas chromatogram of the same amino acids mixture. Carbowax 20 M 1 %. Chromosorb 80/100 mesh. Carrier N_2 14 cc/min, H_2 23 cc/min, air 300 cc/min. Program 2.5°/min.

the stationary phases used, only the neopentyl-glycol succinate has been reported in the literature[14,17] for separation of amino acids. EGSS-X and Carbowax 20 M, not previously employed for this type of analysis, gave more satisfactory results. Neopentyl-glycol succinate does not permit the separation of glycine from leucine. With EGSS-X it was possible to separate these two amino acids, but methionine and aspartic acid emerged in a single peak. Only when working with Carbowax 20 M was it possible to obtain a fair separation of all the 11 amino acids.

The peak pertaining to isoleucine includes also allo-isoleucine, contained as impurity in the sample of isoleucine.

The two amino acids are resolved only with Carbowax 20 M.

Figs. 3, 4 and 5 show typical chromatograms of amino acid derivatives after separation with the 3 stationary phases.

Furthermore, neopentyl-glycol succinate under our experimental conditions can only be used for a very limited number of analyses, while EGSS-X and Carbowax 20 M have the advantage that they permit up to one hundred separations.

SUMMARY

Eleven amino acids (alanine, valine, isoleucine, leucine, glycine, proline, threonine, methionine, aspartic acid, phenylalanine, and glutamic acid) were esterified in the carboxyl group with *n*-butanol in the presence of a catalyst resin and then transformed into their N-trifluoroacetyl derivatives.

The amino acid derivatives were satisfactorily separated by means of gas chromatography using Carbowax 20 M as stationary phase.

The experimental conditions necessary to obtain the best results are described.

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COLUMN CHROMATOGRAPHY OF SOME SULFUR-CONTAINING AMINO ACIDS

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In previous papers[1,2] we have reported methods for the chromatographic analysis of cysteic acid, cysteinesulfinic acid, taurine and hypotaurine. Some reactions for the determination of oxidation level of sulfur compounds have also been investigated[3,4]. The experience thus gained enabled us to make some improvements to the column chromatographic method of SPACKMAN, STEIN AND MOORE[5], so as to render it applicable to the detection of some sulfur compounds, with the aid of the automatic Amino Acid Analyzers.

In the present paper we report the chromatographic behaviour of the following compounds, whose inter-relationships are evident from their chemical formulae: cysteic acid (CA), cysteinesulfinic acid (CSA), taurine (TAU), hypotaurine (HYP), thiotaurine (TIOT), alanine-thiosulfonic acid (ATS), S-sulfo-cysteine (S-CYS) and S-sulfo-cysteamine (S-CYSA).

$CH_2–SO_3H$ | $CHNH_2$ | $COOH$	$CH_2–SO_2H$ | $CHNH_2$ | $COOH$	$CH_2–SO_3H$ | $CH_2–NH_2$	$CH_2–SO_2H$ | $CH_2–NH_2$
CA	CSA	TAU	HYP
$CH_2–SO_2–SH$ | $CHNH_2$ | $COOH$	$CH_2–S–SO_3H$ | $CHNH_2$ | $COOH$	$CH_2–SO_2–SH$ | $CH_2–NH_2$	$CH_2–S–SO_3H$ | CH_2NH_2
ATS	S-CYS	TIOT	S-CYSA

EXPERIMENTAL

Cysteic acid, cysteinesulfinic acid and taurine were commercial products. Hypotaurine and thiotaurine were prepared according to CAVALLINI *et al.*[6–8]. Alanine-thiosulfonic acid, S-sulfo-cysteine and S-sulfo-cysteamine were prepared according to DE MARCO *et al.*[9,10].

All the other compounds or reagents used were of commercial origin. The chromatographic apparatus used was the Model 120 Beckman-Spinco Amino Acid Analyzer.

The long column (150 cm), filled with ion exchange resin type 150 A of Beckman-Spinco, was equilibrated with a solution made up as follows: 0.1 *M* citric acid, 0.2 *M* NaCl, 0.2% Brij 35 solution (50 g + 100 ml H_2O) and 0.01% caprylic acid. The compounds to be analyzed were dissolved in the same solution. Chromatographic runs were performed at 30°.

All the other conditions (ninhydrin solutions, flow rate, washing of the columns, etc.) were as described for the standard runs with the Amino Acid Analyzer.

RESULTS

Fig. 1 shows that when citric acid is used as eluent a very good resolution of CA, CSA, TAU, urea and HYP is obtained.

This is the major improvement in respect to the standard chromatographic conditions for the Amino Acid Analyzer. In fact, when the pH 3.25 buffer is employed, CA and CSA are eluted together, as are also urea and HYP.

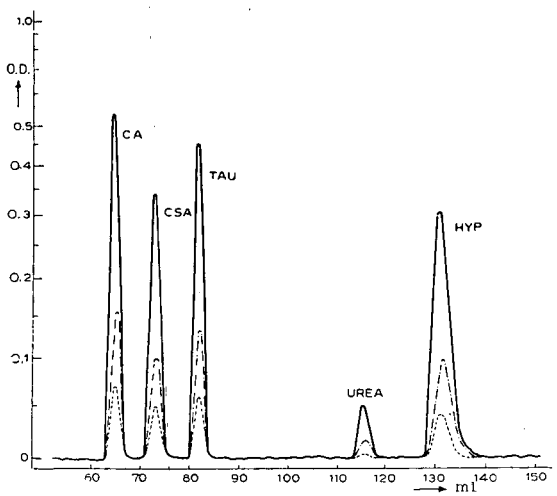

Fig. 1. 0.5 μmoles each of cysteic acid (CA), cysteinesulfinic acid (CSA), taurine (TAU), hypotaurine (HYP) and 1 μmole of urea chromatographed on the long column (150 cm) of the Beckman Spinco Amino Acid Analyzer, using 0.1 *M* citric acid–0.2 *M* NaCl as eluent. Temp. 30°. ——— recording of the unsuppressed 570 mμ photocell, —·—·—· recording of the suppressed 570 mμ photocell, – – – recording of the 440 mμ photocell.

As regards the other compounds studied, S-sulfo-cysteine and ATS are eluted, under our conditions, together with CA; and S-sulfo-cysteamine and thiotaurine together with taurine (Fig. 2).

Nevertheless these compounds may be easily recognized and distinguished from each other by the following procedures.

Detection of S-sulfo-compounds

The presence of the two S-sulfo-compounds may be inferred, in the first instance, by looking at the ratio between the absorbancy at 570 mμ and at 440 mμ. These compounds give a characteristic brown ninhydrin color (clearly evident on

paper chromatograms), which results in a 570:440 mμ ratio of about 2.5 as calculated from the diagrams obtained by repeated chromatographic analyses.

This value is very different from the 570:440 mμ ratios we have calculated for all the other compounds tested under the same experimental conditions, and which vary from 7 to 8.

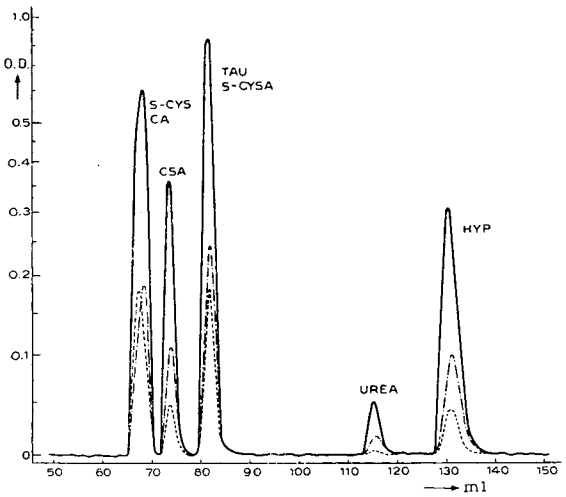

Fig. 2. Chromatography of 0.5 μmoles of the compounds listed in Fig. 1 together with 0.5 μmoles of S-sulfo-cysteine (S-CYS) and S-sulfocysteamine (S-CYSA). All the other conditions as in Fig. 1.

Therefore if S-sulfo-compounds are present together with CA or TAU, the 570:440 mμ ratios of the corresponding peaks are decreased.

Fig. 2 shows the chromatographic pattern of a solution containing S-sulfo-cysteine and S-sulfo-cysteamine besides the other compounds shown in Fig. 1.

With regard to the peak due to CA the asymmetry in the recording of the normal 570 mμ photocell is clearly evident, and there is a correspondingly higher absorption at 440 mμ. This results in a partial separation of the peaks recorded by the 440 mμ and the suppressed 570 mμ photocells. It is clearly evident that we are dealing with two different compounds, the first one, at the left side of the peak, is S-sulfo-cysteine.

As regards the peak due to taurine, the 570:440 mμ ratio calculated from Fig. 2 is 4.55, whereas from the curves in Fig. 1 it is 7.4.

Therefore a low value for the ratio between the absorbancy at 570 and 440 mμ is highly indicative of the presence of S-sulfo-compounds. Further, to ensure that one is dealing with these compounds, they may be destroyed by treatment with an excess of thiol: the S-sulfo-compounds being split into the corresponding sulfide and inorganic sulfite, according to the following reactions[11]:

$$R\text{–}S\text{–}SO_3H + R'\text{–}SH \rightleftharpoons H_2SO_3 + R\text{–}S\text{–}S\text{–}R'$$

$$R\text{–}S\text{–}S\text{–}R' \xrightarrow[R'\text{–}SH]{\text{excess}} R\text{–}SH + R'\text{–}S\text{–}S\text{–}R'$$

The usefulness of this reaction was checked as follows. 2 μmoles of S-sulfo-cysteine and 40 μmoles of cysteamine were dissolved in 1 ml of 1 N acetic acid. After standing for 60 min at room temperature the solution was taken to dryness on a boiling water bath under reduced pressure, to eliminate SO_2, which may interfere in the ninhydrin reaction. Then 2 ml of the citric acid solution used for the column chromatography were added to the test tube and 1 ml of the solution, corresponding to 1 μmole of S-sulfo-cysteine, was put on the column. After 120 ml of citric acid solution had been passed the elution was continued with the pH 4.25 buffer prepared

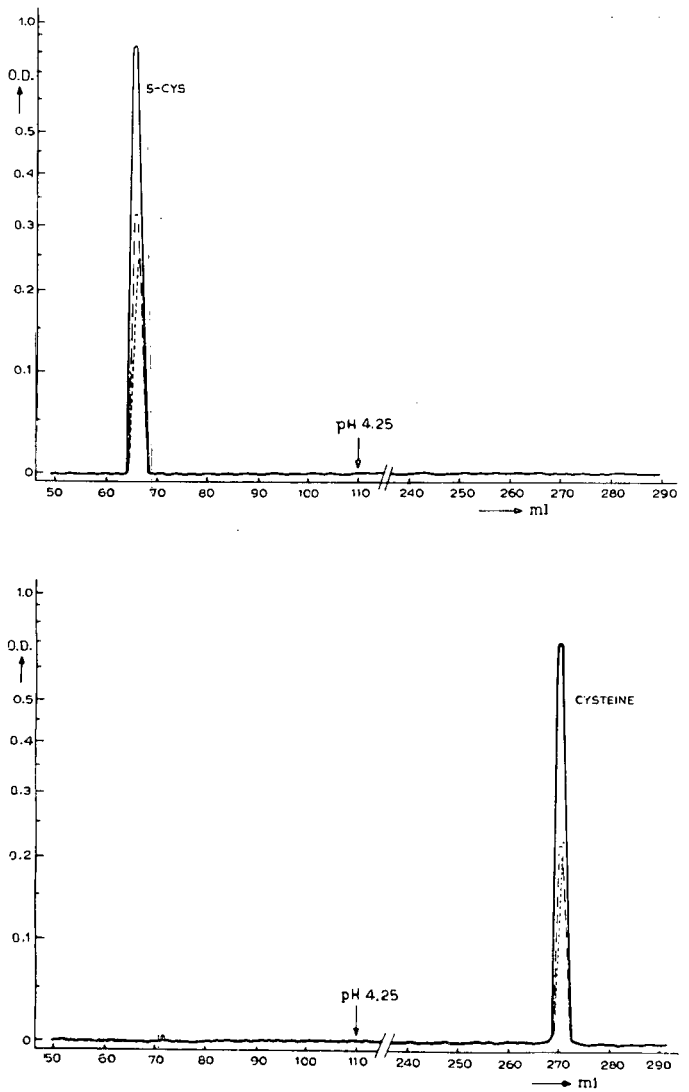

Fig. 3. Chromatography of 1 μmole of S-sulfo-cysteine before (top) and after (bottom) treatment with excess of cysteamine. All the other conditions as in Fig. 1. At the arrow the eluting solution was exchanged for pH 4.25 citrate buffer.

according to SPAKMAN *et al.*[5] to remove cysteine, whose elution pattern had been checked in preliminary experiments performed under the same conditions with the pure compound.

Fig. 3 shows the results obtained: S-sulfo-cysteine has completely disappeared and cysteine is evident (the excess of cysteamine was not eluted from the column, but washed out with NaOH).

S-Sulfo-cysteamine, also, when treated with an excess of cysteamine under the same conditions shows similar behavior, *i.e.* is absent (obviously in this case no cysteine was detected).

Detection of thiosulfonates

A property peculiar to ATS and TIOT is that, unlike the other compounds studied, they are cyanolysable according to the reaction:

$$R\text{–}SO_2SH + HCN \longrightarrow R\text{–}SO_2H + HCNS.$$

Their presence in the solution to be analyzed may then be detected by a positive cyanolysis reaction[12,13]. But this reaction does not permit the identification of the compound with which one is dealing. It is, however, possible by performing column chromatographic analyses before and after the cyanolysis reaction, since ATS is transformed by cyanolysis into CSA and TIOT into HYP.

The validity of this reaction and the experimental conditions have been checked as follows. Two μmoles of ATS were dissolved in 1 ml 1 *N* NH_3, added to 1 ml 0.1 *M* KCN, and left for 30 min in a boiling water bath. Then the solution was acidified with 5 *N* HCl and taken to dryness to remove cyanide (which if present emerges from the column after 90 ml, giving an atypical ninhydrin-positive peak). Two

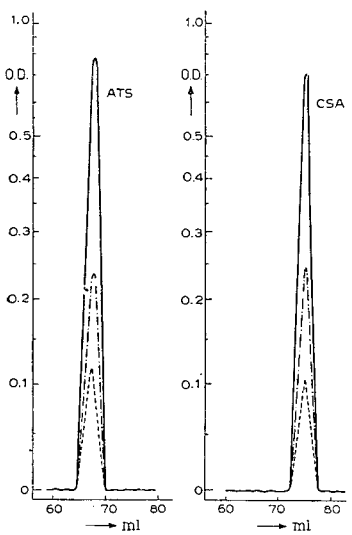

Fig. 4. Chromatography of 1 μmole of alaninethiosulfonic acid (ATS) before (on the left) and after (on the right) the cyanolysis reaction, by which it is transformed into cysteinesulfinic acid (CSA). All the other conditions as in Fig. 1.

milliliters of the citric acid solution were then added to the test tube, and 1 ml was put on the column. Fig. 4 shows the result obtained, *i.e.* the shift of the peak from 65 to 75 ml, indicating the complete transformation of ATS into CSA.

In the same way a solution of thiotaurine, after cyanolysis, shows only the presence of hypotaurine.

Quantitative determinations showed that no destruction of thiosulfonates occurred during cyanolysis, one mole of the latter giving rise to one mole of the corresponding sulfinate.

CONCLUSIONS

By using 0.1 *M* citric acid as the first buffer, good separations of the following compounds, using the long column of the Amino Acid Analyzer, may be obtained: cysteic acid, cysteinesulfinic acid, taurine, urea and hypotaurine.

The S-sulfo-derivatives and the thiosulfonate esters of cysteine and cysteamine, which have chromatographic positions indistinguishable from the corresponding sulfonic acid, may be detected by taking advantage of some of their properties: in the case of the S-sulfo-compounds the higher adsorbancy at 440 mμ after ninhydrin reaction, and the cyanolysis reaction in the case of the thiosulfonate esters.

Methods are described for destroying both types of compounds, thus making possible their quantitative estimation from the difference in the peak areas before and after their elimination.

As a concluding remark the rapidity of the chromatographic method here described is stressed, as it requires only a few hours for a complete screening of the sulfur amino acids here mentioned.

SUMMARY

A method is described for the chromatographic separation in the Automatic Amino Acid Analyzer of the following sulfur-containing amino acids: cysteic acid, cysteinesulfinic acid, taurine, hypotaurine. The chromatographic behavior of S-sulfo-cysteine, S-sulfo-cysteamine, alaninethiosulfonic acid and thiotaurine was also studied, and methods are reported for the identification of these compounds which are not well separated from the corresponding sulfonic acids.

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GRADIENT ELUTION IN COLUMN CHROMATOGRAPHY

THE USE OF CONTINUOUS LINEAR GRADIENTS TO APPROXIMATE CURVED GRADIENTS BY MEANS OF LAYERED SOLUTIONS*

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The theory of gradient elution and methods to establish many different shapes of gradients have been described[1,2]. One of the simplest devices for establishing gradients is composed of two cylindrical containers connected at the base (Fig. 1).

As illustrated, the cylinders contain solutions of concentrations A and B. The left-hand cylinder is the mixing chamber, from which the eluant flows into the column. If the cross-sectional areas of the cylinders $A_2 > A_1$, the shape of the gradient will be convex upwards, if $A_1 > A_2$, concave upwards, and if $A_1 = A_2$, linear, with the initial concentration of eluant $= A$ and final concentration $= B$. These relationships are given in the following equation[2]:

$$E = B - (B - A)\,(1 - V)^{A_1/A_2} \qquad (1)$$

where:

E = concentration of eluant,
B = concentration of solution in right-hand cylinder,
A = concentration of solution in left-hand cylinder,
V = fraction of *total* original volume eluted from apparatus,
A_2 = cross-sectional area of right-hand cylinder,
A_1 = cross-sectional area of left-hand cylinder.

When compounds of a similar nature are to be separated on a column using a linear gradient, emergence at the bottom of the column is often of an exponential type, *i.e.* initial eluate peaks are quite close together and final eluates are increasingly far apart. It often becomes necessary to arrange an exponential gradient to achieve a linear separation. If, in such a separation, several peaks appear close together, a change in the shape or slope of the elution curve becomes necessary: a change that will affect all of the eluted peaks in a way that does not necessarily represent an improvement.

At present, an automatic device is available which will effect changes in one portion of an elution curve without unduly distorting the remainder[3]. Such devices are relatively complex and expensive. The following will illustrate a somewhat

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simpler means of producing the same effect. This method takes advantage of certain facts:

(A) When $A_2 = A_1$ in eqn. 1, the exponential term disappears, making calculations quite simple.

(B) It is possible to layer solutions of different density one on top of the other in order of decreasing density.

(C) When such layers are established with sharp interfaces, the boundaries and density differences act as diffusion barriers.

(D) It is possible to establish differences by varying the concentration of the non-interfering substance X, and once a series of layers is established, to vary the concentration of the eluting substance Y (or of several substances), independently.

Thus, by using cylinders of identical cross-sectional area and establishing a series of density layers, one may elute continuously, following a series of continuous linear gradients.

EXPERIMENTAL

Density differences were accomplished by making solutions of sucrose (9 %, 6 % and 3 %). The concentration of eluting substance was approximated by methylene blue in sucrose of different optical densities.

Layers were established in cylinder B as follows: 400 ml of 9 % sucrose were poured into cylinder B. A cylindrical polyethylene float, of $^1/_4$ in. less internal diameter than cylinder B and 1 in. thick, was connected to a separatory funnel by means of flexible tubing, perforated just above the float. 400 ml of 6 % sucrose were added to the separatory funnel, and then allowed to flow slowly into cylinder B. When the addition was complete, a length of tubing was lowered to the 300 ml marker, and fluid was aspirated until a glassy interface could be seen. This process was then repeated with the top layer. Cylinder A was filled to the same depth as B, the two cylinders were connected together, the magnetic mixer was started under A, and the resulting "eluant" was led into a Packard fraction collector set to collect fractions of approximately 10 ml, using a drop counter.

Fractions were collected in 18 × 150 mm culture tubes, and the optical density at 650 mμ was read directly on the tube in a Coleman Junior spectrophotometer.

RESULTS

The calculated and observed results of three experiments are shown in Tables I–III.

CONCLUSIONS

In general we believe that the theoretical approach to this problem is verified by the experiments performed. Two types of deviations can be observed:

(1) Scatter in the optical density readings. This we believe to be due to the scatter in total diameter and wall thickness of the culture tubes used.

(2) Approach to, but not reaching calculated limits. This deviation we believe to be due to the large hold up volume of the rubber tubing used to connect the two

TABLE I

EXPERIMENT 1

Left-hand cylinder: 900 ml distilled water, optical density (OD) 0.		
Right-hand cylinder	*Slope (OD)*	
	*Calculated**	*Observed*
Layer 1: 300 ml 9% sucrose, OD 0.60	0–0.20**	0–0.20
Layer 2: 300 ml 6% sucrose, OD 0.23	0.20–0.22	0.20–0.23
Layer 3: 300 ml 3% sucrose, OD 0.84	0.22–0.84	0.23–0.77

* Sample calculation: $E = 0.60 - (0.60-0)\ (1-{}^1/_3)$
$0.60 - 0.40$
0.20.

** First linear slope of 600 ml.

TABLE II

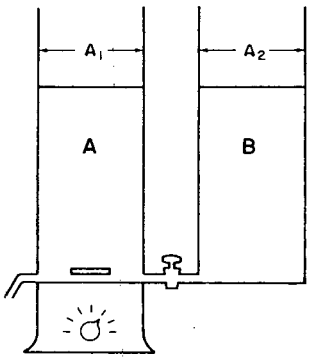
EXPERIMENT 2

Left-hand cylinder: 900 ml distilled water, OD 0.		
Right-hand cylinder	*Slope (OD)*	
	Calculated	*Observed*
Layer 1: 300 ml 9% sucrose, OD 2.40	0–0.80	0–0.71
Layer 2: 300 ml 6% sucrose, OD 0.95	0.80–0.90	0.71–0.80
Layer 3: 300 ml 3% sucrose, OD 0.90	0.90–0.90	0.80–0.80

TABLE III

EXPERIMENT 3

Left-hand cylinder: 900 ml distilled water, OD 0.		
Right-hand cylinder	*Slope (OD)*	
	Calculated	*Observed*
Layer 1: 300 ml 9% sucrose, OD 1.04	0–0.34	0–0.33
Layer 2: 300 ml 6% sucrose, OD 0	0.34–0.21	0.33–0.24
Layer 3: 300 ml 3% sucrose, OD 1.02	0.21–1.02	0.24–0.95



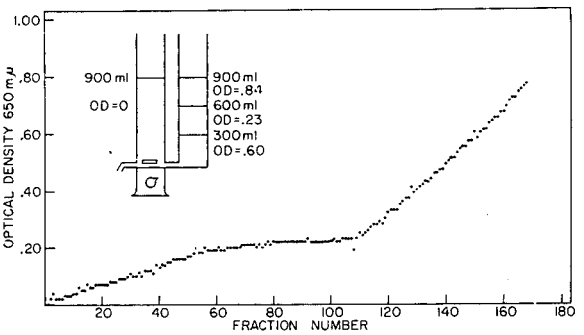


Fig. 1. Diagram of gradient elution apparatus.

Fig. 2. Experiment 1.

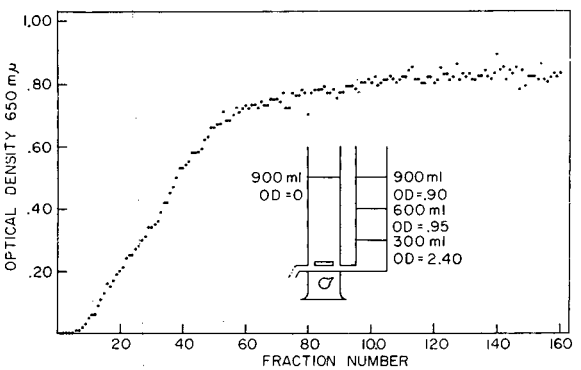


Fig. 3. Experiment 2.

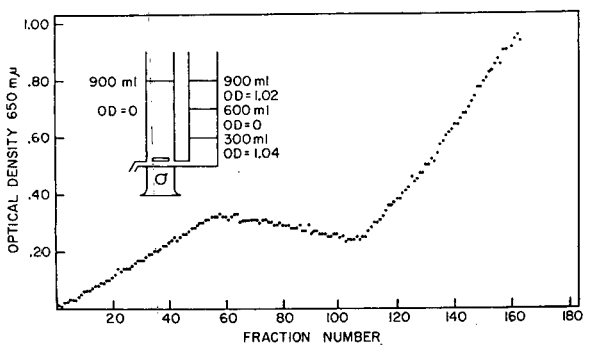


Fig. 4. Experiment 3.

cylinders and to connect the cylinder to the fraction collector, and to the fact that the exit tubes from the cylinders used were not on the bottom of the cylinders, so that the cylinders were not completely emptied.

It has been noted[1] that when solutions of unequal density are used in the two different cylinders, all slopes will be non-linear, since neither the heights, nor the flow rates are the same. However, we have noted very little difference in the heights of the columns of liquid and very little deviation from linearity in the slopes.

The most obvious use of the layering method is where organic solvents are involved, as in silicic acid chromatography. Density differences are often very large and can be utilized to advantage. Moreover, such solvents would tend to attack or erode the plastic containers used in automatic variable gradient machines.

SUMMARY

A method is described and tested experimentally to produce a series of continuous linear gradients for column chromatography. This effect is achieved by layering solutions of different densities on top of each other in a cylindrical glass gradient apparatus.

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A PREPARATIVE METHOD FOR THE ISOLATION OF BRAIN CEREBROSIDE, SULFATIDE AND SPHINGOMYELIN

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INTRODUCTION

Methods for the separation and purification of one or at the most two of the three major sphingolipids of brain on a preparative scale are available but no specific method has been published by which all three can be prepared from a single lipid extract. This paper describes a procedure specifically designed for the preparative fractionation and purification of sphingomyelin, cerebroside and sulfatide from ox brain.

Among the earlier methods available are the solvent fractionations of the ether insoluble lipid of brain by which BLIX[1] prepared pure sulfatide and KLENK and his co-workers[2,3] prepared cerebroside and sphingomyelin. Chromatographic preparations previously reported include the separation of cerebroside and sphingomyelin from sphingolipid preparations of brain[4,5] and the separation and partial purification of cerebroside and sulfatide from a chloroform–methanol extract of brain[6]. All of these procedures are based on chromatography on silicic acid columns eluted with mixtures of chloroform and methanol. LONG AND STAPLES[6] also report a fractionation on alumina columns. SVENNERHOLM AND THORIN[7] purified sulfatide from a combined cerebroside–sulfatide fraction obtained by chromatography of brain lipids on silicic acid. Final purification was carried out on a column of diethylaminoethyl cellulose. RADIN and co-workers prepared cerebroside from a chloroform–methanol extract of brain[8] and an ether insoluble lipid fraction from spinal cord[9] by chromatography on a magnesium silicate column followed by purification with mixed bed ion exchange resin. Magnesium silicate has also been used to purify a sulfatide fraction obtained by solvent fractionation of brain lipids[10]. ROUSER *et al.*[11–14] have outlined at a number of symposia the separation of cerebroside, sulfatide and sphingomyelin consequent to the fractionation of total brain lipids on diethylaminoethyl cellulose, silicic acid, and magnesium silicate columns. Full technical details of these procedures or adequate characterization of the fractions obtained have not been reported. An adaptation of the procedure using chromatography on magnesium silicate and diethylaminoethyl cellulose has been used for the preparation of sulfatide from human brain[15]. Preparations of sphingolipids from sources other than nervous tissue include the purification of sphingomyelin from red blood cells[16], plasma[17], and from a commercial beef heart sphingomyelin preparation[18].

EXPERIMENTAL

Reagent and solvents

All solvents were reagent grade and used without further purification. Mallinckrodt silicic acid, 100 mesh, reagent grade; Merck reagent grade aluminum oxide, suitable for chromatographic adsorption; and Florisil, Floridin Co., Tallahassee, Florida; were used for chromatography adsorbents. Fines were removed from the Florisil by suspending 100 g in 500 ml of distilled water and decanting after 10 min. This is repeated until the supernatant is clear. The Florisil is then dried at 100° over night and stored in a closed container.

Analytical procedures

Phosphorus was determined by the method of FISKE AND SUBBAROW[19] after digesting with perchloric acid. Carbohydrates were assayed by a modification of the procedure of RADIN *et al.*[9] which is described in detail elsewhere[20]. Sulfate and sphingosine were assayed by the procedures described by LONG AND STAPLES[6]. Nitrogen was assayed by the direct Nesslerization procedure of LANG[21] except the digestion mixture was diluted with 3 ml of water and 1 ml aliquots reacted with 1 ml of Nessler's reagent. Total fatty acids were determined by titrating with 0.05 *N* methanolic KOH. Free fatty acids for the titration were obtained by first refluxing the sample with 2 *N* methanolic sulfuric acid for 8 h, extracting with petroleum ether and then saponifying the ether extract with 2 *N* KOH in 50 % ethanol for 2 h at 100°. The fatty acids were finally extracted into petroleum ether after reacidifying the hydrolysate with H_2SO_4. The petroleum ether extract was washed repeatedly with water until neutral and aliquots taken to dryness and dissolved in methanol before titrating. Total weight of the lipids was determined by drying aliquots under vacuum at 80° in tared, aluminum dishes. Choline was assayed by adapting features of several assays[22–25] with certain modifications to increase the level of sensitivity. The lipid to be assayed and containing up to 300 μg of choline is hydrolyzed with 2 ml of aqueous saturated $Ba(OH)_2$ in a closed, screw capped tube for a minimum of 10 h at 100°. The hydrolysate is filtered on Whatman No. 1 filter paper and 0.5 ml of the filtrate pipetted into a 12 ml conical centrifuge tube. 0.5 ml of an aqueous saturated ammonium reineckate solution is mixed with the sample and the mixture is allowed to stand 3 h at 4°. The remainder of the procedure is carried out at 4° except as indicated. The tube is centrifuged at approximately 2,000 r.p.m. for 10 min and the supernatant solution drawn off with a Pasteur pipet. The reineckate remaining in the tube is washed three times by suspending in 0.5 ml of ice cold *n*-propanol saturated with choline reineckate. The suspension is centrifuged and the supernatant solution discarded after each washing. The reineckate is then dissolved in 5 ml of acetone and recentrifuged to remove a small amount of suspended material. After closing the tube with a cork to avoid evaporation of the acetone, the solution is allowed to come to room temperature in the dark and then read at 327 mμ.

Paper and thin-layer chromatography

Hydrolysates of the lipids were chromatographed for the detection and identification of carbohydrates with the ethyl acetate–pyridine–water solvent system of JERMYN AND ISHERWOOD[26]. A 2 h, 100°, 3 N sulfuric acid hydrolysate which had been

passed through a column of AG-3 anion exchange resin to remove the sulfuric acid was used. Thin-layer chromatography of the lipids for identification purposes and as one criterion of purity was carried out as previously described[27].

Preparation of brain sphingolipids

Ox brains were obtained from 30 to 60 min after slaughter and carried in ice to the laboratory. The brains were freed of adhering membrane and blood clots and the lipids extracted with chloroform–methanol[28]. The sphingolipids were separated from this extract by first removing the solvents under vacuum on a rotary evaporator. The lipids were then suspended in diethyl ether, 50 ml of ether for every 21 g of lipid. The suspension is allowed to stand at 4° for 90 min, transferred to 250 ml stainless steel centrifuge bottles and centrifuged 20 min at 5,000 r.p.m. The supernatant solution is decanted and the insoluble residue resuspended in one half the volume of ether used in the initial extraction. This suspension is allowed to stand 90 min and centrifuged as before. The procedure is repeated once more using one quarter of the original volume of ether. The final residue or sphingolipid fraction is approximately 29 % of the original weight of lipid.

This crude sphingolipid preparation is freed of acyl lipids by treating essentially under the mild alkaline conditions described by MARINETTI[29]. The lipids are dissolved in chloroform, 25 ml/g of lipid, and 10 ml of methanol and 2.5 ml of 0.5 *N* sodium methoxide in methanol per g of lipid is added. The mixture is incubated at 37° for 30 min and neutralized with 1 ml of methyl formate for every 35 ml of the reaction mixture. Water is then added in an amount equal to 5 % of the total volume and the mixture passed through a Sephadex column as described by WELLS AND DITTMER[20]. 1 g of Sephadex is used for every 10 ml of the mixture. This latter procedure removed the water soluble reagents and reaction products. 94 % of the original weight of the crude sphingolipid preparation is recovered.

Alternatively, a sphingolipid preparation obtained by the method of CARTER *et al.*[30] was treated by the mild alkaline hydrolysis procedure and Sephadex as described above. A yield of 91 % of the crude sphingolipid preparation is obtained.

Chromatographic fractionation of brain sphingolipids

Columns containing from 1 to 150 g of silicic acid have been used. The silicic acid with one half its weight of Hyflo Super-Cel (Johns-Manville) is suspended in chloroform and the column packed as described by HANAHAN *et al.*[31]. The column is washed with $CHCl_3$, 10 ml/g of silicic acid, and the sphingolipid run on the column dissolved in $CHCl_3$. It is imperative that enough $CHCl_3$ to completely dissolve the sphingolipids be used at this point. Failure to do so causes greatly reduced flow rates and poor resolution. 20 ml of $CHCl_3$ for each gram of sphingolipid is satisfactory. Up to 20 mg of lipid per g of silicic acid can be chromatographed. The column is eluted in succession with the equivalent of 10 ml of $CHCl_3$; 25 ml of $CHCl_3$–CH_3OH (11:1) with 0.5 % H_2O (v/v/v); 30 ml of $CHCl_3$-CH_3OH (6:1) with 1.0 % H_2O (v/v/v) and 20 ml of $CHCl_3$ CH_3OH (1:4) (v/v) per g of silicic acid. The effluent is collected in the equivalent of 3.5 ml/g of silicic acid in each fraction and at a flow rate of 3.5 ml/h per g of silicic acid.

The location of the sphingolipids in the fractions collected is determined by thin-layer chromatography. 0.5 ml samples of each fraction is taken to dryness

in vacuo at 35°, dissolved in approximately 30 μl of $CHCl_3$–CH_3OH (2:1) (v/v) and spotted on a silica gel thin-layer plate. All 23 fractions are spotted on one 20 × 20 cm plate. Development is carried out in $CHCl_3$–CH_3OH (4:1) containing 2 % water and 2 % pyridine (v/v/v/v). A general detection spray, Rhodamine 6G, and a specific phosphate spray[27] are used in succession for detection of the lipids. A typical separation is shown in Fig. 1. On the basis of the thin-layer plate, the fractions containing each of the sphingolipids are combined and then taken to dryness under vacuum.

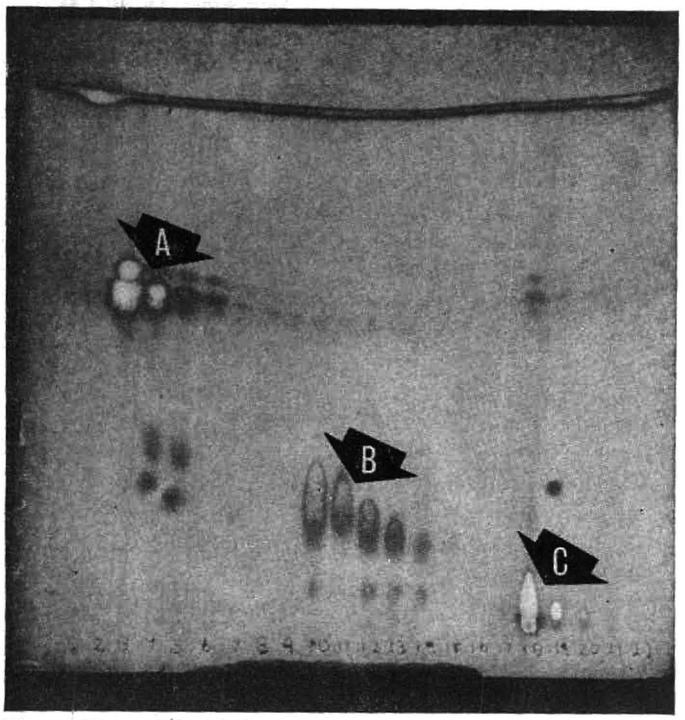

Fig. 1. Silica gel G thin-layer plate of sample from fractions of sphingolipids chromatographed on a 150 g silicic acid column. The three major fractions have been identified as A, cerebroside; B, sulfatide; C, sphingomyelin. The plate was developed in chloroform–methanol–water–pyridine (40:10:1:1) (v/v/v/v). Detection of the compounds was made with Rhodamine 6G[27].

Final purification

As can be seen on the thin-layer plate, Fig. 1, and as has been shown by chemical analysis, the fractions obtained by chromatography on silicic acid are not completely homogeneous and generally further purification is necessary. When the ether insoluble or sphingolipid fraction prepared from a $CHCl_3$–CH_3OH extract as described above is used, the cerebroside fraction obtained from silicic acid requires no further purification. With the sphingolipid preparation of CARTER *et al.*[30] the cerebroside is found to contain small amounts of phospholipid which may be removed by eluting the cerebroside from a Florisil column with $CHCl_3$–CH_3OH (2:1) as described by KISHIMOTO AND RADIN[8]. For our purposes, 1 g of Florisil was used for every 115 mg of cerebroside and the column was eluted with 6 ml of $CHCl_3$–CH_3OH per g of Florisil. The phospholipids present are retained on the column.

Regardless which sphingolipid preparation is used, it is found necessary to remove trace amounts of phosphorus containing material from the sulfatide. Purification is carried out by chromatography on Florisil as used for the cerebroside except no more than 35 mg of lipid per g of Florisil is used. Finally, a slight yellowish tint of the preparation is removed by dissolving the sulfatide in a minimum volume of $CHCl_3$ and precipitating with two volumes of acetone. The mixture is allowed to stand at 4° for 30 min and then centrifuged. Solutions of the sulfatide in $CHCl_3$ after this treatment are colorless.

Sphingomyelin as obtained from the silicic acid column is contaminated with traces of sulfatide and is also yellow in solution. Both the sulfatide and yellow color are removed by chromatography on alumina by a modification of the system described by RHODES AND LEA[36]. One gram of alumina is used for every 42 mg of crude sphingomyelin. The sphingomyelin in 1 ml of $CHCl_3$–CH_3OH (3:2) (v/v) is eluted through the column with the equivalent of 5 ml of the same solvent per g of alumina.

RESULTS AND DISCUSSION

Recovery of sphingolipids

Various criteria were used to determine the yield of purified sphingolipids including weight and analysis of galactose, phosphorus and sphingosine nitrogen. In a typical preparation using a 150 g silicic acid column, 2.08 g (90 %) of 2.32 g of sphingolipid prepared from a $CHCl_3$ CH_3OH extract was recovered in the three fractions isolated. This 2.08 g consisted of 1.226 g cerebroside, 0.310 g sulfatide and 0.548 g sphingomyelin. After final purification a yield of 0.286 g (95 %) sulfatide and 0.446 g (81 %) of sphingomyelin was obtained. Analysis of galactose and phosphorus before and after the final purification step showed that 5 % of the sulfatide and 2 % of the sphingomyelin is lost. The nature of the contaminant which accounts for the discrepancy between the weight loss and phosphorus loss in the sphingomyelin purification was not identified.

In a preparation using the ethanol insoluble or sphingolipid preparation of CARTER *et al.*[30] a closer check was made on the recovery by following the total sphingosine and galactose content as well as weight throughout the fractionation. Using 2.78 g of sphingolipid on a 150 g column, 2.41 g (86.5 %) was recovered in the three pooled fractions. This was distributed with 1.430 g of cerebroside, 0.442 g of sulfatide and 0.534 g of sphingomyelin. After the final purification 1.35 g (94.5 %) of cerebroside, 0.331 g (74.8 %) of sulfatide and 0.384 g (86.8 %) of sphingomyelin were obtained. On the basis of galactose and phosphorus, 94 % of the cerebroside, 90 % of the sulfatide and 95 % of the sphingomyelin were recovered in the final purification step. The weight of lipid recovered from the silicic acid column indicates that as much as 14 % of the sphingolipid may be lost during this step of the fractionation. Analysis of sphingosine and galactose indicates otherwise. In this preparation, a total of 37.5 mg of sphingosine nitrogen and 380 mg of galactose was placed on the column. The three fractions obtained from the column contained a total of 34.9 mg of sphingosine nitrogen and 376 mg of galactose. This corresponds to a recovery of 93.2 % of the total sphingolipid and 98.8 % of the cerebroside and sulfatide. After the final purification the three fractions contained a total of 34.7 mg of sphingosine nitrogen and 362 mg of galactose which corresponds to an over all recovery of 92.6 % of the total sphingo-

lipids and 95.3 % of the cerebroside and sulfatide combined. The higher recoveries indicated by the sphingosine and galactose assays as compared with those obtained on the basis of weight are consistent with the presence of lipids other than sphingolipids in the original sphingolipid preparation of which some are eluted from the silicic acid. Both fatty acids or fatty acid methyl esters and lyso plasmalogens would be expected to remain in this fraction after the mild alkaline methanolysis treatment used in the preparation. The exact nature of the contaminating material has not, however, been characterized. In summary, recoveries of 92–95 % of the sphingolipids can be expected.

Very few recoveries have been reported for chromatographic preparations of sphingolipids. The 5–10 % losses of lipid sulfate previously reported[6–7] for chromatographic preparations of sulfatide are in the range of the losses reported here. SCHWARZ *et al.*[5] give a fairly complete report on the recovery of cerebroside and sphingomyelin after silicic acid chromatography of the sphingolipids of human brain by a fractionation method similar to that reported here. They report recoveries of 100 % of

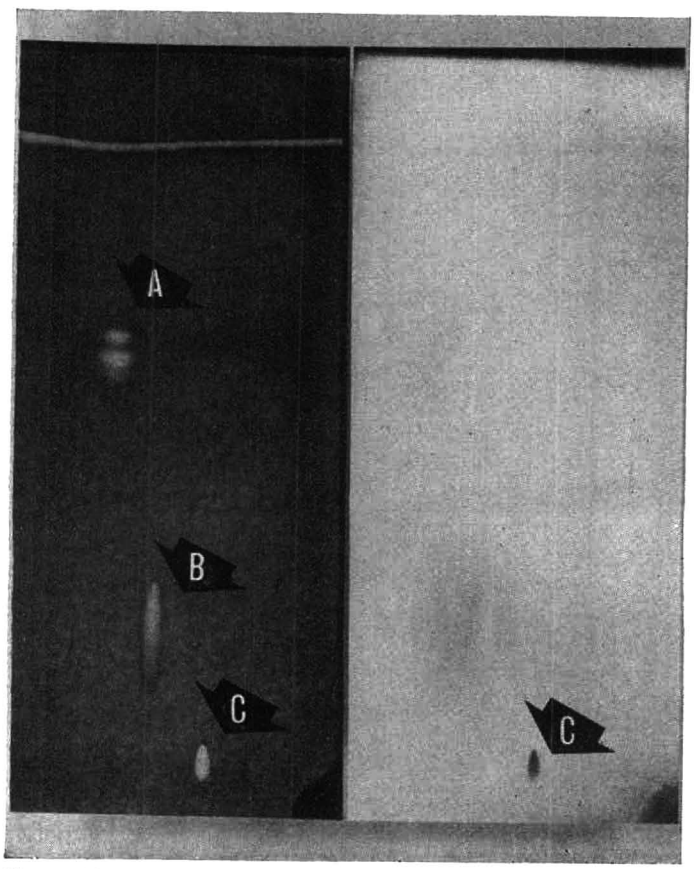

Fig. 2. Silica gel thin-layer plate of purified sphingolipid preparations. The three sphingolipids are: A, cerebroside; B, sulfatide; C, sphingomyelin. The photograph at the left was made after spraying with Rhodamine 6G and that at the right is of the same plate sprayed with a specific phosphate reagent[27].

TABLE I

CHEMICAL COMPOSITION OF PURIFIED SPHINGOLIPIDS

Compound	*Composition (% weight)*														
	Nitrogen			*Phosphorus*			*Galactose*			*Sulfur*			*Choline*		
	Found		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*
	I	*II*		*I*	*II*		*I*	*II*		*I*	*II*		*I*	*II*	
Cerebroside	1.69	1.70	1.71	<0.005	<0.005	0	21.6	21.8	21.9	—	—	0	—	—	0
Sulfatide	1.57	1.56	1.57	<0.005	<0.01	0	20.3	20.0	20.1	3.66	3.51	3.59	—	—	0
Sphingomyelin	3.49	3.50	3.48	3.88	3.86	3.84	0	0	0	—	—	0	14.6	—	15.0

Compound	*Molar ratios*														
	P/N			*Galactose/N*			*Sulfur/N*			*Choline/N*			*Fatty acid/N*		
	Found		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*
	I	*II*		*I*	*II*		*I*	*II*		*I*	*II*		*I*	*II*	
Cerebroside	—	—		0.98	0.99	1.00	—	—		—	—		—	1.02	1.00
Sulfatide	—	—		1.01	0.99	1.00	1.03	0.99	1.00	—	—		—	1.01	1.00
Sphingomyelin	0.505	0.505	0.500	—	—	—	—	—		0.496	—	0.500	—	0.51	0.50

the weight, 92–100 % of hexose and 83–89 % of phosphate. No sulfatide is accounted for in any of their fractions. Taking into consideration the inconsistency between the recovery of total weight and hexose and phosphate, the recoveries are probably of the same order as that obtained here.

Characterization of products

Chromatography of the fractions on thin-layer plates and chemical analysis were used as criteria of the quality of the fractions obtained. Chromatograms on silica gel plates (Fig. 2) show no gross contamination in any of the preparations. The detection reagents used are sensitive to as little as 0.005 μmoles of lipid. Paper chromatography of acid hydrolysates of the cerebroside and sulfatide fractions show that the only carbohydrate present is galactose. In addition no glycerol could be detected on these chromatograms. A relatively complete chemical analysis of significant constituent groups of each compound obtained from sphingolipids prepared by two different extraction procedures is given in Table I. Theoretical values for lignoceryl cerebroside, lignoceryl sulfatide and behenyl sphingomyelin have also been tabulated as a basis of comparison. Both on the basis of the percent weight of the various constituents and the molar ratios of the constituents, all of the compounds prepared by this method are highly pure.

Further efforts to characterize the sulfatide obtained by this method supports recent reports[32,33] that the sulfate is not on the number 6 carbon of the galactose as originally reported by THANNHAUSER *et al.*[34]. Periodate oxidation under the conditions described by CARTER *et al.*[35] failed to show any oxidation of the sulfatide over a 12 h period. Under the same conditions, 2.08 moles of periodate per mole of cerebroside were reduced in 2 h. This observation confirms the periodate oxidation study of YAMAKAWA *et al.*[32] and supports their view that the sulfate is on the number 3 carbon.

Generally in the past preparations of the various sphingolipids have been characterized by analysis of various constituents and/or carbon, hydrogen and nitrogen analyses. Where this type of data has been given[1–3,7–10,15,35] the preparations obtained here compare very favorably. In conclusion this method permits the separation of all three of the major sphingolipids from a mixture in a yield of 92–95 % and of a quality equal or better than that obtained by previously reported methods for preparing one or at the most two of the sphingolipids from a mixture.

ACKNOWLEDGEMENTS

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SUMMARY

Ox brain sphingolipids are separated into cerebroside, sulfatide and sphingomyelin fractions by chromatography on a silicic acid–Hyflo Super-Cel column. Elution is carried out with mixtures of chloroform–methanol–water. Depending on the method by which the sphingolipids are prepared, it is necessary to free the cere-

broside of small amounts of contaminating lipid by chromatography on Florisil. Regardless of the source, the sulfatide and sphingomyelin require further purification. Final purification of the sulfatide is carried out by chromatography on Florisil followed by precipitation from chloroform with acetone. Final purification of sphingomyelin is accomplished by chromatography on alumina. Final yields of 92–95 % of the sphingolipids in a brain sphingolipid preparation are obtained. Chemical analysis of the products indicates purities of the order of 99 %. Choline of sphingomyelin is assayed by a new micro assay based on the formation of choline reineckate.

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CHARACTERIZATION OF POLYNUCLEAR AZA HETEROCYCLIC HYDROCARBONS SEPARATED BY COLUMN AND THIN-LAYER CHROMATOGRAPHY FROM AIR POLLUTION SOURCE PARTICULATES

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INTRODUCTION

Two recently developed thin-layer chromatographic systems give entirely different separation patterns for the polynuclear aza heterocyclic hydrocarbons[1] and thus make possible the separation of aza compounds from complex mixtures. In addition, a column chromatographic system for the separation of basic compounds from complex mixtures makes possible the collection of large partially separated fractions[2]. Extensive characterization work can be done with these large fractions. With the recent development of a procedure for the direct spectrophotofluorometric examination of thin-layer chromatograms[3], it is now possible to characterize the unknowns on the chromatogram without tedious extraction or elution. The analytical procedure has been standardized with coal tar pitch[4].

In the work reported here the separation–characterization procedure is applied extensively to gaseous effluents trapped from various air pollution sources. Examples of the evidence on which the characterizations are based are given.

EXPERIMENTAL

Reagents

Most of the aza heterocyclic hydrocarbons were obtained from Aldrich Chemical Company of Milwaukee or K and K Laboratories, Plainview, Long Island. A few of these compounds were synthesized and purified by literature procedures. 11H-Indeno-(1,2-*b*)quinoline was obtained from Dr. J. SMOLICZ.

Equipment

An Aminco-Bowman spectrophotofluorometer was used with the following settings: sensitivity 50, slit arrangement No. 2, and phototube RCA type 1P21. The instrument was equipped with an Aminco solid-sample accessory to obtain the fluorescence spectra of spots on thin-layer chromatograms. Details for the direct spectrophotometric analysis of aromatic compounds on thin-layer chromatograms were presented in a previous paper[3].

Thin-layer chromatography

Either glass plates less than 0.1 in. thick or plastic sheets ranging from 0.015 to 0.06 in. thick can be used. Ordinary window glass works nicely; a glass cutter must be used to cut out the spots for spectrofluorometric examination. Our best results were obtained with sheets of 0.015 in.-thick vinyl plastic VSA-3310 Clear 31 Matte 06, which are easily cleaned and coated with cellulose or alumina. Following development, spots are readily cut out with scissors. Window glass is less expensive than the glass usually used in thin-layer chromatography, and the plastic sheets are much less expensive. The main difficulty with the plastic material is its solubility in some organic solvents. Although the solvent systems used in this research did not affect the vinyl plastic, the possible effects of solvents should be explored for each plastic material. With the Teflon type of plastic, of course, all types of solvent systems can be used.

Aluminium oxide G and MN-cellulose powder 300 G (Brinkmann Instruments, Long Island) in 250-μ layers were used as the adsorbents.

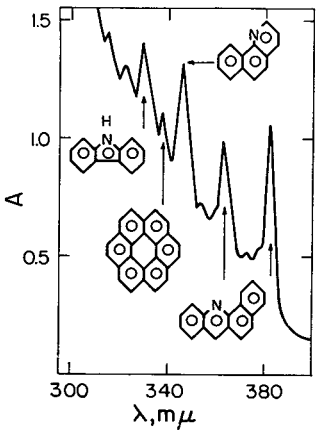
Analytical procedures

The collection of particulates from urban atmospheres polluted by coal-tar-pitch fumes[5] and from the effluent gases emanating from incinerators and the stacks of residential coal-burning furnaces[6] has been described. The particulate matter and other condensed material from about 5 to 20 m^3 of effluent gas or from a 2- to 24-h collection of coal-tar-pitch-polluted atmosphere was Soxhlet-extracted with benzene for about 6 to 8 h. The basic fraction was obtained from about 0.4 to 1 g of the benzene-soluble material from incinerators or stacks or from about 0.1 to 0.4 g of the coal-tar-pitch sample; it was then chromatographed on an alumina column with 100-ml volumes of pentane solutions containing 8, 16, 24, 32, 40, 48 and 56 % ether and 5, 10, 15, 20, 25, 30, 35 and 40 % acetone followed by 100-ml volumes of ether and then methanol[2]. The fractions, consisting of 15-ml volumes of eluent, were separated further on thin-layer plates coated with cellulose. Dimethylformamide–water (35:65) was used as the developer.

In some cases the basic fraction was separated with the thin-layer system of alumina plus pentane–ether (9:1, v:v). In a few instances these steps were followed by extraction of appropriate spots, then by further separation with a thin-layer system of cellulose dimethylformamide–water (35:65).

DISCUSSION

Occasionally after the column chromatographic separation of a benzene-soluble fraction preliminary to analysis for polynuclear aromatic hydrocarbons, the presence of aza heterocyclic hydrocarbons can be ascertained in the absorption spectrum of the benzoperylene or coronene fraction. This phenomenon is shown in Fig. 1 for a column chromatographic subfraction of a benzene-soluble fraction of a coal-tar-pitch-polluted air sample. Bands at 330, 338 and 346 mμ are indicative of carbazole, coronene and benzo(*h*)quinoline, respectively. Bands at 363 and 382 mμ are indicative of benz(*c*)acridine. When this sample was chromatographed on a cellulose thin-layer plate with water–dimethylformamide as the developer, the four main components of this fraction were readily separated (Fig. 2). Direct spectrophoto-



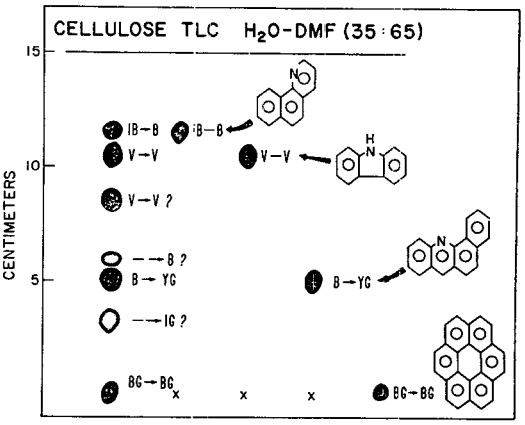


Fig. 1. Ultraviolet absorption spectrum of the carbazole fraction obtained through alumina column chromatographic separation[8] of an airborne particulate sample polluted with coal-tar-pitch fumes.

Fig. 2. Thin-layer chromatogram of the carbazole column chromatographic fraction, benzo(*h*)-quinoline, carbazole, benz(*c*)acridine, and coronene. Fluorescent colors of spots: B = blue; G = green; l = light; V = violet; Y = yellow.

fluorometric examination of the separated spots under neutral, alkaline and acid conditions characterized the four compounds unequivocally.

A few of the aza compounds could be characterized, following column chromatography of the basic fraction, by means of the similarity of the longer wavelength bands of the absorption spectrum of the appropriate fraction in neutral and acid solutions to that of the pure compound. Acridine can be characterized in this fashion.

In another method of characterization used to identify the unknowns unequivocally the basic fraction was thin-layer chromatographed twice, first with the alumina pentane–ether (9:1) system and then with the cellulose/dimethylformamide–water (35:65) system. The separation of the acridine spot is shown in Fig. 3. It must be emphasized that the final green-fluorescing acridine spot still contained other compounds. For these mixtures it can be stated as a general rule that after two separations each spot has a group of compounds with a common R_F value on alumina and another common R_F value on cellulose. Thus, the R_F values by themselves do not give proof of identity. With the high order of selectivity of fluorometric methods, it is a fairly simple matter to characterize the spots on the plate directly, if standards are available. Selectivity can be enhanced considerably through use of quenchofluorometric techniques[7]. The thin-layer chromatogram of the first column chromatographic fraction containing recognizable aza compounds is shown in Fig. 4. Alkylbenzo(*h*)quinoline, benz(*c*)acridine, and alkylbenz(*c*)acridine were characterized by R_F values, direct fluorometric analysis on the plate under neutral and acid conditions, and other fluorometric and phosphorimetric techniques, many of which have been described in a previous paper[3]. In some cases additional information was obtained by extracting the spot from the thin-layer chromatogram, evaporating the solvent, and then obtaining the spectra in appropriate solvent systems. Figs. 5 and 6 present examples of such supplementary characterization techniques. In Fig. 5 the extract of a spot with the same R_F value as acridine is characterized readily through its fluorescence spec-

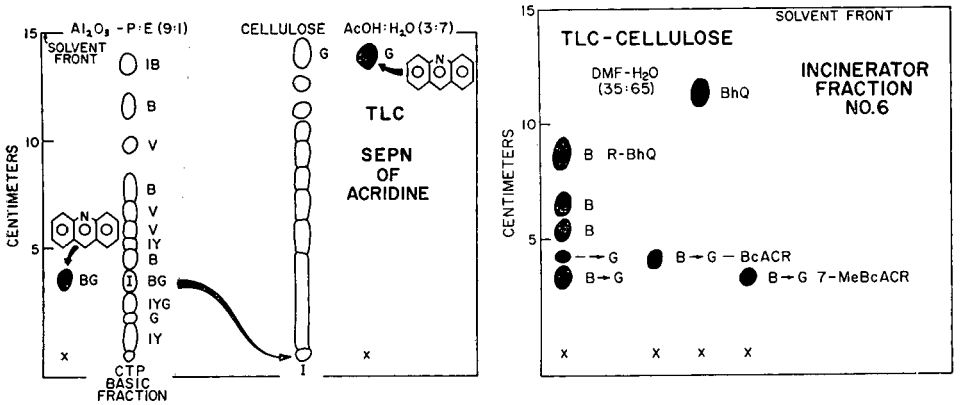


Fig. 3. Thin-layer chromatogram on alumina of the basic fraction of a coal-tar-pitch-polluted air sample followed by separation of the acridine fraction, I, on a cellulose thin-layer chromatogram. For explanation of letters see legend to Fig. 2.

Fig. 4. Thin-layer chromatogram of a column chromatographic fraction obtained from the basic fraction of the particulates collected from incinerator effluents, benzo(*h*)quinoline, benz(*c*)acridine, and 7-methylbenz(*c*)acridine. For explanation of letters see legend to Fig. 2.

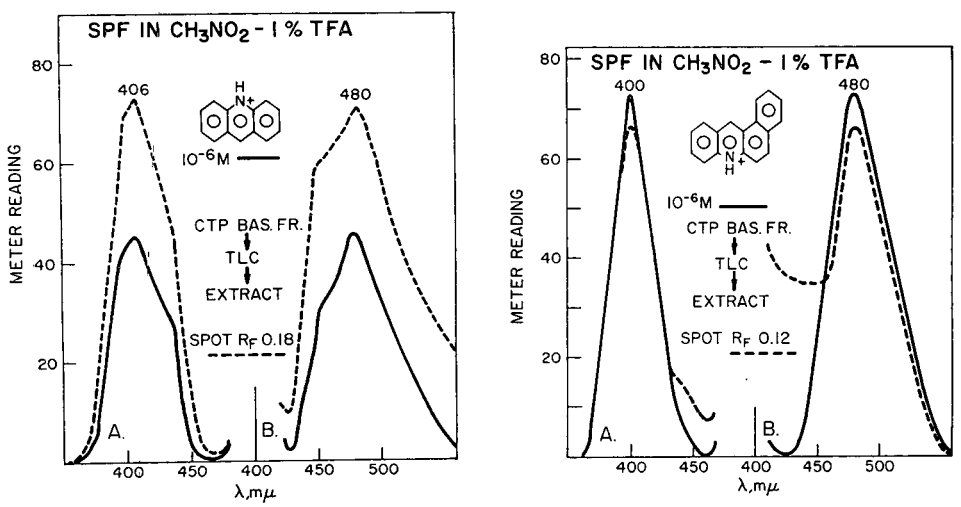


Fig. 5. Fluorescence excitation (A) and emission (B) spectra of acridine (———) and an extract of the spot, R_F 0.18, obtained from an alumina thin-layer chromatogram (pentane–ether, 9:1, v:v) of the basic fraction of a coal-tar-pitch-polluted air sample (– – –). Meter multiplier reading (MM) 0.01.

Fig. 6. Fluorescence excitation (A) and emission (B) spectra of benz(*a*)acridine (———) at MM 0.03 and an extract of the spot, R_F 0.12, obtained from an alumina thin-layer chromatogram (pentane–ether, 9:1, v:v) of the basic fraction of a coal-tar-pitch-polluted air sample (– – –) at MM 0.01.

trum in acidic nitromethane. In alkaline or acidic pentane the spectrum in solution or on the plate was not clear-cut because of the presence of bands from other compounds.

The acidic nitromethane solvent system quenches the fluorescence of practically all aromatic compounds except the aza heterocyclic and fluoranthenic hydrocarbons. The fluorescence spectra of the spot R_F 0.12 in Fig. 6, dissolved in pentane or acidic pentane, show the presence of benzo(*f*)quinoline and unknown compounds in addition to benz(*a*)acridine. A solution of the extracted spot, R_F 0.12, in alkaline nitromethane does not fluoresce. This is in line with the non-fluorescence of benz(*a*)acridine and benzo(*f*)quinoline in this solvent. In acidic nitromethane, however, the fluorescence of the extract of this spot is identical to that of the benz(*a*)acridine salt (Fig. 6).

For some compounds the fluorescence spectra on an adsorbent are different from those in solution. This difference can be used to strengthen the evidence for the characterization of a compound. For example, the emission spectrum of benz(*c*)-acridine shows three sharp bands in solution, while the same spectrum on the plate shows a broad doublet at 400 and 410 mμ. This type of evidence, *e.g.* solution spectra, is shown in Figs. 7 and 8, in which the fluorescence spectrum of an extracted spot with the same R_F value as benz(*c*)acridine is compared to the spectra of that standard in pentane and acidic pentane solutions. This technique has been used to characterize many of the compounds separated by chromatography from various air pollution source effluents.

All compounds were characterized further by their positions in the effluent from an alumina column chromatogram, their R_F values on cellulose thin-layer chromatograms, and their fluorescence excitation and emission spectra (taken

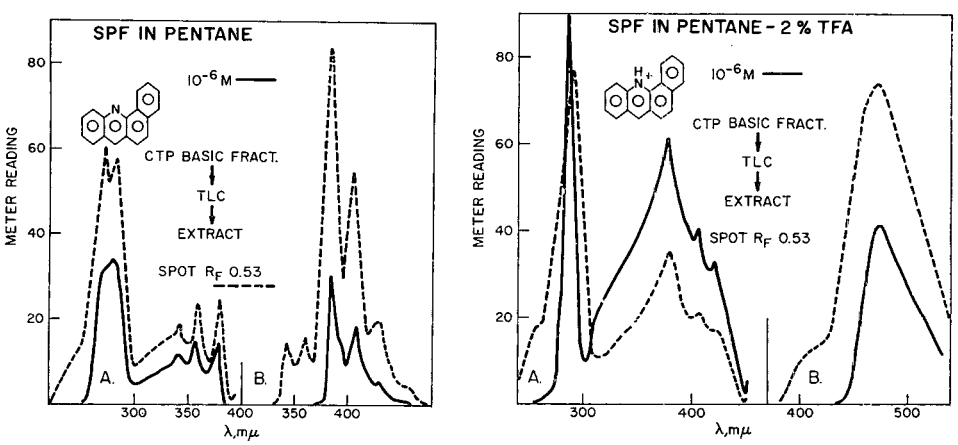


Fig. 7. Fluorescence spectra of benz(*c*)acridine at MM 0.03 (———). A. Excitation spectrum at emission wavelength 384 mμ. B. Emission spectrum at excitation wavelength 280 mμ. An extract of the spot, R_F 0.53, obtained from an alumina thin-layer chromatogram (pentane–ether, 9:1, v:v) of the basic fraction of a coal-tar-pitch-polluted air sample at MM 0.01 (– – –). A. Excitation spectrum at emission wavelength 402 mμ. B. Emission spectrum at excitation wavelength 280 mμ.

Fig. 8. Same fractions as in Fig. 7 but in acidic pentane at MM 0.01. Excitation spectra at emission wavelength 473 mμ and emission spectra at excitation wavelength at 287 mμ.

directly from the chromatogram) in the dry state, wet with ammonium hydroxide, and wet with aqueous trifluoroacetic acid. All these properties of an unknown had to be identical to those of a standard before the characterization was termed unequivocal.

RESULTS

Table I shows the various aza heterocyclic hydrocarbons that have been characterized in coal tar pitch, coal-tar-pitch-polluted air, incinerator effluents from two different petroleum refineries, and the effluent from a domestic coal-burning furnace. Although the R_F evidence indicates that the alkyl group in many of the compounds is a methyl group, positive identification of the alkyl group and its position on the molecule could not be made because of a lack of standards.

The 25 aza heterocyclic hydrocarbons and the 8 polynuclear aromatic hydrocarbons that were extracted into the basic fraction are shown in Figs. 9a and 9b. The analyzed sample contained a large quantity of alkylated polycyclic hydrocarbons, as shown by the few that slipped into the basic fraction. These figures show the type

TABLE I

AZA HETEROCYCLIC HYDROCARBONS FOUND IN AIR POLLUTION SOURCE EFFLUENTS

No.	*Compound**	*Coal-tar-pitch*	*Coal-tar-pitch air sample*	*Pet. refinery I incinerator effluent*	*Pet. refinery II incinerator effluent*	*Coal combustion stack effluent*
1	R_aBenzo(*h*)quinoline	*	*	*	*	*
2	Benzo(*h*)quinoline	*	*	*	*	*
3	R_bBenzo(*h*)quinoline	*	*	*	*	*
4	R_cBenzo(*h*)quinoline	*	*	*	*	*
5	Benz(*c*)acridine	*	*	*	*	*
6	R_aBenz(*c*)acridine	*	*	*	*	*
7	R_bBenz(*c*)acridine	*	*	*	*	*
8	Dibenz(*ah*)acridine	*	*	*	*	*
9	5H-Indeno(1,2-*b*)pyridine ?				*	*
10	11H-Indeno(1,2-*b*)quinoline	*	*	*		*
11	Indenoquinoline I ?					*
12	Indenoquinoline II ?					*
13	Indenoquinoline III ?					*
14	Phenanthridine	*	*	*	*	*
15	R Phenanthridine					*
16	Acridine	*	*	*	*	*
17	Indeno(1,2,3-*ij*)isoquinoline	*	*	*	*	*
18	Benzo(*f*)quinoline	*	*	*	*	*
19	R_aBenzo(*f*)quinoline	*	*			*
20	R_bBenzo(*f*)quinoline	*	*			*
21	Benzo(*lmn*)phenanthridine	*	*			*
22	Benz(*a*)acridine	*	*	*	*	*
23	R_aBenz(*a*)acridine	*	*			*
24	R_bBenz(*a*)acridine	*	*			*
25	Dibenz(*aj*)acridine	*	*	*		*

* R_a, R_b and R_c represent simple alkyl groups differing in position of substitution, size (methyl or ethyl) or number (1 or 2).

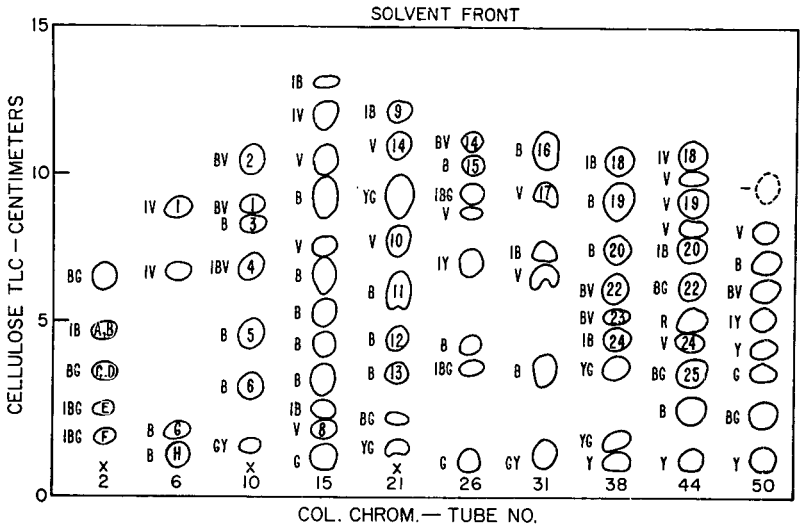


Fig. 9a. Thin-layer chromatograms (cellulose/dimethylformamide–water, 35:65) of the alumina column chromatographic fractions of the basic fraction of particulates collected from the chimney effluent of a domestic coal-burning furnace. The aza heterocyclic compounds, numbers 1 to 25, are identified in Table I. A = anthracene; B = phenanthrene; C = pyrene; D = fluoranthene; E = an alkylpyrene; F = another alkylpyrene; G = benzo(*a*)pyrene; and H = benzo(*k*)fluoranthene.

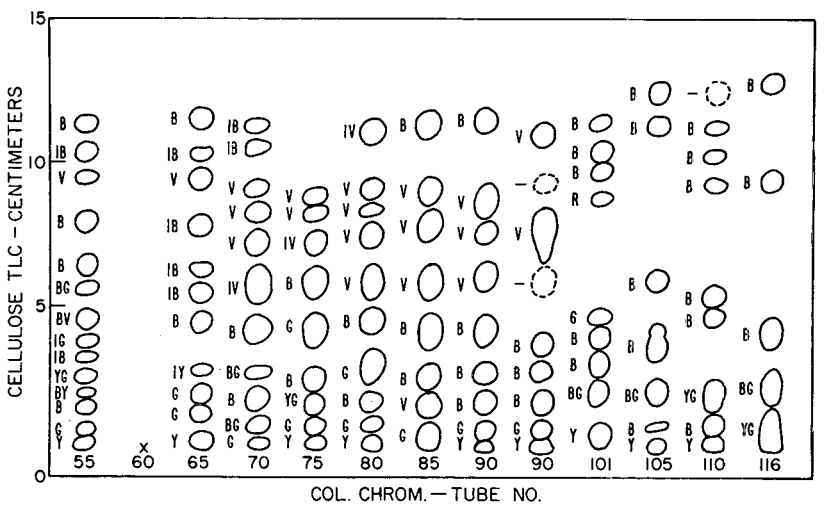


Fig. 9b. Continuation of Fig. 9a.

of separation possible with thin-layer chromatography of about every fifth column chromatographic tube. Each spot contained several compounds, most ofw hich were not identified. Not one identified compound was found in the thin-layer chromatograms in tubes No. 50 through 116. These two figures are a reminder of the vast number of unknown compounds present in the basic fraction of these mixtures.

SUMMARY

By column and thin-layer chromatography approximately 200 spots have been obtained in the analysis of various air pollution sources. With the help of direct spectrophotofluorometric analysis of the spots on the thin-layer chromatograms 25 aza heterocyclic and 8 polynuclear aromatic hydrocarbons were characterized according to the parent ring structure. Eleven of the aza heterocyclic hydrocarbons were identified unequivocally.

The following carcinogens have been found in air pollution source effluents: dibenz(*a h*)acridine and dibenz(*a j*)acridine. In addition, alkyl derivatives of benz(*a*)-acridine and benz(*c*)acridine were obtained, some of which could be carcinogenic.

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THE APPLICATION OF ULTRAVIOLET REFLECTANCE SPECTROSCOPY TO THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

The availability of improved instrumentation and techniques has resulted in an increased interest in the employment of spectral reflectance as a means of chemical analysis[1–5]. More recently a reliable method has been devised for the determination of the components of dye mixtures resolved on thin-layer plates by spectral reflectance[6], and a similar procedure has been applied successfully to the analysis of amino acids separated on chromatoplates[7,8]. Thus far, however, recourse to this technique has been restricted to the visible portion of the spectrum, and, hence, to the analysis of substances which are either colored or which can be converted to a colored species by the use of a suitable chromogenic reagent.

Because of the large number of substances having characteristic ultraviolet spectra that may be employed in their analysis, it was felt that the application of ultraviolet reflectance spectroscopy to thin-layer chromatography would prove to be an invaluable analytical tool, especially for those dealing with the pharmaceutical and biochemical sciences. The feasibility of this approach was ascertained by employing it to determine the composition of mixtures of aspirin (acetyl salicylic acid) and salicylic acid which had been separated on silica gel plates. This particular system was selected for study not only because the spectra of the two compounds were suited for the purpose at hand, but also because it presented no difficulties in the matter of locating the compounds at the conclusion of the resolution. Both appeared as yellowish-brown spots when the plates were dried.

EXPERIMENTAL

The aspirin and salicylic acid, which were of Merck U.S.P. and Chase U.S.P. purity, respectively, were dried over sulfuric acid for 24 h before use. 0.10 *M* stock solutions of the compounds in chloroform were used to prepare the dilution series employed in this study. Solutions were applied as spots by means of 5 and 10 μl micropipets. The 20 × 5 × 0.35 cm plates were coated with adsorbent by distributing a Merck silica gel G–water mixture (6:12) with a glass rod which rested on two thicknesses of masking tape affixed to the ends of the plates. This technique gave a uniform coating 0.5–0.7 mm thick. After the plates had been allowed to "set" for 20 min at room temperature, they were dried at 110° for 1 h and stored in a desiccator.

After spotting, the plates were dried by letting them stand at room temperature

for 10 min and then developed by the ascending technique with the use of a hexane–glacial acetic acid–chloroform mixture (85:15:10). R_F values observed for salicylic acid and aspirin were 0.35 and 0.2 respectively. Although in preparative work the spots may be removed from the chromatoplates directly after their development, in the analysis of the mixtures the plates were dried at a temperature of 90° in an oven for 2 h.

The reflectance spectra of the compounds were obtained with a Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment. All other measurements were made with a similarly equipped Beckman Model DU Spectrophotometer. The cells used to hold both the analytical samples and the reference material consisted of a circular quartz plate, which had a diameter of 22 mm, superimposed on a 40 × 40 × 1 mm piece of white paperboard. The quartz disk was held in place by means of a 40 × 40 × 3 mm plastic plate which was affixed to the backing paper with two pieces of masking tape. A circular window, 19 mm in diameter, in the upper surface of the plate opened into a concentric circular well, 24 mm in diameter, that was deep enough to accommodate the quartz disk. These data are presented schematically in Fig. 1, where a sketch of the assembled cell is also given.

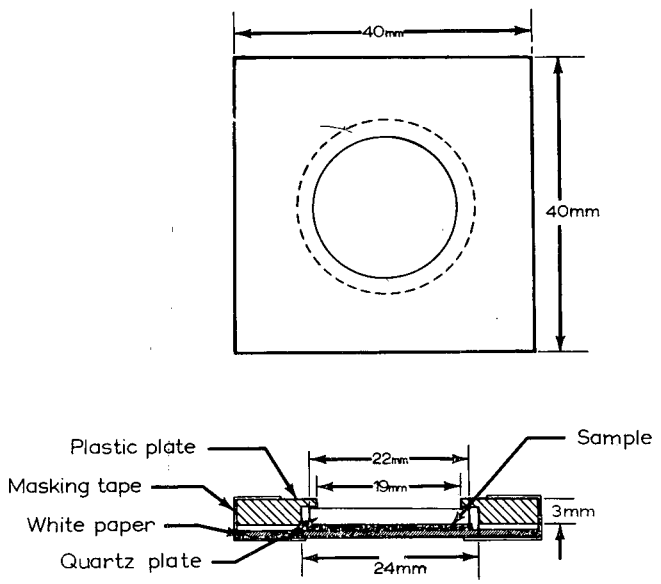

Fig. 1. Dimensions of cell elements and sketch of assembled cell.

The 70 mg which comprised the analytical samples were weighed to the nearest ±0.2 mg and ground in a small agate mortar for two periods of 20 sec each to insure homogeneity and uniform particle size. The material was then introduced into the cell and carefully compressed between the quartz disk and the paperboard by rotating the former until a thin layer having an approximate diameter of 22 mm and an approximate thickness of 20 mg/cm² was obtained. The reference standard in all cases consisted of silica gel adsorbent from the plate under investigation.

RESULTS AND DISCUSSION

Optimum conditions for drying of chromatoplates

The procedure employed in the drying of the chromatoplates following their development was decided upon after a study of the effect of drying times and temperatures upon the stabilities of salicylic acid and aspirin adsorbed on silica gel. When samples containing 5 μmoles were prepared according to the procedure given above and then subjected to different drying conditions, the reflectance spectra presented in Figs. 2 and 3 were obtained. As may be seen in Fig. 2, the general form

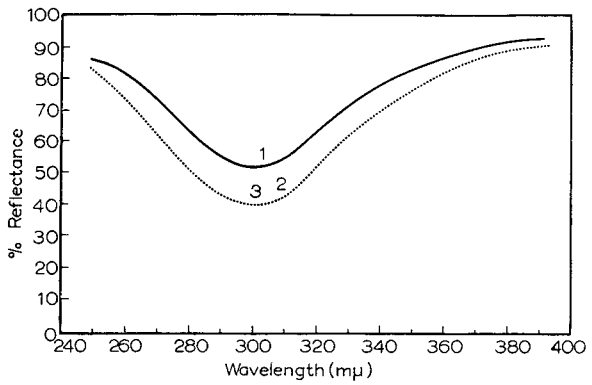

Fig. 2. Reflectance spectra obtained for salicylic acid adsorbed on silica gel G at indicated intervals after spotting: (1) After 15 min at room temperature; (2) after an additional hour at 90°; (3) after still another hour at 90°.

of the spectrum for salicylic acid, which exhibited an absorption maximum at 302 mμ, was unaffected by variations in drying time and temperature. The spectrum for aspirin, on the other hand, underwent substantial changes. A consideration of Fig. 3, which shows the aspirin spectra, discloses that the position of the absorption maximum

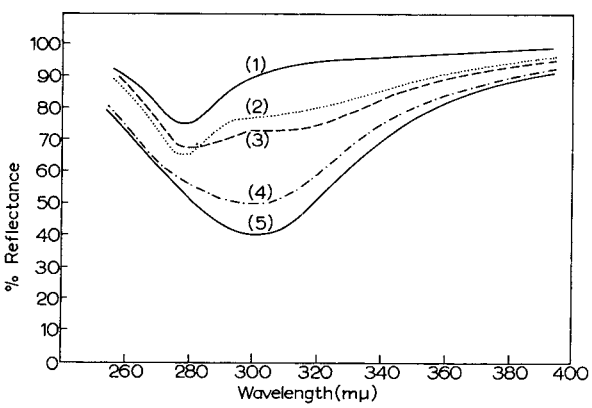

Fig. 3. Reflectance spectra obtained for aspirin adsorbed on silica gel G at indicated intervals after spotting: (1) After 15 min at room temperature; (2) after 2 more hours at room temperature; (3) after an additional 5 min at 90°; (4) after an additional 10 min at 90°; (5) after still another 10 min at 90°.

varied between 278 mμ and 302 mμ. Since aspirin is known to form salicylic acid upon hydrolysis and since the reflectance spectrum of the decomposition product of aspirin is identical with that obtained for salicylic acid, this shift of the absorption maximum may be ascribed to the conversion of aspirin into salicylic acid.

By making use of this conversion, it was possible to devise a more sensitive method for the determination of aspirin, since the decomposition product absorbs more strongly than aspirin itself. The experimental conditions under which the conversion was induced were selected after studying the effect of varying drying times and temperatures upon the % reflectance at 302 mμ—the absorption maximum for both salicylic acid and the decomposition product of aspirin—of 1 μmole of salicylic acid and 1 μmole of aspirin adsorbed on silica gel. A drying period of 2 h at 90° was found to be most convenient and expeditious.

Preparation of analytical samples

The precautions that had to be observed during the sample preparation were essentially the same as those suggested by FRODYMA AND FREI[6,7,9] for reflectance in the visible region of the spectrum. The optimum sample thickness for measurements with ultraviolet radiation was arrived at by determining the per cent reflectance at 302 mμ of samples consisting of 3 μmoles of salicylic acid per 70 mg of adsorbent but of different thicknesses. Constant reflectance readings were obtained when the sample thickness exceeded 16 mg/cm^2 with white backing paper, and 22 mg/cm^2 with brown plastic backing material. For values less than these, a gradual increase in per cent reflectance accompanied each increase in sample thickness. It was decided to employ a thickness of 18 mg/cm^2 in conjunction with white paperboard as this would allow for possible losses of material that might occur during the packing of the analytical sample in the cell. Inasmuch as the samples used in this study had a diameter of 22 mm, this thickness corresponded to an optimum sample size of 70 mg.

As was also the case with work in the visible region, the factors limiting the precision of the technique were associated with the packing of the sample in the cell. When the per cent reflectance at 302 mμ of samples containing from 0.5 to 2.0 μmoles of salicylic acid per 70 mg of silica gel was determined, the differences observed between readings for the same sample which had been repacked were of the same order as those obtained for replicate samples and in no case exceeded 0.6 reflectance units. Although uniformity of sample thickness was not as critical as in the visible, probably because the thickness was 2 mg/cm^2 in excess of the minimum needed for constancy of reflectance, the extent of the pressure applied when the material was compressed between the quartz disk and the backing paper did affect the reproducibility somewhat. When the pressure exerted was excessive, a decrease in the apparent reflectance of a salicylic acid–silica gel sample amounting to 1 to 2 % was noted. For pressures less than excessive, any derivations observed were within instrumental limitations.

The determination of aspirin and salicylic acid

Calibration curves were prepared for both aspirin and salicylic acid by applying different concentrations of the two compounds to thin-layer plates which were then developed, dried, and prepared for the measurement of reflectance according to the method outlined in the experimental section. In both cases the absorbance at 302 mμ

was determined and then plotted as a function of the square root of the concentration. Three of the plots obtained with salicylic acid and two obtained with aspirin are presented in Fig. 4. Although the data graphed there were gathered at different times and with the use of different dilution series of the two compounds, the five plots are remarkably similar and almost coincide in the concentration range below 3.0 μmoles. This suggests the possibility of employing a single calibration curve for the determination of both substances. The close coincidence of the plots obtained for a single compound also indicates that it is unnecessary to run a set of standards alongside each unknown in routine analysis.

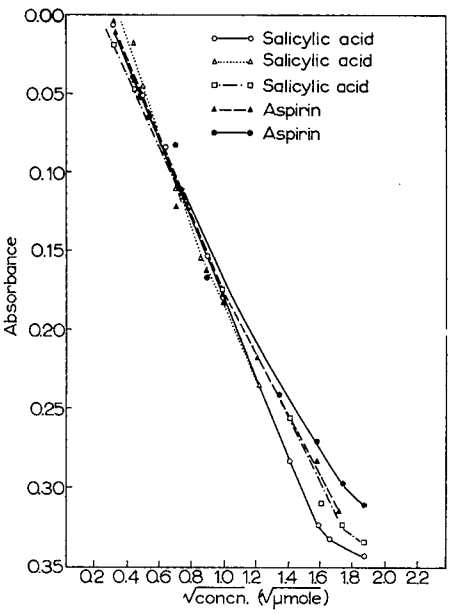

Fig. 4. Absorbance at 302 mμ of salicylic acid (trial 1, ○—○; trial 2, △···△; trial 3, □·—·□) and aspirin (trial 1, ●---●; trial 2, ▲– – –▲) adsorbed on silica gel as a function of the square root of concentration.

The precision that can be anticipated when aspirin and salicylic acid are resolved on chromatoplates and then analyzed by means of ultraviolet reflectance spectroscopy was determined by running through the procedure outlined above with four one μmole replicates of each compound. This standard deviation of the reflectance readings obtained for these different samples was found to be 0.37 reflectance units for salicylic acid and 0.47 reflectance units for aspirin. There was no difference between any pair of readings which exceeded 0.8 reflectance units with salicylic acid and 1.1 reflectance units with aspirin.

CONCLUSIONS

The components of salicylic acid–aspirin mixtures resolved on thin-layer plates can be determined by carrying out ultraviolet spectral reflectance measurements on spots removed from the dried plates and packed in an appropriate cell. Calibration

curves obtained for the two compounds by means of this procedure were so similar and so reproducible that for routine analyses a single curve that needed to be checked only periodically sufficed for the analysis of both salicylic acid and aspirin. The method provided quantitative data having a standard deviation amounting to 0.37 reflectance units for salicylic acid and 0.47 reflectance units for aspirin. A linear relationship between absorbance and the square root of the concentration was observed with spots containing up to 3.0 μmoles of either compound.

This technique should be applicable to the analysis of a large number of complex organic compounds which absorb in the ultraviolet and which are resolvable on thin-layer plates. Especially promising is the employment of this technique in conjunction with another involving the incorporation of fluorescent and phosphorescent materials in the adsorbent layer[10–13].

SUMMARY

A method which can be employed for both preparative and analytical purposes was devised for the resolution of salicylic acid–aspirin mixtures on chromatoplates. Ultraviolet reflectance spectroscopy was then used to determine the amounts of resolved material by carrying out measurements on spots removed from the dried plates and packed in a suitable cell.

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THIN-LAYER CHROMATOGRAPHY OF 2-SUBSTITUTED FLUORENES

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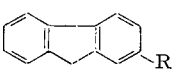
The separation of aromatic hydrocarbons by thin-layer chromatography on silica gel[1], alumina[1,2] and acetylated cellulose[3–5] has been reported many times. In the present investigation, the influence of 2-substituents on the migration of fluorene in thin layers was studied. Four types of adsorbents and six developing solvents have been used. In addition, the five isomeric monomethylfluorenes were compared, and as expected, it was practically impossible to distinguish between them by thin-layer chromatography.

Table I shows that acetylated cellulose is much less effective than silica gel or alumina in distinguishing between the variously substituted fluorenes. One has the impression that cellulose acetate "senses" only the aromatic ring system; indeed, it has been useful in separating various polycyclic aromatic hydrocarbons[6]. On alumina and silica, on the other hand, it is the functional groups which determine the adsorbability of the compound. The sequence of elution of the substituents obtained from our results is practically identical with that established by BROCKMANN AND VOLPERS[7] in other cases; the nitrile group is similar to the carbomethoxy radical, and

TABLE I

R_F VALUES OBTAINED BY TLC OF 2-SUBSTITUTED FLUORENES 

R	*Silica gel G*						*Alumina G*	*Acetylated cellulose*	*MN 300G/Ac.*
	*1**	*2*	*3*	*4*	*5*	*6*			
—H	0.61	0.78	0.95	0.98	0.95	0.97	0.58	0.74	0.85
—OH	—	0.02	0.14	0.15	0.12	0.32	—	0.68	0.84
$-NH_2$	0.02	0.04	0.16	0.23	0.14	0.31	0.02	0.30	0.70
$-OOCCH_3$	0.02	0.04	0.34	0.45	0.28	0.48	0.02	0.54	0.81
$-COOCH_3$	0.03	0.09	0.48	0.60	0.39	0.65	0.03	0.60	0.85
—CN	0.04	0.10	0.61	0.74	0.53	0.73	0.04	0.56	0.78
$-NO_2$	0.15	0.22	0.71	0.85	0.59	0.90	0.10	0.50	0.75
$-OCH_3$	0.16	0.36	0.80	0.91	0.84	0.92	0.20	0.64	0.82
—F	0.60	0.80	0.91	0.93	0.92	0.94	0.56	0.72	0.84
—Cl	0.64	0.84	0.93	0.95	0.94	0.96	0.57	0.74	0.85
—Br	0.65	0.84	0.93	0.95	0.95	0.97	0.58	0.76	0.86
—I	0.65	0.85	0.94	0.94	0.95	0.97	0.58	0.76	0.86
$-CH_3$	0.62	0.81	0.96	0.95	0.96	0.97	0.61	0.76	0.87

* The numbers refer to the solvent systems used (see p. 528).

the various halogens behave practically identically, the fluorine atom perhaps occupying a somewhat separate position.

EXPERIMENTAL

Chromatography was carried out on glass plates (20 × 20 cm) coated with a layer of 0.25 mm thickness of silica gel G, alumina G or acetylated cellulose.

Preparation of silica gel G and alumina G plates

The suspension required for five plates was prepared by vigorously shaking 30 g of silica gel G and 60 ml of water, or 50 g of alumina G and 100 ml of water, in a stoppered conical flask for 30 sec; it was then immediately transferred to a Desaga thin-layer applicator which was drawn across the plates to give a uniform layer. The plates were allowed to dry at room temperature for 30 min and then heated for 30 min at 120°. After cooling, they were kept in a vacuum desiccator.

Preparation of acetylated cellulose plates

Whatman's cellulose chromatography powder was acetylated by SPOTSWOOD's method[8].

The suspension required for 5 plates was prepared by shaking 35 g of acetylated cellulose and 60 ml of methanol. It was then spread on the plates with a Desaga thin-layer applicator to give a 0.5 mm layer. The plates were dried in air.

Preparation of MN300G/Ac. plates (Macherey and Nagel Co., Düren, Rhineland, Germany)

A mixture of 20 g MN300G/Ac. (acetylated cellulose containing plaster of Paris) with 100 ml of methanol and 10 ml of distilled water was vigorously shaken for 1 min. The suspension was then spread on the plates with a Desaga applicator (thickness 0.5 mm) and the plates were dried for 10 min at 60°.

Materials

Fluorene, m.p. 116° (from ethanol)*, and 2-hydroxyfluorene, m.p. 170° (from aqueous alcohol) were commercial samples. The following fluorenes were available from the collection in the Department: 1-methyl- (from methanol), m.p. 85°; 2-methyl- (from ethanol), m.p. 104°; 3-methyl- (from ethanol), m.p. 88°; 9-methyl- (from petroleum ether), m.p. 46°; 2-fluoro- (from ethanol), m.p. 100°; 2-methoxy- (from aqueous ethanol), m.p. 113°, and 2-acetoxy- (from ethanol), m.p. 132°.

The other substances were prepared by known methods: 4-methylfluorene[12] (from methanol), m.p. 70°; 2-chlorofluorene[13] (from ethanol), m.p. 98°; 2-bromofluorene[14] (from ethanol), m.p. 110°; 2-iodofluorene[15] (from ethanol), m.p. 128°; 2-cyanofluorene[16] (from petroleum ether), m.p. 105°; 2-aminofluorene[17] (from aqueous ethanol), m.p. 127°; 2-nitrofluorene[17] (from acetic acid), m.p. 158°.

For the preparation of 2-carbomethoxyfluorene (from ethanol), m.p. 120°, fluorenone-2-carboxylic acid[18] was reduced by means of sodium amalgam to fluorene-2-carboxylic acid by a similar procedure to that used by BERGMANN AND IKAN[19] for the 1-isomer. The acid was then esterified by Fischer's method.

* The solvents in parentheses were those used for crystallization.

Development

The samples were dissolved in chloroform for the silica gel and the alumina plates, and in methanol for the acetylated cellulose plates and then spotted on the plates with micropipettes along a line 2 cm from the edge of the plate.

The following solvent systems were used for silica gel:

(1) *n*-Heptane
(2) Carbon tetrachloride
(3) Carbon tetrachloride–ethyl acetate, 19:1
(4) Carbon tetrachloride–ethyl alcohol, 19.8:0.2
(5) Benzene
(6) Benzene–ethyl acetate, 19:1.

For alumina G the solvent *n*-heptane was used, a mixture of methanol–ether–water (4:4:1) was used for the acetylated cellulose plates[3,4] and a mixture of ethanol–toluene–water (17:4:4) for MN 300G/Ac[5].

The solvents were allowed to travel up the plate to a height of 15 cm. The plates were then taken out of the tanks, and dried in air.

Detection

The dried plates were observed under U.V. light and also sprayed with the following reagents:

(a) 1,3,5-Trinitrobenzene (T.N.B.), 10 % in acetone.
(b) Tetracyanoethylene (T.C.E.), 10 % in benzene[9,10].
(c) Antimony pentachloride, 10 % in chloroform.
(d) Formaldehyde, 2 ml (37 %) in 100 ml of conc. sulfuric acid[11].

TABLE II

DETECTION OF 2-SUBSTITUTED FLUORENES ON THIN LAYERS*

2-R-Fluorene	*U.V.*		*Reagents***			
	Acetylated cellulose	*Silica gel G*	*T.N.B.*	*T.C.E.*	*$SbCl_5$*	*HCHO/ H_2SO_4*
—H	W	V	Y	DV	LG	BGr
—OH	V	V	O	BGr	Br	G
—NH_2	V	V	Br	Y	DG	LV
—$OOCCH_3$	GY	LGr	LY	P	DY	Y
—$COOCH_3$	V	V	Y	Br	LP	Gr
—CN	Y	V	—	LP	DY	Y
—NO_2	LY	Y	Y	LY	Y	LY
—OCH_3	P	V	LO	BGr	LGr	B
—F	Y	V	LY	P	DY	BGr
—Cl	Y	V	Y	G	DY	BGr
—Br	LG	V	Y	G	DY	BGr
—I	LG	Br	Y	G	DY	BGr
—CH_3	Y	V	Y	V	B	BGr

* B = blue, Br = brown, D = dark, G = grey, Gr = green, L = light, O = orange, P = pink, V = violet, W = white, Y = yellow.
** On silica gel G layers.

RESULTS

The R_F values for the 2-substituted fluorenes are summarized in Table I, and the colors observed in Table II. It will be seen from Table I that the following mixtures are easily separated: (a) 2-nitro- and 2-amino-fluorene, (b) 2-hydroxy- and 2-acetoxy-fluorene, and (c) 2-hydroxy- and 2-methoxy-fluorene.

The results obtained for the monomethyl isomers are shown in Table III.

TABLE III

R_F VALUES OBTAINED BY TLC OF METHYLFLUORENES

Methyl-fluorenes (substituted position)	*Silica gel G*						*Alumina G*	*Acetylated cellulose*	*MN 300G/Ac.*
	*1**	*2*	*3*	*4*	*5*	*6*			
1-	0.60	0.80	0.98	0.96	0.97	0.97	0.60	0.76	0.86
2-	0.62	0.81	0.96	0.95	0.96	0.97	0.61	0.76	0.87
3-	0.62	0.81	0.98	0.96	0.96	0.98	0.60	0.77	0.87
4-	0.60	0.82	0.95	0.94	0.95	0.97	0.61	0.73	0.86
9-	0.62	0.81	0.95	0.94	0.95	0.98	0.62	0.72	0.84

* The numbers refer to the solvent systems used (see p. 528).

SUMMARY

The possibility of separating 2-substituted fluorenes by thin-layer chromatography has been studied, using silica gel G, alumina G and acetylated cellulose plates. The isomeric monomethylfluorenes cannot be separated by this method.

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THIN-FILM ELECTROPHORESIS*

PART II. FREEZE-DRYING OF ELECTROPHEROGRAMS

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Surprise has recently been expressed that the use of thin-film materials prepared for chromatography, for example, alumina, kieselguhr and silica gel, has not become at all common for electrophoretic separations[1]. In fact, the separations so far described appear to be restricted to amines and amino acids[2–4], naphthols[4], phenols and phenol carboxylic acids[5], dyes[4,6,7], and certain inorganic systems[8,9].

The limited applications of the method do not have regard to the advantages claimed over paper electrophoresis, namely, improved resolution, increased speed and facile removal of substances for quantitative estimation. Reasons for this can be related to the fact that, as with chromatography on thin-film materials, there are factors which affect reproducibility differently from similar methods on paper. One such factor concerns the drying of thin-film electropherograms, a feature not recorded in Part I[7] since migration distances were measured on the wet films immediately electrophoresis was completed.

On drying electropherograms in the usual way at 100–110°, it has been observed that, apart from becoming more diffuse, zones frequently migrate haphazardly, thus leading to inconsistent readings of electrophoretic migration distances. This haphazard zone-migration during the drying stage is particularly serious when colourless materials having a high solubility in the electrolyte are studied, since the location of the zones with the appropriate detection reagent will not necessarily bear any relation to their position at the end of the electrophoresis.

To overcome the serious disadvantage of zone-migration that occurs by using conventional methods of drying, freeze-drying of electropherograms is proposed and recommended.

Experiments illustrating the distinct advantages of freeze-drying of electropherograms have been carried out for electrophoretic separations of mixtures of food colours, whose positions may be noted at all stages during the electrophoresis and drying stages.

EXPERIMENTAL AND RESULTS

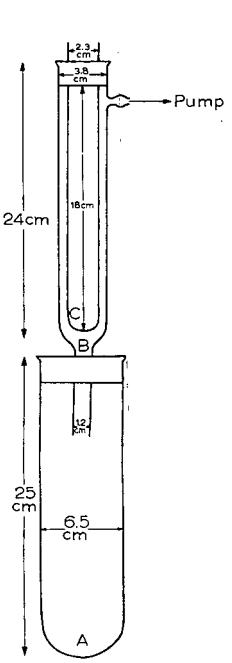
Design of apparatus for freeze-drying

Two sets of apparatus were assembled for the freeze-drying experiments. The first, shown in Fig. 1, is suitable for drying electropherogram plates of 20 cm long

* For Part I of this series, see ref. 7.

and 5 cm wide, while the second, shown in Fig. 2, is suitable for drying 20 × 20 cm plates. The second apparatus may, therefore, be used for the full-size plates now accepted as normal in thin-film chromatography and electrophoresis, and which are suitable also for two-dimensional work.

The apparatus shown in Fig. 1 is composed of a tube, A, into which is fitted a condenser, B, incorporating a cold finger, C, and vacuum line. In the larger-scale apparatus (Fig. 2), the condenser, B, is now fitted to a vacuum desiccator of internal diameter 30 cm. The remaining dimensions of both sets of apparatus are given in the diagrams.



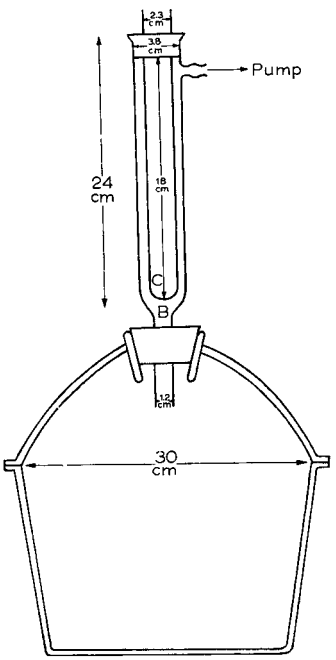


Fig. 1. Apparatus for freeze-drying electropherogram plates of 20 cm × 5 cm.

Fig. 2. Apparatus for freeze-drying electropherogram plates of 20 cm × 20 cm.

Operation of the freeze-drying apparatus

Immediately the electrophoresis is terminated, the glass plate supporting the thin-film electropherogram is placed on a bed of powdered solid carbon dioxide where the water absorbed in the electropherogram freezes in about 10–20 sec.

The glass plate with the thin-film electropherogram is placed in tube A of the apparatus shown in Fig. 1 or, for larger plates, in the desiccator compartment of the apparatus shown in Fig. 2. In either case, the cold finger incorporated in the condenser, B, has been previously charged with an acetone–solid carbon dioxide mixture. The condenser is then fitted to the tube, A, or to the vacuum desiccator in the alternative apparatus.

The apparatus is then evacuated and ice is soon observed on the condensing surface of the cold finger, C. The freeze-drying is continued until the electropherogram

is free from water, a process which normally takes between 20 and 30 min as indicated by a pronounced whitening of the film.

On releasing the vacuum, the electropherogram is ready for development with the appropriate detecting reagents in the usual way.

Appraisal of the freeze-drying process for drying electropherograms

To assess the advantages of the freeze-drying process for drying electropherograms over the conventional methods of drying at elevated temperatures, mixtures of food colours were taken and subjected to electrophoresis in duplicate on alumina G (Merck), silica gel G (Merck) and kieselguhr (Shandon) using the procedure previously described[7] with a 0.05 *M* aqueous solution of borax as electrolyte.

In each case, the positions of the zones at the end of the electrophoresis stage were noted. Of each duplicate electrophoresis run, one was subjected to drying at 105° and the other dried by the freeze-drying process.

Zone-migration was absent in *all* cases where the electropherograms were freeze-dried. On the other hand, where the electropherograms were dried at 105°, haphazard migration of zones was observed in all instances, the extent of zone-migration being up to 15 mm for kieselguhr, up to 5 mm for silica gel G and up to 3 mm for alumina G. In extreme cases, zones have been observed to interchange positions on drying at 105°.

Freeze-drying experiments using liquid nitrogen as the freezing medium

Attempts to freeze the water in the electropherograms by dipping in liquid nitrogen resulted in the frequent fracturing of the glass plates. The use of perspex plates overcame this problem, but the quality of the thin-layer film deteriorated owing to cracking and flaking.

DISCUSSION

As indicated above, zone-migration on thin-film electropherograms during drying at elevated temperatures can lead to serious errors. This is particularly true when the thin-film support has a low adsorptive capacity as is the case with kieselguhr. Similar behaviour can also be expected where the material being subjected to electrophoresis has a low adsorption affinity.

Where, however, the adsorptive capacity of the thin-film support is greater, for example, silica gel and alumina, or where the material being subjected to electrophoresis has a high adsorption affinity, the migration of zones during drying is considerably less in magnitude, as confirmed by the observations noted above for silica gel and alumina. High adsorptive capacity also explains why the phenomenon of zone-migration is not very apparent in the drying of paper electropherograms.

However, due to their adsorptive capacity, paper and thin-film supports of alumina cause tailing and widening of zones during electrophoresis[7]. Both these characteristics give rise to poor resolution. On the other hand, tailing is greatly diminished with supports of low adsorptive capacity, such as kieselguhr[7] and cellulose acetate[10]. It is clear, therefore, that the use of such low adsorption materials has distinct advantages, providing the attendant difficulties of zone-migration during the drying stage can be eliminated as in the freeze-drying method described above.

A further feature of freeze-drying is that the zones remain narrow and discrete instead of diffusing to cause widening as is frequently the case on drying at elevated temperatures.

The poor reproducibility of R_F values in thin-film chromatography has recently been featured by several authors[11–15]. While, in the light of the above, it might be suggested that zone-migration could be one of the responsible factors, preliminary experiments, even with slow drying developing solvents, indicate that zone-migration is of the order of a millimeter or less[16]. However, further study with developing solvents of varying dielectric constant is required before this factor can be finally dismissed as irrelevant.

Of course, while zone-migration during drying is completely eliminated by freeze-drying, it is possible that a certain amount of migration may take place on spraying dry electropherograms (or chromatograms) with detecting reagents. Experiments to establish whether or not migration does occur under these circumstances are now in hand.

CONCLUSION

Zone-migration during the drying stage is clearly an operative factor accounting for the limited application of electrophoresis on thin-film materials prepared for chromatography. The difficulties and problems brought about by the phenomenon can be entirely resolved by freeze-drying the electropherograms instead of drying at elevated temperatures.

In addition, freeze-drying is recommended as a convenient and simple method of detecting zone-migration whenever the phenomenon is suspected.

Further work is now proceeding on the electrophoretic separation on thin films of materials of biochemical interest.

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SUMMARY

Freeze-drying as a means of overcoming the zone-migration that occurs during drying of thin-film electropherograms at elevated temperatures is described and recommended.

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CHROMATOGRAPHY OF POLYAMINES

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This study of the chromatographic separation of polyethylene polyamines was undertaken when distillation failed to separate a complex mixture formed by the reaction of allyl bromide with triethylenetetramine. In the separations reported here the unsubstituted polyamines, ethylenediamine, diethylenetriamine, triethylenetetramine and tetraethylenepentamine, were used as model substances in artificial mixtures. There are apparently no previous reports on the separation of mixtures of these polyamines by chromatography, and the high polarity of the compounds makes them an interesting group for chromatographic studies.

PURIFICATION OF THE POLYAMINES

The polyethylene polyamines mentioned above are all commercially available, and are obtained by fractional distillation of the reaction products of ethylene dichloride and ammonia. Although distillation separates the linear polyamines from each other, cyclic compounds are present too, and gas–liquid chromatography has revealed 7 major components in the commercial tetraethylenepentamine[1]. In earlier studies the pentamine and tetramine were often purified by fractional distillation, but further purification may have occurred in preparation of crystalline derivatives for analysis. Chromatography of the free bases is necessary to prove their purity.

In this laboratory the polyamines were distilled through a TODD column[2], and the middle fraction of each polyamine was examined by gas–liquid chromatography. Ethylenediamine was obtained pure, and diethylenetriamine contained only a trace of impurity, which was removed by a single crystallization of the sulphate. The tetramine and pentamine still showed six components, and several recrystallizations of their sulphates[1] were necessary for complete purification.

GAS–LIQUID CHROMATOGRAPHY

When attempts were made to analyse mixtures of the pure polyamines by gas–liquid chromatography, the system used for testing the purity of individual polyamines[1] was found to be unsatisfactory because of pronounced tailing. Many techniques have been proposed in the literature to overcome the tailing and irreversible adsorption of polar compounds. Of these, treatment of the packing *in situ* with hexamethyldisilazane[3], treatment with potassium hydroxide[4], and the use of "Teflon" powder as a support[5] were tried. The last technique gave the best results with the

polyethylene polyamines, but even here it was necessary to use larger amounts of sample than usual in order to avoid loss of minor components by irreversible adsorption.

The use of derivatives of amines has been recommended to avoid these difficulties[6], but the derivatives have lower vapour pressures than the free amines, and tetraethylenepentamine itself has a very low vapour pressure. For ethylenediamine and diethylenetriamine the Schiff's bases made by the use of acetone as a solvent gave satisfactory results, but for triethylenetetramine severe tailing occurred. The tetramine is known to react with 3 molecules of benzaldehyde[7] or salicylaldehyde[8], and a similar reaction may occur to some extent with acetone.

The triamine, tetramine and pentamine were chromatographed as free bases at a column temperature of 155° (Fig. 1). A lower temperature was required to separate ethylenediamine from the solvent used (ethanol), but thin-layer chromatography afforded a more convenient method for the detection of ethylenediamine.

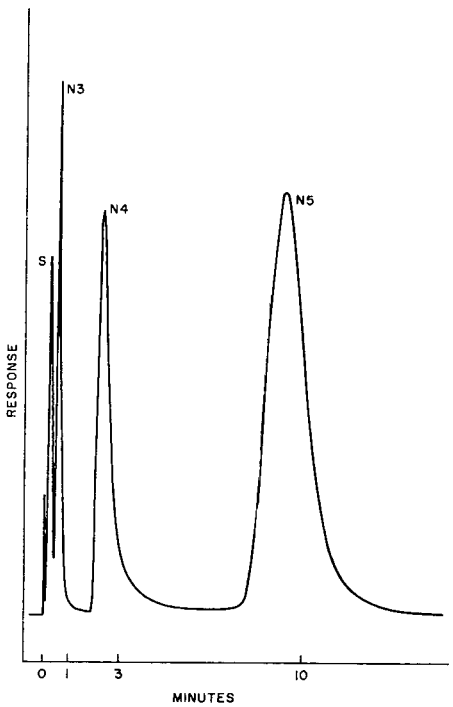

Fig. 1. Gas chromatogram of diethylenetriamine (N3), triethylenetetramine (N4) and tetraethylenepentamine (N5). The solvent (S) is ethanol.

THIN-LAYER CHROMATOGRAPHY

Some allyl derivatives of polyamines could be separated by paper chromatography with a solvent system reported previously for allylamines[9], but the unsubstituted polyamines had very low R_F values, and an impracticably long time was required for their separation. Since all attempts to increase the R_F values by altering the solvent led to streaking, thin-layer chromatography was investigated.

The polyamines were very strongly adsorbed even in systems designed for strongly adsorbed amines[10]. After many experiments with different adsorbents and solvents it was found that silica gel thin-layers and development with 25 % ammonia solution–ethanol (2:1) gave the best results. The R_F values are shown in Table I. These were measured from the front of the spot, because this was clearly defined although slight tailing occurred.

TABLE I

R_F VALUES OF POLYAMINES ON THIN-LAYER CHROMATOGRAMS*

	Compound			
	$C_2H_8N_2$	$C_4H_{13}N_3$	$C_6H_{18}N_4$	$C_8H_{23}N_5$
R_F at 10°	0.44	0.41	0.32	0.27

* These values refer to silica gel thin layers and the solvent system aqueous (25 %) ammonia solution–ethanol (2:1).

When a mixture of polyamines was chromatographed, ethylenediamine (which gave a black spot with iodine vapour) was clearly distinguished from the rest (which gave a brown colour), but the higher polyamines moved together as an unresolved streak. However, impurities in any one of the higher polyamines could be detected with this system, which compared favourably with gas chromatography for this purpose. In the same way, substituted polyamines, which showed less tailing and higher R_F values than the original polyamines, could be separated from each other. For the separation of polyamines on a larger scale, ligand-exchange chromatography and ion-exchange chromatography were considered.

LIGAND-EXCHANGE CHROMATOGRAPHY

The use of cation-exchange resins in the nickel or copper form for the separation of ligands was first discussed by HELFFERICH[11]. He showed that 1,3-diaminopropan-2-ol could be absorbed from a very dilute solution containing excess ammonia, and could be eluted from the nickel form of the resin by concentrated ammonia solution. Although many uses were indicated for this technique, and it seems promising for the chromatography of ligands, it has serious limitations. One difficulty is that the metal may be displaced from the resin by the eluent or by one of the ligands to be separated. Thus mono-ethanolamine was found to form an uncharged complex with copper, and this caused a large proportion of the copper to be displaced from a sulphonic acid resin[12]. Another difficulty is that ammonia, which is the most convenient eluent for the separation of amines, may not be able to displace strongly bound ligands. Thus WALTON AND LATTERELL[13] found that 1,2-diamines were very difficult to displace from the nickel form of Dowex 50X8 with an ammonia solution.

Some values for the stability constants of the 1:1 complexes of the polyethylene polyamines with copper, nickel and silver are collected in Table II. These have been selected from a compilation[14] of published values. The stability constants of the complexes of the different ligands with copper or nickel are very different from one another, whereas the acid dissociation constants of the ligands are similar. This

would indicate that ligand-exchange is better than ion-exchange for the separation of these polyamines. However, the values of the stability constants of the polyamines are very high in comparison with that of ammonia, and therefore elution is difficult. It should be noted that 1,3-diaminopropan-2-ol, which HELFFERICH successfully eluted with concentrated ammonia[11], is an exceptional diamine in that it forms a nickel complex with a stability constant one hundred times smaller than that of the ethylenediamine–nickel complex.

TABLE II

STABILITY CONSTANTS OF POLYAMINE COMPLEXES[14]

Ligand (L)	*Log K_1 (Cu^{2+})*	*Log K_1 (Ni^{2+})*	*Log K_1 (Ag^{+})*	*pK of HL^{+}*
Ammonia	4.0	2.8	3.4	9.61
Ethylenediamine	10.7	7.5	4.7	10.17
Diethylenetriamine	16.0	10.7	6.1	9.94
Triethylenetetramine	20.4	14.0	7.7	9.92
1,3-Diaminopropan-2-ol	9.7	5.6	5.8	9.68

In a series of experiments with the resin Amberlite IRC-50 the polyamines were found to be strongly absorbed by the copper and nickel forms, and none were eluted by concentrated ammonia from the copper form. Ethylenediamine was eluted in a wide band from the nickel form, but the triamine, tetramine and pentamine could not be recovered without stripping the metal from the resin. The chelating resin Dowex A-1 gave similar results. When the silver form of Amberlite IRC-50 was used, appreciable amounts of silver were eluted, as had been found with a sulphonic acid resin[13], and there was no separation of the eluted polyamines.

ION-EXCHANGE CHROMATOGRAPHY

Mixtures of the polyamines were absorbed on various cation-exchange resins, and elution was attempted with hydrochloric acid or ammonia solutions. Ammonia eluted the polyamines efficiently, but there was no separation on a carboxylic acid resin. On sulphonic acid resins ethylenediamine could be obtained pure in the first elution peak, but the other polyamines were eluted together in the second peak (Fig. 2). This behaviour is not surprising in view of the similarity of the pK values for diethylenetriamine and triethylenetetramine given in Table II.

In acidic solutions where the polyamines are fully protonated, separations should be possible, because the cation of highest valency would be most strongly held. In fact these cations were so strongly held that only ethylenediamine could be eluted satisfactorily with molar hydrochloric acid. For this experiment the highly swollen resin Bio-rad AG-50X2 was used, because the affinity of the cations for the resin decreases as the swelling of the latter increases. Attempts to separate the polyamines by elution of their copper complexes with potassium hydroxide solution, and by absorbing them on the copper form of a resin before removing the copper with acid and then eluting with ammonia, were all unsuccessful.

Separation of a mixture of the triamine, tetramine and pentamine was finally achieved on a column of Bio-rad AG-50X8 by developing the chromatogram with sufficient 2 *M* hydrochloric acid, and then eluting the polyamines with dilute ammonia solution. The elution curve (Fig. 3) shows that the separation was not quantitative,

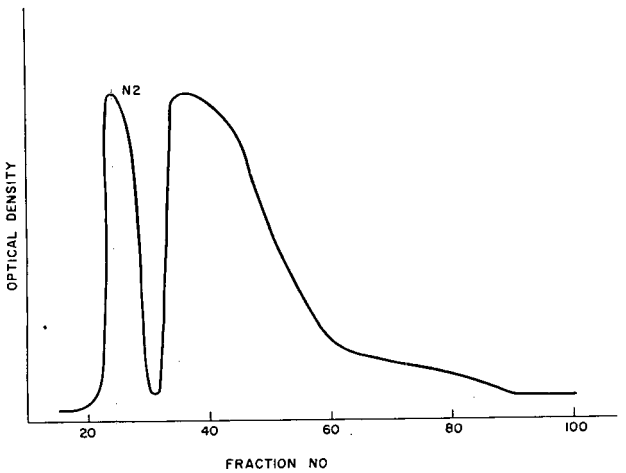

Fig. 2. Ion-exchange chromatography of ethylenediamine (N2). The second peak is a mixture.

but fractions taken from a suitable portion of each peak were shown to be pure by gas–liquid chromatography. A longer column or longer development with acid might improve this result, but tailing is likely to prevent a quantitative separation. The substituted polyamines are less strongly held and can be separated more easily.

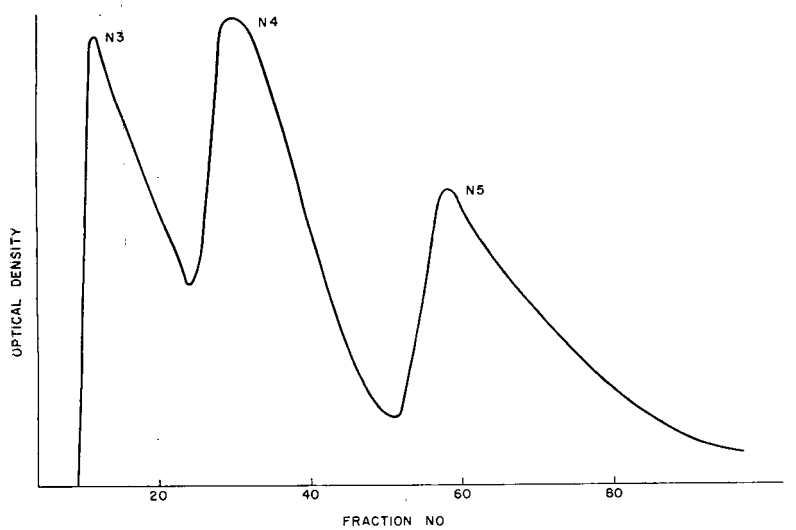

Fig. 3. Ion-exchange chromatography of diethylenetriamine (N3), triethylenetetramine (N4) and tetraethylenepentamine (N5).

EXPERIMENTAL

Gas–liquid chromatography

The instrument used was the Barber-Colman Model 10 with ionization detector. The 3 ft., 0.25 in. diameter, column was packed with "Haloport F" (F and M Scientific Corp.) coated with 5 % SE-30 silicone rubber gum (G.E.C.). The temperature was 155°, and the flow rate 60 ml/min of argon at 40 p.s.i. Samples (0.5 μl) of a 5–10 % solution of the polyamine in ethanol were injected.

Thin-layer chromatography

Layers of silica gel on microscope slides[15] were used, and the chromatograms were developed with 25 % ammonia solution–ethanol (2:1). The dry slides were placed in a small jar containing a few iodine crystals to reveal the spots.

Ion-exchange chromatography

A 30 cm column, 1.2 cm diameter, of Bio-rad AG-50X8 was used. The resin was washed with water and loaded with a dilute solution containing 0.1 ml of each polyamine. A 2 *M* solution of hydrochloric acid (600 ml) was allowed to flow through the column at 0.5–1.0 ml/min. The resin was then washed with water, and the polyamines were eluted with 6 % ammonia solution (500 ml), the eluate being collected in 5 ml fractions.

In this procedure ethylenediamine would be eluted by the hydrochloric acid. To recover ethylenediamine from a mixture the development with acid was omitted, and during elution with ammonia the ethylenediamine came off first in a sharp peak (Fig. 2).

The absorption at 210 mμ was used in plotting elution curves, and the fractions were examined by thin-layer chromatography, or by gas chromatography after the water and ammonia had been removed.

SUMMARY

The separation of polyethylene polyamines by various forms of chromatography has been studied. Ethylenediamine is easily separated from the higher polyamines by thin-layer chromatography and by ion-exchange chromatography. Diethylenetriamine, triethylenetetramine and tetraethylenepentamine can be separated by gas–liquid chromatography on Teflon powder coated with 5 % SE-30, and on a larger scale by ion-exchange chromatography. The latter technique involves absorption on AG-50X8, development with 2 *M* hydrochloric acid, and elution with 6 % ammonia solution.

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DISTRIBUTION OF WEAK BASES BETWEEN ANHYDROUS ACETONITRILE AND CROSSLINKED POLYSTYRENESULFONIC ACIDS. SEPARATION OF BASICITY, CAPACITY AND SELECTIVITY EFFECTS. NONAQUEOUS ION EXCHANGE CHROMATOGRAPHY

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INTRODUCTION

Little is known about the behavior of ion exchange resins under strictly anhydrous conditions. In principle it should be possible to fractionate mixtures of weak bases, B ($pK_a^{BH^+} < 0$), on anhydrous, strong acid exchangers in contact with non-basic or poorly basic solvents. Fig. 1 shows that such chromatographic separations are indeed realizable. Instead of developing this aspect of the subject, we have preferred to concentrate on quantitative separation and evaluation of the contributing factors by precise measurement of the equilibrium distribution of base between solution and resin phases. This has been carried out over a wide concentration range as a function of base and resin structure and of the solvent. The first article of this series[1] described experiments of this type in aqueous dioxane as solvent.

Three factors may be anticipated to determine the extent of sorption of base in such systems. The first is the resin capacity, which cannot be taken as the true capacity measured in water, C_0, since the resin is generally swollen to a much smaller extent (or indeed not measurably swollen) in non-water-like solvents than in water[1,2]. We will designate by C the *effective capacity* displayed by the resin toward a given base in a given solvent. The second factor is the *base strength*. If the observed distribution equilibrium, $B_{solution} \rightleftharpoons B_{resin}$, is identified with reaction (1), as the demonstrated absence of base sorption other than *via* salt formation allows us to do in the

$$B + res^-H^+ \underset{}{\overset{K}{\rightleftharpoons}} res^-, BH^+ \qquad (1)$$

solvents selected for study, this is separable into two parts, (2) and (3), whose sum is (1)**.

$$B + H^+ \overset{K_B}{\rightleftharpoons} BH^+ \qquad (2)$$

$$BH^+ + res^-H^+ \overset{K_{sel}}{\rightleftharpoons} res^-BH^+ + H^+ \qquad (3)$$

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** Solvent has been omitted from the equations for the sake of clarity. By res^-H^+ is meant $ArSO_2OH\cdots S$, $ArSO_2O^-\cdots HS^+$ or $ArSO_2O^- + SH^+$ (S = solvent), depending on the actual state of ionization and dissociation of the sulfonic acid groups, which is poorly known[3].

The contribution of base strength is made through (2) and is measured by the equilibrium constant K_B. The third factor is the *selectivity* of the resin for BH^+ (relative to H^+) measured by the selectivity coefficient K_{sel} for the ion exchange equilibrium (3).

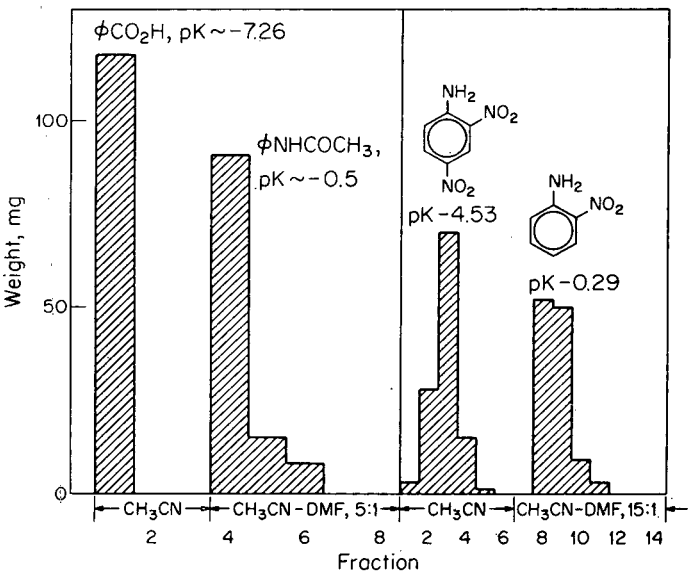


Fig. 1. Chromatography of weak base mixtures on anhydrous Bio-Rad AG50W-X8, minus 400 mesh. $pK_a^{BH^+}$ values taken from E. M. ARNETT, *Prog. Phys. Org. Chem.*, 1 (1963) 223. Percentage recovery of bases: benzoic acid, 99%; acetanilide, 97%; 2,4-dinitroaniline, 100%; *o*-nitroaniline, 98%.

By appropriate analysis of the data for sorption of base over a wide concentration range, it is possible to determine both C and $K = K_B K_{sel}$ precisely. Separation of the relative contributions of K_B and K_{sel} has been approached by study of series of bases in which first K_{sel}, then K_B is held constant. In previous studies[2] of res$^-H^+$–base equilibria in non-aqueous media, no attempt was made to separate the factors determining the distribution of base into the resin.

EXPERIMENTAL

Swelling measurements were made as previously[1] reported. Melting points are corrected, boiling points uncorrected. Infrared spectra were recorded on a Beckman IR-4 instrument.

Materials

Reagent grade acetonitrile was distilled twice from phosphorus pentoxide, the second time employing a 2-ft., glass helices-packed column and collecting a constant boiling center cut. For a typical batch this possessed b.p. 80.5° (740 mm), n_D^{25} 1.3413 [reported[4] b.p. 80.8° (740 mm); n_D^{25} 1.3416]. Karl Fischer titration revealed less than 0.008% water. The material was homogeneous to gas–liquid chromatography.

γ-Phenylbutyranilide appears not to have been previously described. It was prepared from the acid by the method of SHRINER, FUSON AND CURTIN[5] using chloroform as solvent, and purified by chromatography on alumina and four crystallizations from aqueous ethanol, m.p. 96.4–97.4°.

Analysis. Calculated for $C_{16}H_{17}NO$: C, 80.30; H, 7.16; N, 5.85. Found: C, 80.60, 80.62; H, 7.07, 7.14; N, 6.00, 5.93.

Lauranilide was prepared from lauroyl chloride and aniline in benzene, m.p. 78.2–79.3° after three crystallizations from aqueous ethanol. The remaining bases were commercially available and were crystallized at least three times from appropriate solvents; melting points were in agreement with literature values. All bases were sublimed at *ca.* 10^{-4} mm shortly before use.

The resins used were purified grades of Dowex 50W, obtained from Bio-Rad Laboratories, Richmond, Calif., as AG 50W. These were first air-dried to about 20 % water, then weighed into 1 ml ampules (*ca.* 0.1–0.15 g) and dried to constant weight at 75–80° and a final pressure of *ca.* 10^{-4} mm (6–8 h). The ampules were sealed under vacuum, recovered and weighed. Potentiometric titration of the dry material in water containing potassium chloride gave the following values for the capacity, C_0, of the 8 % divinylbenzene resin: 5.20, 5.21, 5.23 mequiv. H^+/g resin. The vacuum dried resin contained no more than 0.042 mmole of water per mequiv. of $-SO_3H$ groups, as determined by Karl Fischer titration in which 1 g of resin under 10 ml of methanol was titrated potentiometrically. This method gave reasonably good results for resin samples containing known amounts of added water (*e.g.*, added, 13.1 mg; found, 12.8 mg), and the water content of the air-dried resin determined by this method agreed well with that obtained by weight loss on exhaustive drying: found (drying), 21.5 %; found (Karl Fischer), 20.5, 21.0 %.

Desulfonation of Bio-Rad AG 50W-X8

A mixture of 3.11 g of the air-dried resin and 15 ml of 12 *N* sulfuric acid was rocked in a sealed tube (evacuated to 10 mm) at 170° for 59 h. After washing the product with 2 l of deionized water, exhaustive drying and titration indicated a capacity of 3.50 mequiv. H^+/g resin. This partially desulfonated product was converted to material with a capacity of 1.48 mequiv. H^+/g resin by repetition of the above procedure at 185°.

Equilibrium measurements

An evacuated ampule containing a weighed resin sample was placed in an 18 × 150 mm heavy-wall Pyrex test tube. After the neck of the latter had been constricted, it was flushed with dry nitrogen at 50–60° and the neck plugged with glass wool. Freshly prepared solutions of the base were weighed into the tube, solvent and solution transfers being made with hypodermic syringes from stoppled containers, and the tube was capped with an efficient drying tube, cooled to —78° and sealed. On warming to room temperature, the inner, resin-containing ampule was broken by shaking, and the tube was rocked in a constant temperature bath at 25.0° ± 0.1° until equilibrium was attained. The tip of the tube was cracked and the solution was rapidly filtered by means of a hypodermic needle and syringe equipped with a Swinny filter adapter containing 1.3 cm circles of glass fiber and Whatman No. 40 filter papers and a Teflon gasket. The filtrate was immediately analysed using a Cary

Model 11 or Model 14 spectrophotometer to measure absorbance at a visible or ultraviolet absorption maximum, applying a small correction for absorption due to soluble impurities in the resin. Extinction coefficients used were averages of at least two determinations. Beer's law was verified for several of the bases. The amount of base taken into the resin phase was computed from the initial and final concentrations. Attainment of equilibrium was assured for each system by extending the equilibration time until invariance of the base sorption was attained, then employing equilibration times well in excess of this requirement. In the previous work[1,6] this method was shown to be adequate by checking against results obtained by approaching equilibrium from both directions.

Control experiments

Soluble acid in the resin amounted to no more than $3.8 \cdot 10^{-3}$ mequiv./g resin as determined by equilibrating pure acetonitrile with resin, filtering and titrating the filtrate potentiometrically.

Possible sorption of free base, independent of salt formation, was excluded by equilibrating (1) various of the bases with a salt form of the resin, and (2) the less basic acetophenone with the hydrogen form, finding in each case no base entering the resin phase. The data are given in Table I.

TABLE I

ABSENCE OF SORPTION OF BASES FROM ACETONITRILE IN THE ABSENCE OF RESIN PHASE SALT FORMATION

Base	*Concentration*[a] *(molal)*	*Resin state*	*Resin wt. (g)*	*log I/I_0*[b] *initial*	*log I/I_0 final*
Acetanilide	$4.1 \cdot 10^{-3}$	Na	0.1178	1.68	1.68
p-Nitro-acetanilide	$2.3 \cdot 10^{-4}$	Na	0.0991	2.61	2.61
Acetophenone	$6.5 \cdot 10^{-2}$	H	0.1183	2.55	2.54

[a] Volume of solution = 10.0 ml.
[b] Optical density at 282, 320 and 317 mμ, respectively.

Recovery of unchanged acetanilide from the resin phase was demonstrated by applying 0.314 g of the amide in 25 ml of acetonitrile to a column of 5.74 g of dry resin. Acetonitrile eluted no solid, but elution with 75 ml of acetonitrile–dimethylformamide (15:1) removed 0.300 g (96 %) of acetanilide, m.p. and mixed m.p. with the starting material 114.5–116°, after sublimation in 96 % recovery.

A study of the infrared absorption spectrum of benzamide in acetonitrile showed that the concentration dependence of the 3355, 3155, 1661 and 1626 cm^{-1} bands of the solid disappeared before concentrations as low as 0.01 molal were reached. The amide I frequency, 1686 cm^{-1}, observed at this concentration is characteristic of the monomer[7]. Thus, as expected from the behavior of carboxylic acids in acetonitrile[8], the concentration range employed in the distribution measurements is free of complications due to association.

Chromatography

After drying at 80° in vacuum, 10 g of the X-4, minus 400 mesh resin was transferred, still under vacuum, to a dry box. The resin was slurried with dry acetonitrile and poured into a column so arranged that on removal from the dry box the resin was never in contact with the atmosphere. The sample solution was introduced through a rubber stopple with a hypodermic syringe, and solvent additions were made from reservoirs by means of dry nitrogen pressure.

RESULTS

The correct formulation of the equilibrium constant K for reaction (1) is a formidable problem. First, it is known[6,9] that acid–base equilibria in acetonitrile often involve one or more extra molecules of acid whose function is anion stabilization via hydrogen bridging:

$$B + nHA \rightleftharpoons BH^+, A(HA)_{n-1}^-$$

with $n = 2$ or 3. Second, both res$^-$H(solvent)$^+$ and res$^-$BH$^+$ are undoubtedly associated electrolytes. Finally, there is no good way to correct for salt effects in the resin phase. The previous results in 80 % aqueous dioxane[1], however, showed that a classical mass law expression held accurately in that solvent without apparent disturbance from the latter two difficulties. In the present system too, it was found that the data could be analysed well within the experimental precision, allowing only for simultaneous reactions of 1:1 and 2:1 stoichiometry*:

$$B + \text{res}^-H^+ \overset{K_1}{\rightleftharpoons} \text{res}^-BH^+ \tag{4}$$

$$B + 2\text{res}^-, H^+ \overset{K_2}{\rightleftharpoons} (\text{res}^-, H^+)_2 \cdot B \tag{5}$$

$$K_1 = \frac{X^r_{\text{res}^-BH^+}}{X^r_{\text{res}^-H^+}} \cdot \frac{1}{X_B} \qquad K_2 = \frac{X^r_{(\text{res}^-H^+)_2 \cdot B}}{(X^r_{\text{res}^-H^+})^2} \cdot \frac{1}{X_B} \tag{6}$$

Here X_B is the base concentration in the solution phase (mole fraction units), and X^r denotes mole fractions in the resin phase. Setting R = weight of resin (g), C = effective capacity (mequiv./g), A_{obs} = measured number of mmoles of base sorbed, A_1 and A_2 = number of mmoles of res$^-$BH$^+$ and (res$^-$H$^+$)$_2$·B formed respectively, and $x = 1 + 1/KX_B$ and finally substituting into (6) and solving simultaneously one finds:

$$A_{obs} = A_1 + A_2 \qquad A_1 = [C^2R^2/(4K_2X_B(x-1)^2 + x^2)]^{1/2}$$
$$A_2 = (CR - xA_1)/2 \tag{7}$$

* The general form of the observed m_B *vs.* Rm_B/S plots could alternatively be produced by assuming a small dissociation constant, K_D, for res$^-$H$^+$. Attempts at exact fitting to a three-parameter (K_D, K_B, C) expression showed, however, that a single value of K_D was incapable of accommodating the data for different bases, while for acetanilide the variance for the best fit was about an order of magnitude greater than that for the K_1, K_2, C model actually used. Introduction of a second association constant for res$^-$BH$^+$ to give a four-parameter expression was considered unjustified.

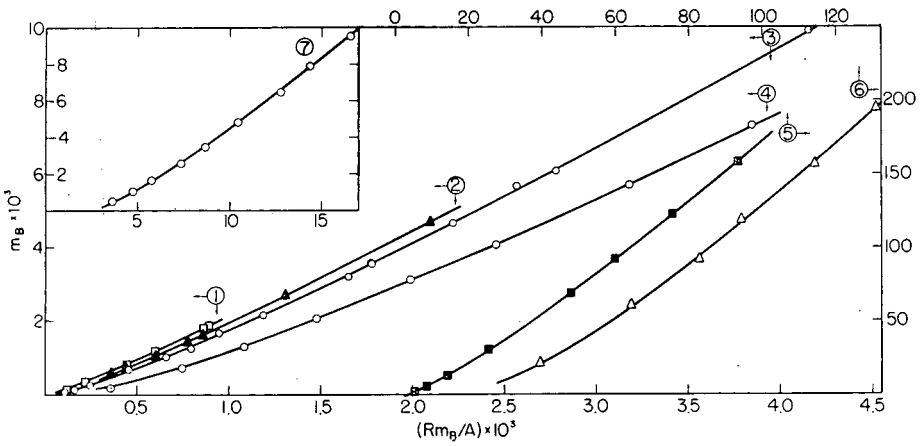


Fig. 2. Distribution of substituted acetanilides and of *o*-nitroaniline between anhydrous acetonitrile and crosslinked polystyrenesulfonic acid at 25.0°: (1) *p*-methoxyacetanilide; (2) *p*-methylacetanilide; (3) acetanilide; (4) *p*-chloroacetanilide; (5) *o*-nitroaniline (ordinate and abscissa are $m_B \times 10^5$ and $Rm_B/A \times 10^5$); (6) *m*-nitroacetanilide (ordinate and abscissa are $m_B \times 10^4$ and $Rm_B/A \times 10^4$); (7) acetanilide reacting with desulfonated resin; arrows indicate the appropriate scales; curves are calculated from Eqn. (7) using the constants given in Table II.

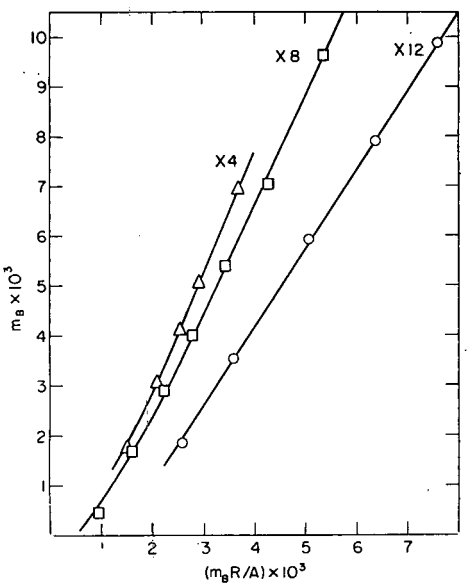


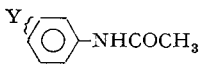
Fig. 3. Distribution of lauranilide between anhydrous acetonitrile and polystyrenesulfonic acids of various crosslinkings, plotted as in Fig. 2.

Fitting of the data to (7) was accomplished by an iterative least squares method using an IBM 7090 computer. Results are shown in Figs. 2, 3 and 5 in the form of $X_B R/A$ *vs.* X_B plots which are linear when reaction (5) is negligible[1]. The equilibrium constants, K_1 and K_2, and effective capacities, C, thus determined are summarized in Tables II, III and V; the estimated precision of these quantities is $\pm 10\,\%$ for K_1 and K_2, 5 % for C.

DISCUSSION

Basicity dependence

The *m*- and *p*-substituted acetanilides



of Table II constitute a reaction series in which a remote substituent, Y, has been varied to produce a spectrum of basicities without change in the functional group undergoing protonation and with only minor change in the overall size and character of the molecule. Within such a series K_{sel} should be essentially constant and the observed variation of K_1 should be solely due to the variation in basicity. Since pK_a^{BH+} values are not available for these bases, comparison has been made in Fig. 4

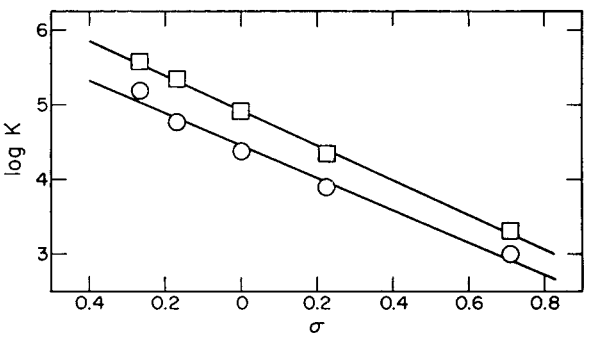


Fig. 4. Equilibrium constants for Eqns. (4) and (5) *vs.* HAMMETT σ for *m*- and *p*-substituted acetanilides. □, K_2 values; ○, K_1 values.

with the HAMMETT σ values which are expected to correlate with pK_a^{BH+} with high precision in such a series[10]. For both K_1 and K_2 the correlation is good*, the least squares equations being:

$$\log K_1 = 4.46 - 2.14\,\sigma$$

$$\log K_2 = 4.93 - 2.33\,\sigma$$

This indicates not only that K_{sel} is indeed constant within the reaction series but also that our analysis of the data gives K values possessing the chemical significance attributed to them in eqns. (4) and (5).

* The correlation coefficients for K_1 and K_2 are 0.992 and 0.999. The precision of fit is well within the experimental uncertainty of the K values; that for K_2 must be regarded as fortuitously good.

TABLE II

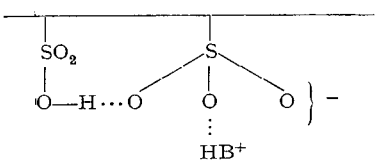
EQUILIBRIUM CONSTANTS AND EFFECTIVE CAPACITIES FOR THE REACTION OF SUBSTITUTED ACETANILIDES AND SOME OTHER BASES WITH HYDROGEN RESINATE[a] IN ACETONITRILE AT 25.0°

Base	$K_1 \times 10^{-3}$	$K_2 \times 10^{-3}$	C (*mequiv./g*)
p-Methoxyacetanilide	153	373	2.53
p-Methylacetanilide	57.2	221	2.85
Acetanilide	23.8	81.8	3.07
p-Chloroacetanilide	7.70	22.6	3.12
m-Nitroacetanilide	1.01	2.03	3.12
Acetanilide[b]	3.97	4.90	1.01
o-Nitroaniline	83.6	172	2.29
Benzamide	52.6	7.80	3.50

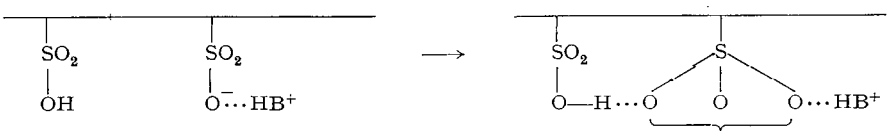
[a] Bio-Rad AG50W-X8, 200–400 mesh.
[b] The desulfonated resin, $C_0 = 1.48$, described in the experimental section was used.

K_2 values

The product of reaction (5) can be represented as:



In a linear polymer, interaction of this type should be highly favorable. This situation finds a kinetic analog, for example, in the participation of adjacent carboxylate groups in the alkaline hydrolysis of substituted carbophenoxy groups attached to a polyacrylate chain, where a considerable rate enhancement is observed[11]. The introduction of cross-links, as in the present system, is expected to restrict the conformational mobility of the polymer, reducing the number of attainable orientations favorable to reaction (5) and decreasing K_2 relative to K_1. This is illustrated in Table III where K_2/K_1, which measures the process:



(8)

is seen to decrease drastically with increasing cross-linking, such that reaction (5) is no longer detectable for the 12 % divinylbenzene resin. The average value of K_2/K_1 for the substituted acetanilides, 3.0, can be compared with that reported[9] (~ 1700) for sulfuric acid reacting with indicator bases in acetonitrile solution. X_B *vs.* RX_B/S curves calculated allowing reaction (5) only for adjacent $-SO_3H$ moieties were not appreciably different from the simpler model. Increase in the size of the base (Tables III, V) appears to decrease K_2/K_1, presumably by imposing further steric restrictions on interaction with the second acid moiety.

TABLE III

DISTRIBUTION OF BENZAMIDE AND LAURANILIDE BETWEEN ACETONITRILE AND RESINS OF VARIOUS CROSSLINKING

Resin[a]		*Benzamide*				*Lauranilide*			
X[b]	C_0 (*mequiv. per g*)	$K_1 \times 10^{-3}$	$K_2 \times 10^{-3}$	C	K_2/K_1	$K_1 \times 10^{-3}$	$K_2 \times 10^{-3}$	C	K_2/K_1
4	5.26	32.2	173	4.54	5.38	6.73	8.40	2.88	1.25
8	5.21	52.6	78.0	3.50	1.48	11.9	4.70	2.25	0.395
12	4.98	32.0	~ 0	2.99	~ 0	11.4	~ 0	1.58	~ 0

[a] Bio-Rad AG50W, 200–400 mesh.
[b] Nominal crosslinking, % DVB.

This model has the corollary that K_2/K_1 should diminish for a resin in which the average distance between sulfonic acid groups is increased. Such a material was prepared by partial desulfonation[12] of the original resin to a capacity of 1.48 mequiv./g (28 % of the initial capacity), and its reaction with acetanilide was studied (Table II, Fig. 2). The ratio K_2/K_1 drops by about a factor of three. It is not expected that K_2/K_1 would become negligible except in the limit of extremely small concentration of $-SO_3H$ groups on the matrix, since examination of molecular models suggests that interaction with the first, second or third sulfonate moiety down the chain should be possible.

The slightly larger sensitivity to basicity (ρ value -2.33 *vs.* -2.14) of K_2 relative to K_1 corresponds to slight increase in K_2/K_1 with increasing basicity of ArNHAc (Table II). This is equivalent to decrease in reaction (8) with increasing acidity of BH^+, a reasonable result in view of the increase in $-SO_3^- \cdots HB^+$ hydrogen bond strength expected to accompany increase in BH^+ acidity[13].

Effective capacity dependence

The effective capacities, C, manifested by the resin toward acetanilide or benzamide in acetonitrile (Tables II, III) are approximately 60 % of the full capacity, C_0. On the other hand, in 80 % aqueous dioxane the C values observed for reaction with substituted anilines[1] were equal to C_0. Approximate values of C for aniline in anhydrous dioxane (1.1 mequiv./g) and acetonitrile (2.7 mequiv./g) were also observed[1]. Table IV shows how decreased effective capacity accompanies decreased swelling of res^-BH^+ as a function of solvent.

It is clear from Table III that increase in the degree of crosslinking of the resin produces a strong decrease in effective capacity toward a given base. Further, comparison of C values from Tables III and V shows that C also decreases with increasing molecular volume of the base.

These three quantities—solvent swelling, crosslinking and ion size—are considered to be, in the order given, the principal determinants of C. A physical model accounting for these effects is readily developed. GLUECKAUF[15] has presented strong experimental evidence for wide non-uniformity of crosslinking within polystyrene-sulfonic acid exchanger beads. Such a distribution of crosslink density would indeed be expected[16] to arise from copolymerization of a pair of monomers differing slightly[17] in reactivity. Apparently, in water and 80 % aqueous dioxane, the polymer network

TABLE IV

EFFECTIVE CAPACITIES AND RESIN SWELLINGS FOR RES$^-H^+$ AND RES$^-BH^+$ IN SEVERAL SOLVENTS

Solvent	*Swelling, %*		C/C_0	*Reference*
	res $^-H^+$	*res* $^-BH^+$		
Water	122	28[a]	1.0	1
80% aqueous dioxane	108	26[a]	1.0 ± 0.03	1
Acetonitrile	45	11[b]	0.52[c], 0.56[d], 0.67[e]	3 and f
Dioxane	56	~ 0[g]	0.21	3 and f

[a] B = *p*-toluidine.
[b] B = triethylamine.
[c] For aniline.
[d] Average for *m*- and *p*-substituted acetanilides.
[e] For benzamide.
[f] Present work.
[g] Ref. 14.

is sufficiently extended to permit access to all exchange sites, including those in the localities of highest crosslinking, by all but the very largest species[18]. Under conditions of low swelling, however, there appear to be volume elements in which the effective pore diameter is so small that diffusion into such volume elements by molecules of the size employed in the present work is effectively prohibited. The sum of these then constitutes the unavailable fraction of sites in our definition of effective capacity.

It is of interest to compare the values of C/C_0 observed in the present work with those obtained for a complex *anion* formation equilibrium, res$^+$A$^-$ + HA $\rightleftharpoons$ res$^+$AH$\cdots$A$^-$, also occurring in acetonitrile[6]. In the latter case, with B = *m*- and *p*-substituted benzoic acids, *i.e.*, molecules of the same general size and shape as $ArNH_2$, $ArCONH_2$ and $ArNH_2$, values of C/C_0 near 0.95 were observed for the 4% divinylbenzene resin. Greater swelling of the anion exchanger is not the source of this large effective capacity, since the swelling of the res$^+$benzoate$^-$ used was only 15% in acetonitrile (in water, 112%). It thus appears that the structure of the anion exchanger in acetonitrile is for some reason much more open than that of the corresponding cation exchangers. The absence of hydrogen bonding properties of the $-N^+(CH_3)_3$ exchange groups may play a role in this system.

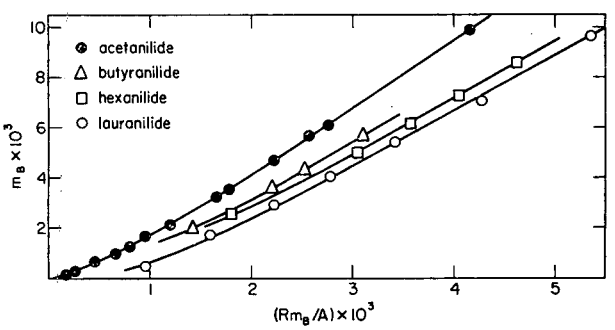


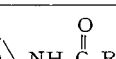
Fig. 5. Distribution of various anilides between acetonitrile and crosslinked polystyrenesulfonic acid, plotted as in Fig. 2.

Ion selectivity dependence

In order to obtain information on the effect of ion structure on the ion exchange equilibrium (3), a series of bases was studied in which K_B was held approximately constant so as to make K a measure of the variation of K_{sel}. These results are summarized in Table V and Fig. 5.

TABLE V

EQUILIBRIUM PARAMETERS FOR THE DISTRIBUTION OF VARIOUS ANILIDES BETWEEN ACETONITRILE AND HYDROGEN RESINATE[a] AT 25.0°

Base, 	$K_1 \times 10^{-3}$	$K_2 \times 10^{-3}$	C (*mequiv./g*)
R = CH_3	23.8	81.8	3.07
R = n-C_3H_7	9.16	19.9	3.01
R = n-C_5H_{11}	9.04	25.3	2.86
R = $-(CH_2)_3-$ 	7.89	8.21	2.59
R = n-$C_{11}H_{23}$	11.9	4.70	2.25

[a] Bio-Rad AG50W-X8, 200–400 mesh.

The extent of reaction decreases markedly through the series, but this is primarily the result of the expected decrease in effective capacity C, displayed by the resin toward ions of increasing size, and the decreased importance of K_2.

The most interesting conclusion from Table V is the essential constancy of K_{sel} (K_1)for the structure variations studied (excluding the change from acetanilide to butyranilide which includes a sizable difference in basicity). This result contrasts with the selectivity behavior in water[19–24] where the preference of the resin for organic ions increases with increasing lipophilic character of the ion, introduction of aromatic nuclei producing especially large selectivity enhancement*.

Whether the selectivity order in water is more properly attributed to Van der Waals forces operating between organic ions and the resin matrix[19] or to the effect of the increasingly large ions on solvent–solvent forces[25], selectivity from these sources should be largely eliminated when the difference in solvent character between solution and resin phases is reduced by substitution of an organic solvent for water. Within the accuracy of our necessarily complicated analysis of the data, this is what we have observed in acetonitrile.

Relation to selectivity in ion exchange resin catalysis

HAMMETT and co-workers[26–32] have studied the rates of resin-catalysed ester hydrolysis as a function of substrate and resin structure in comparison with the homogeneous hydrochloric acid-catalysed reaction. They express their results in terms of the efficiency of the hydrogen resinate catalyst, $q = k_{res^-H^+}/k_{HCl}$, where k is the appropriate specific rate constant. Then[26,27],

$$-RT \ln q = F^{\ddagger}_{res^-H^+} - F^{\ddagger}_{HCl} - F^{\circ}_{res^-H^+} + F^{\circ}_{HCl} \qquad (9)$$

* For sulfonated crosslinked polystyrene exchangers this is true only for the lower degrees of crosslinking. However, in acetonitrile there is no marked dependence of K_{sel} on crosslinking (Table III).

where F° and $F^\ddagger$ are the standard free energy of the reactant and transition states respectively. It is instructive to write the chemical equilibrium for which (9) gives the free energy change:

$$\text{res}^-, H_3O^+ + (B \cdot H_3O^+)^\ddagger, Cl^- \overset{K_{sel}}{\rightleftharpoons} \text{res}^-, (B \cdot H_3O^+)^\ddagger + H_3O^+, Cl^- \qquad (10)$$

This is an (hypothetical) ion exchange equilibrium for an organic ion *vs.* hydronium ion; hence (9) may be written:

$$-RT \ln q = \Delta F_{sel} = -RT \ln K_{sel} \qquad (11)$$

and

$$q = K_{sel} \qquad (12)$$

Thus the kinetic studies are closely analogous to ion selectivity measurements *via* ion exchange equilibria proper[19–24], or base–res$^-$H$^+$ equilibria as in the present work. For ester hydrolyses in water as solvent, $q = K_{sel}$ was found to be > 1, and it increased with increasing hydrocarbon chain length of the ester, in complete analogy with the known selectivity behavior in water (including the reversal with increasing crosslinking)[19–24].

In 70 % aqueous acetone[26,27], on the other hand, q was always less than unity and decreased strongly with increasing lipophilic character in the ester. This was traced[26] to a negative entropy change for (10), this ΔS_{sel} becoming more negative with increasing ester chain length. HASKELL AND HAMMETT[26] concluded quite reasonably that this resulted from loss of internal entropy on the part of the ester due to constrainment of molecular motion on entering the resin phase, this loss increasing with increasing ground state entropy of the ester.

Thus the 70 % aqueous acetone selectivity behavior corresponds neither to that observed in water nor in acetonitrile, although the constrainment of molecular motion argument should apply equally well to at least the latter solvent*. One might therefore wish to seek a possible supplementary or alternative interpretation of the 70 % acetone results based upon the resin's preferential sorption[34] of water from the mixed solvent. One can estimate from data of DAVIES AND OWEN[34] that the liquid sorbed by the resin of HASKELL AND HAMMETT[26] contained *ca.* 90 mole % water *vs.* 64 mole % in the solution phase. Equation (10) then corresponds to transporting the lipophilic ion from a less to a more aqueous medium. This should become energetically more unfavorable with increasing molal volume[25,35,36] of the ester and could conceivably give rise to the selectivity order observed. Furthermore, this should be predominantly an entropy effect[35] as required by HASKELL AND HAMMETT's results. Such a model, however, would have its own serious difficulties since the high concentration of sulfonic acid groups, the presence of the resin matrix and even the $\sim$ 10 % of acetone**

* A rationale for nonapplicability of the constrainment order in water was given by BERNHARD AND HAMMETT[28] on the basis of strong, cancelling constrainment of motion in the ground state ester by solvation forces in that solvent. This, however, seems less convincing to the present writer than the analogy with ion exchange equilibria described above. Note that the C and K_2 variations cannot be involved in 70 % acetone, since the resin is fully swollen (*e.g.* 103 % for the AG 50W-X8 resin) and the presence of water eliminates reaction (5).

** ARNETT and co-workers[37] have found very large maxima in the heat of solution of large organic ions and molecules in the region $X_{H_2O} = 0.85$–0.9 for such mixed solvents.

make it impossible to estimate whether the structural condition of this resin phase water is such that the usual thermodynamic consequences of dissolving a large organic ion in water would be observed in this case.

Chromatography

The separations shown in Fig. 1 are essentially displacement chromatograms based upon (large) differences in basicity of the components. Experimental refinements would undoubtedly allow more subtle separations. The equilibrium measurements allow the prediction that first basicity, then ion size (sieving accompanying variation in C with ion size) will be important determinants of base retention, with other selectivity factors having little influence.

Several further observations were made. In very poorly basic solvents (benzene, methylene chloride) bases with $pK_a^{BH^+} \lesssim -6$ are not appreciably retained by the resin. Changes of solvent which produce swelling changes (*e.g.*, benzene to acetonitrile) ruin the column. Sizable evolution of heat accompanies introduction of solvents more basic than acetonitrile (*e.g.*, N,N-dimethylformamide). This may ruin the column unless the transition is made very gradually or external cooling is applied. Finally, some solvents leach traces of red polymer from the resin; sublimation of eluted fractions is a good method of eliminating this material.

SUMMARY

Distribution of weak bases between anhydrous acetonitrile and crosslinked polystyrenesulfonic acid ion exchange resins was studied at 25.0° over a wide concentration range. Analysis of the data afforded equilibrium constants and effective resin capacities for a variety of bases. Equilibrium constants so obtained correlate well with basicity for a series of *m*- and *p*-substituted acetanilides. Modification of the hydrocarbon portions of the base has little effect on the selectivity of the resin for the conjugate acid cation. This result is compared with the different behavior known for ion exchange equilibria with water solutions and deducible from selectivity effects in resin-catalysed ester hydrolyses in aqueous acetone. A physical model explaining the observation of reduced effective capacities, C, of the resins in contact with non-aqueous solvents was developed and the measured C values were correlated with structure. Principal determinants of C are solvent, crosslinking and ion size. Application of these results to chromatography on anhydrous ion exchangers in non-aqueous media is discussed and preliminary chromatographic results are presented.

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ANION-EXCHANGE BEHAVIOR OF SCANDIUM, RARE EARTHS, THORIUM AND URANIUM IN MAGNESIUM NITRATE SOLUTION

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INTRODUCTION

The behavior of rare earths, thorium and uranium in nitric acid media on anion exchangers has been well established and some useful separations of these elements have been reported. General information on the adsorbability of these elements on a strongly basic anion exchanger from nitric acid media has recently been provided by ICHIKAWA *et al.*[1] and FARIS AND BUCHANAN[2]. These authors have presented the distribution coefficients of elements as a function of nitric acid concentration in the form of the periodic table and useful separations are possible by the proper control of nitric acid concentrations. DANON[3,4] has investigated the adsorption of thorium on Dowex 1 from nitric acid solution and effected the separation of thorium from yttrium and rare earths. CARSWELL[5] has also made use of the anionic character of thorium in nitric acid solution in separating thorium and uranium by anion exchange at elevated temperatures. FRITZ AND GARRALDA[6] have reported the anion-exchange separation of thorium, using 6 *M* nitric acid, from many elements, including the rare earths and uranium. Anion-exchange behavior of uranium in nitric acid solutions has been investigated by several workers[5,7–9]. They showed that uranium has a maximum value for the distribution coefficient of about 20, with approximately 8 *M* acid. With respect to the adsorbabilities of the rare earths, ICHIKAWA[10] and DANON[3,4] have reported that the lanthanides are slightly adsorbed by anion-exchange resins, such as Dowex 1, from concentrated nitric acid solutions. In general the distribution coefficients for most metals are rather low in nitric acid solution and, accordingly, high concentrations of the acid must be employed in most cases to ensure good separation. This necessarily causes damage to the resin exchangers.

To obtain the distribution coefficients for uranium of the order of 1000, media of low acid with high salting strength have been suggested. The enhanced adsorbability of uranium in high nitrate salt solution has been used for the separation of iron[11] and as a general separation method prior to the peroxide colorimetric determination of uranium[12]. The effectiveness of nitrate salts in increasing the uptake of uranium on the anion-exchange resin Deacidite FF is in the order $Al > Ca > Li > NH_4$[13]. DANON[14] has shown that praseodymium is adsorbed more strongly on Dowex 1 from nitrate solutions than from acid media. The increase of adsorption with the nature of the salting cation is in the order $Al > Fe > Cu > Ca > Li > NH_4 > H$. DANON has also described a procedure for separating trace amounts of actinium and weighable quantities of lanthanum with the anion-exchange resin in 4.4 *M* $LiNO_3$ solution.

MARCUS AND NELSON[15] have investigated the adsorbabilities of several rare earths in lithium nitrate media with a strong base anion-exchange resin and obtained good separations of complex mixtures of lighter rare earths tracers. BUCHANAN AND FARIS[9] recently showed that considerable fractionation of the rare earths occurred in 10 *M* ammonium nitrate solution of low acidity. Adsorbabilities decreased with increasing atomic number ranging from an estimated distribution coefficient of 100 for lanthanum to approximately 4 for lutetium. Except for the extremes, lanthanum and lutetium, they have not given any detailed information on the values for individual rare earths elements.

Because of the marked difference in the behavior of metal ions in nitric acid and nitrate solution, it appeared of interest to investigate the adsorption of metals from magnesium nitrate solutions to provide additional data on nitrate complexing, and to see if any useful separations would be feasible. The metals studied were scandium, the rare earths, thorium and uranium. For those metals which showed appreciable adsorption, the distribution coefficients were measured over a wide range of magnesium nitrate concentration and some separations were carried out.

EXPERIMENTAL

Reagents and apparatus

Stock solution of scandium, 1 mg/ml. Approximately 150 mg of oxide (99.9 % purity) were dissolved in 6 *M* nitric acid, evaporated to dryness, taken up in 0.5 *M* nitric acid and diluted to 100 ml with the same solution.

Stock solution of thorium, 2.5 mg/ml. Approximately 600 mg of thorium nitrate tetrahydrate was dissolved in 0.5 *M* nitric acid and diluted to 100 ml with the same solution.

Rare earths solutions. Appropriate amounts of rare earths oxides (99.9 % purity) were dissolved in a minimum amount of 6 *M* nitric acid and evaporated to dryness. The residues were dissolved in 100 ml of 0.5 *M* nitric acid to give 2–6 mg of each rare earth per ml of 0.5 *M* acid. The strengths of the stock solutions mentioned above were determined by titration with 0.001 *M* EDTA standard solution using Xylenol Orange as indicator.

Radioactive tracers. The rare earth tracer ^{147}Nd (half-life 11.1 days) was produced by irradiating an appropriate amount of its oxide for 6 h at a neutron flux of $5 \cdot 10^{11}$ n/cm^2/sec in a TRIGA MARK II reactor of St. Paul's University, Yokosuka. The irradiated oxide was dissolved in nitric acid and evaporated, the residue being taken up in 0.5 *M* nitric acid to give a convenient activity level for measurement. ^{154}Eu (half-life 16 years) and ^{144}Ce (half-life 284 days) with a high radiochemical purity were obtained through a supplier.

Ion-exchange resin

Dowex 1-X8 ion-exchange resin, 100–200 mesh in particle size, was used. Before use the resin was converted into the nitrate form by transferring it to a larger column and passing enough 3 *M* ammonium nitrate down the column until the effluent was negative to a silver nitrate test. The resin was then thoroughly washed with deionized water, dried at 40° for 3 h and finally stored for over a week in a large desiccator containing a saturated potassium bromide solution.

Ion-exchange column

A conventional column, 1.0 cm internal diameter, 15 cm long, pulled to a tip, and plugged with glass wool at the outlet, was used. Five grams of the dried resin were slurried with water and poured into the column. The resulting bed height was approximately 9.5 cm. The eluant was introduced through a 100 ml separatory funnel whose stem was attached to the top of the column with rubber tubing.

Procedure

Determination of the metal ions. Scandium and thorium were determined by titration with 0.001 *M* EDTA solution at pH 2.5 and 3, respectively, using Xylenol Orange as indicator. The rare earths and uranium were determined spectrophotometrically with arsenazo[3-(2-arsono-phenylazo)-4,5-dihydroxy-2,7-naphthalene-disulfonic acid][16] and thiocyanate[17], respectively, as color reagents. In the column elution experiments for the separation of rare earths, radioactive tracers, with or without carriers, were used throughout the experiment to construct elution curves. Radioactivity measurements were taken with a conventional well-type scintillation counter.

Measurement of distribution coefficients. The values for the weight distribution coefficient, K_d, were determined by either a batch or a column elution method, depending upon the magnitude of the K_d values. K_d values higher than 20 were generally determined by the equilibrium batch method[18], lower values by the column elution method[19].

Column separation. Prior to loading the sample solution on to the top of the column it was pretreated with a 3 *M* magnesium nitrate solution. The sample solution was evaporated to nearly dryness and taken up in 5–10 ml of a 3 *M* magnesium nitrate solution. The sample solution was loaded on to the top of the column and allowed to

TABLE I

SEPARATION OF SCANDIUM AND THORIUM/OR URANIUM

Run	*mg taken*		*mg recovered*		*Remarks*
	Sc	*Th/U*	*Sc*	*Th/U*	
1	1.01	5.31	1.01	5.28	Sc eluted first with 350 ml of 3 *M* $Mg(NO_3)_2$, then Th with 60 ml of 2.4 *M* HCl.
2	0.99	10.8	1.00	10.5	Sc eluted first with 350 ml of 3 *M* $Mg(NO_3)_2$, then Th with 60 ml of 2.4 *M* HCl.
3	1.01	10.5	1.00	10.3	Sc eluted first with 140 ml of 2.5 *M* $Mg(NO_3)_2$, then Th with 30 ml of 2.4 *M* HCl.
4	1.01	31.0	1.03	30.0	The same elution condition used as that of Run 1.
5	9.25	1.20	9.06	1.18	The same elution condition used as that of Run 3.
6	1.01	6.72*	1.03	6.76*	Sc eluted first with 50 ml of 1.5 *M* $Mg(NO_3)_2$, then U with 30 ml of 1 *M* $HClO_4$.
7	1.10	24.0*	1.11	23.7*	Sc eluted first with 350 ml of 3 *M* $Mg(NO_3)_2$, then U with 60 ml of 1 *M* $HClO_4$.
8	1.10	24.0*	1.09	23.3*	Sc eluted first with 130 ml of 2 *M* $Mg(NO_3)_2$, then U with 80 ml of 1 *M* $HClO_4$.
9	1.10	56.0*	1.13	54.5*	Sc eluted first with 130 ml of 2 *M* $Mg(NO_3)_2$, then U with 80 ml of 1 *M* $HClO_4$.
10	8.74	0.920*	8.67	0.931*	Sc eluted first with 160 ml of 2.5 *M* $Mg(NO_3)_2$, then U with 50 ml of 1 *M* $HClO_4$.

* The figures marked with an asterisk refer to uranium, the other figures to thorium.

pass down the column. The less adsorbable elements were eluted with the magnesium nitrate solution, the concentration of which is listed in Table I. Eluants employed for the subsequent elution of individual metal ions are also given in Table I along with the first eluant. The flow rate is kept at 0.3–0.5 ml/min.

RESULTS AND DISCUSSION

Adsorption from magnesium nitrate solution

In Fig. 1 the weight distribution coefficient data are plotted *vs.* the concentration of magnesium nitrate solution. It can be seen that there are marked increases in the adsorption of uranium, thorium, lanthanum and scandium as the concentration of magnesium nitrate increases. Distribution coefficient values for uranium in nitric acid solution are about unity at 2 *M* nitric acid, rising to 20 at 8 *M* nitric acid. The use of

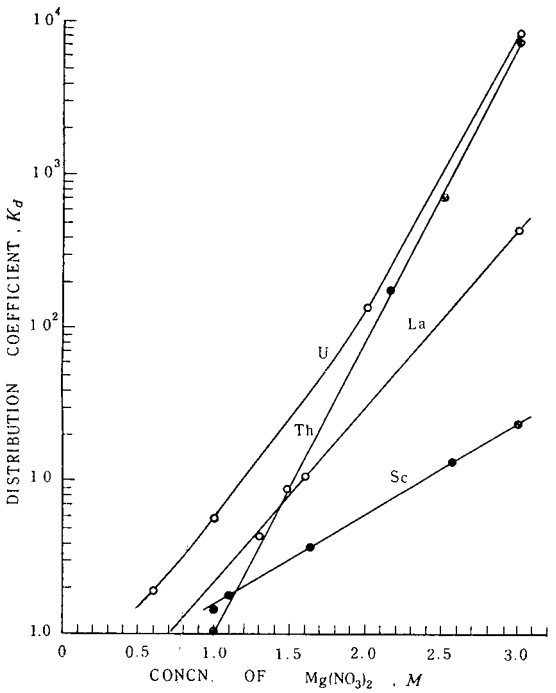

Fig. 1. Distribution coefficients of scandium, lanthanum, thorium and uranium on Dowex 1 anion-exchange resin in magnesium nitrate media.

nitric acid is limited practically to 8 *M* in an ion-exchange system because of the rapid resin deterioration in higher nitric acid concentrations. The value of the distribution coefficient for thorium on Dowex 2 in nitric acid solutions shows a maximum value of 100 near 8 *M* nitric acid[6,8]. In Fig. 2 the distribution coefficients of the rare earths with Dowex 1-X8 in 3 *M* magnesium nitrate solution are plotted against the atomic numbers of the rare earths. It can be seen that pronounced uptake and fractionation of the rare earths occurs in a concentrated magnesium nitrate solution. According to

ICHIKAWA[10], the distribution coefficient values for the rare earths in 8 *M* nitric acid decrease within the group of lighter rare earths with increasing atomic number, reaching a minimum near gadolinium, and then remaining practically constant with further increase in atomic number. The highest value is approximately 8 for lanthanum in 8 *M* acid. A similar trend can be seen in Fig. 2 within the lighter rare earths ranging from lanthanum to gadolinium. However, the distribution coefficient increases regularly with increasing atomic number within the group of the heavier rare earths in a 3 *M* magnesium nitrate solution. Considerable uptake occurs from the nitrate solution as compared to the strong nitric acid solution. It is of interest to note here that there is a distinct correlation between the values of the distribution coefficients and the solubility of the double nitrate salts of the rare earths. The solubility of the double salts of the rare earths first increases with increasing atomic number, in the order[20] La < Ce < Pr < Nd < Sm < Eu < Gd > Er ...., with a maximum at gadolinium.

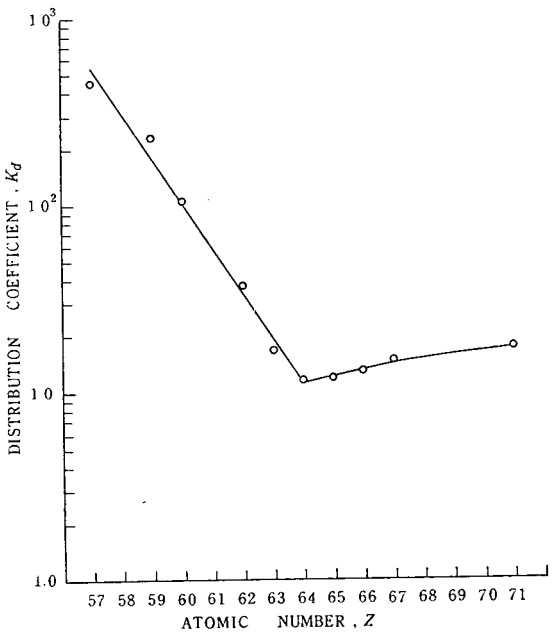

Fig. 2. Distribution coefficients of rare earths as a function of the atomic number.

Thus it can be seen that the minimum affinity to the anion exchanger corresponds to the maximum solubility. The same trend has been found before for the adsorption of rare earths on Dowex 1 from potassium sulfate solution[19].

Comparing the values for the distribution coefficients of the rare earths in nitric acid, ammonium and lithium nitrates, we can assume the order of effectiveness for increasing the adsorbability of the rare earths on a strong anion exchanger by nitrate salting-out to be in the order $Mg > Li > NH_4 > H$. The governing factor may evidently be attributed to a decrease in the effective concentration of water through hydration of the salting-out cation.

For the separation of two ions with an ion exchanger, it is important that the

separation factor, defined by the ratio of their distribution coefficient $\alpha = Kd_1/Kd_2$, is as large as possible in a given ion-exchange system. From Fig. 2 it can be calculated that the separation factor α within the lighter rare earths, ranging from lanthanum to gadolinium, has an average of 1.74, while the value α for a pair of heavier rare earths, from gadolinium to lutetium, is down to an average of 1.06.

Separations

As can be seen from Fig. 1 the differences in the distribution coefficients between scandium and uranium/or thorium are large enough to enable their sharp separation. It would also be feasible to separate scandium from lanthanum. However, scandium cannot always be separated from the rare earth elements because the distribution coefficients of the rare earths are widely different, so that the separation of scandium from rare earths heavier than samarium would only be feasible with great difficulty. In Table I the results for the separation of scandium and thorium/or uranium are listed; the optimal conditions were not necessarily selected. All elutions were carried out at room temperature. As can be seen from Table I, mixtures containing scandium and thorium/or uranium in widely varying ratios can be analyzed successfully. If thorium is present in sample mixtures along with weighable amounts of scandium, thorium should be eluted with a magnesium nitrate solution greater than 2.5 M, otherwise it tends to break through in an earlier fraction, resulting in the contamination of the eluted scandium fraction. This must be attributed to the rather slow approach of thorium to the ion-exchange equilibrium in a magnesium nitrate

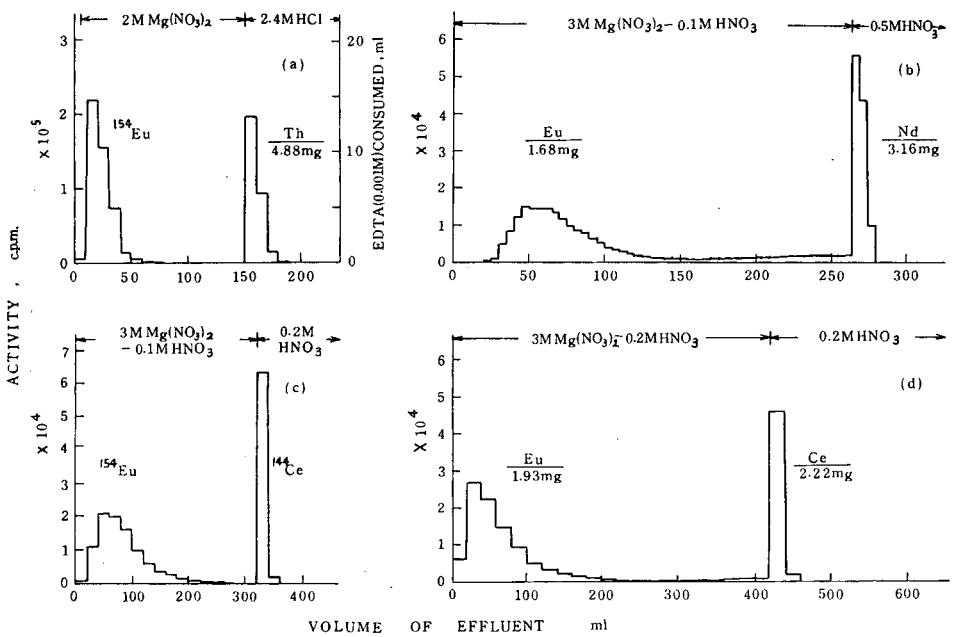

Fig. 3. Typical rare earths separations. (a) Separation of carrier-free ^{154}Eu and thorium. Thorium recovery: 99.3%. (b) Carrier separation of europium and neodymium, labelled with ^{154}Eu and ^{147}Nd, respectively. (c) Separation of carrier-free ^{154}Eu and ^{144}Ce(III). (d) Carrier separation of europium and cerium(III), labelled with ^{154}Eu and ^{144}Ce, respectively.

solution of low concentration. So far as the separation of scandium and uranium is concerned, scandium can be eluted even with a 1.5 *M* nitrate solution without breakthrough of uranium, and consequently it was removed in a sharp band.

In other experiments carrier-free ^{154}Eu was shown to be easily separable from weighable quantities of thorium by feeding the mixture, adjusted to 2 *M* in magnesium nitrate, on to the column and eluting ^{154}Eu with the same solution. Thorium remained strongly adsorbed on the column and was quantitatively recovered by elution with a 2.4 *M* hydrochloric acid solution (Fig. 3a).

Separation of selected pairs of rare earths was also carried out. The results are illustrated in Fig. 3. Each mixture, adjusted to 3 *M* in magnesium nitrate, was loaded on the column. Europium was first eluted by the same solution, while cerium/or neodymium remained adsorbed on the column, being eluted sharply with dilute nitric acid.

Europium tended to tail at increased concentrations of magnesium nitrate. However, a well-defined bell-shaped elution band of europium appeared at an elevated temperature (60°); a detailed investigation on the effect of the temperature on the separation has not been attempted. As can be seen from Fig. 1 the distribution coefficient of lanthanum decreases rapidly with decreasing concentration of magnesium nitrate solution. This behavior of lanthanum would suggest similar behavior with other rare earth elements. To obtain optimal working conditions for the separation of the rare earths we have extended the measurement of distribution coefficients for individual rare earths over a wide concentration range of magnesium nitrate.

SUMMARY

Pronounced adsorption of scandium, rare earths, thorium and uranium in magnesium nitrate solution has been demonstrated with a strongly basic anion exchanger. The adsorbability of these metals increases sharply with increasing concentration of magnesium nitrate solution. Fractionation of the lighter rare earths was possible in a 3 *M* magnesium nitrate solution. The distribution coefficients were found to decrease within the cerium group, reaching a minimum near gadolinium, to increase slightly with further increase of atomic number. Sufficiently large differences in adsorbability to permit the separation of scandium and thorium/or uranium were found and selected pairs of the lighter rare earths were also found to be separable with relatively short columns.

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ION EXCHANGE IN FUSED SALTS

I. CHROMATOGRAPHIC BEHAVIOUR OF VARIOUS METAL CATIONS IN FUSED SALTS ON γ-ALUMINA AND SYNTHETIC INORGANIC ION EXCHANGE MATERIALS

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INTRODUCTION

The selective adsorption of metal ions dissolved in fused salts on various inorganic materials has been recently reported by several authors[1–11].

So far, γ-Al_2O_3[1–4], zirconium phosphate and zirconium oxide[5,6], zeolites[7,8], silica gel[9], glass powder[10] and glass-fiber paper[11] have been tested as adsorption materials and their selectivity has been used to separate mixtures of inorganic ions dissolved in fused alkali chlorides and nitrates.

There is still, however, little information on the mechanism of selective adsorption. According to MARCUS[7,8] Linde Molecular Sieve 4A would retain its ion exchange properties in ionic melts as well as in aqueous solutions. It might be expected, therefore, that ions dissolved in fused salts are also adsorbed by an ion exchange mechanism on other inorganic materials. It might be of interest, both from a theoretical and a practical point of view, to gain further insight into the ion exchange properties of inorganic materials in fused salts, since it is possible to predict, on the basis of the present knowledge of ion exchange mechanism, that these properties will differ considerably from those in aqueous solutions.

The main differences to be expected are as follows:

(a) Marked changes of selectivity should be observed in fused salts when compared with aqueous solutions. In aqueous solutions, the ion exchanger tends to show a preference for ions with a larger crystal radius (*i.e.* a smaller hydrated radius) when the charges of the ions are equal. The opposite should take place in fused salts.

(b) The extent of swelling in aqueous solutions and in ionic melts should differ considerably.

(c) The non-hydrated hydrogen ion is very small relative to other ions. The selectivity of the exchanger material for H^+ ions, in perfectly anhydrous conditions, should therefore be greatly increased.

(d) The efficiency of electrolyte exclusion from ion exchangers decreases as the concentration of the solution increases. This provides strong support for the view that invasion of the ionic solvent takes place in fused salts.

(e) When a cationic exchanger is in contact with a molten salt, all cations present in the melt will compete for the exchangeable sites. A metal ion dissolved at low concentrations in the fused salt will therefore be adsorbed only when its affinity for the ion exchanger is sufficiently high. Furthermore, the metal ion adsorption could be strongly affected by complex formation.

High distribution coefficients for a given metal ion dissolved in fused salts will thus be obtained when the ionic melt has a low complexing power and the metal ion has a high affinity for the ion exchanger.

The present paper reports a systematic investigation of the adsorption properties of various inorganic materials in fused salts. Attempts were made to interpret the mechanism of metal ion adsorption and to confirm the expected differences between the ion exchange properties in molten salts and in aqueous solutions. It seemed convenient to undertake the study of adsorption in molten salts by a chromatographic technique employing glass-fiber paper impregnated with the ion exchangers to be studied, because of the speed and simplicity compared with column chromatography.

EXPERIMENTAL

Chemicals

Finely powdered potassium nitrate (Erba RP) and lithium nitrate (Erba RP) were oven dried at 94° and mixed in molar proportions of $LiNO_3$:KNO_3 = 43:57. This mixture was dried again at 94°, fused in the chromatography vessel and allowed to stand for 1 h at the operating temperature (160°) before starting the experiments. The chromatographic apparatus and technique have been described elsewhere[6].

Solutions (*ca.* 10^{-2} *M*) of the ions to be studied were prepared by dissolving the metal nitrates (Erba RP) in the $LiNO_3$–KNO_3 eutectic. Europium nitrate was prepared by dissolving Eu_2O_3 (Johnson-Matthey, Co., London) in hot conc. HNO_3 and heating to dryness. $Fe(NO_3)_3$ was dissolved in the molten $LiNO_3$–KNO_3 eutectic containing conc. HNO_3 in order to avoid Fe_2O_3 precipitation. Metal ions were detected by spraying suitable reagents on the glass-fiber strips. UO_2(II) and Eu(III) were detected by fluorescence spot tests[12,13]. Solutions of Na(I), Rb(I), and Cs(I) nitrates were labelled with ^{22}Na, ^{86}Rb and ^{137}Cs, respectively.

Preparation of glass-fiber strips impregnated with zirconium phosphate, zirconium oxide and γ-Al_2O_3

The glass-fiber paper employed (Whatman GB, 0.15 mm thick, weight 150 mg/cm^2) was purified by dipping it in HNO_3 (1:1) and rinsing with distilled water. Strips of this paper (12 cm × 1 cm) impregnated with zirconium phosphate were prepared according to the technique reported elsewhere[14] and dried at 160°.

Glass-fiber paper strips impregnated with γ-Al_2O_3 were prepared by immersing first in a 2 *M* $AlCl_3$ solution, then in 1 *M* NH_4OH. After 2 h the strips were rinsed with distilled water, air dried, and then oven dried at 500° for 5 h. X-ray analysis (CuK_α) confirmed the formation of γ-Al_2O_3.

Glass-fiber paper strips impregnated with zirconium oxide were prepared by immersing first in a 4 *M* HCl–0.15 *M* $ZrOCl_2$ solution, then in 32 % NH_4OH. After 2 h the strips were rinsed with distilled water, air dried and then oven dried at 160°.

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TABLE I

CHROMATOGRAPHY OF INORGANIC IONS ON VARIOUS SUPPORTS AND WITH DIFFERENT ELUANTS AT 160°

Ion	*Eluant*	*Glass-fiber paper*	*Glass-fiber paper + ZP**	*Glass-fiber paper + ZO***	*Glass-fiber paper + Al_2O_3*
Na(I)	$LiNO_3$–KNO_3	At liquid front	At liquid front	At liquid front	At liquid front
Rb(I)	$LiNO_3$–KNO_3	At liquid front	At liquid front	At liquid front	At liquid front
Cs(I)	$LiNO_3$–KNO_3	At liquid front	At liquid front	At liquid front	At liquid front
Tl(I)	$LiNO_3$–KNO_3	At liquid front	At liquid front	At liquid front	At liquid front
Pb(II)	$LiNO_3$–KNO_3	At liquid front	At front with comet	At front with comet	At front with comet
	$LiNO_3$–KNO_3 10% NH_4NO_3	At liquid front	At liquid front	At liquid front	At liquid front
	$LiNO_3$–KNO_3 15% KCl	At liquid front	At front with comet	At front with comet	At front with comet
	$LiNO_3$–KNO_3 15% NH_4Cl	At liquid front	At liquid front	At front with comet	At front with comet
Ni(II)	$LiNO_3$–KNO_3	At front with comet	At front with comet	At the starting point with comet	At the starting point
	$LiNO_3$–KNO_3 10% NH_4NO_3	At liquid front	At liquid front	At liquid front	At front with comet
	$LiNO_3$–KNO_3 15% KCl	At liquid front	At the starting point with comet	At the starting point with comet	At the starting point with comet
	$LiNO_3$–KNO_3 15% NH_4Cl	At liquid front	At front with comet	At front with comet	At front with comet

Co(II)	$LiNO_3$–KNO_3	At front with comet	At the starting point with comet	At the starting point	At the starting point
	$LiNO_3$–KNO_3 10% NH_4NO_3	At liquid front	$R_F = 0.66$	At front with comet	At front with comet
	$LiNO_3$–KNO_3 15% KCl	At liquid front	At liquid front	At liquid front	At liquid front
	$LiNO_3$–KNO_3 15% NH_4Cl	At liquid front	At front with comet	At liquid front	At liquid front
UO_2(II)	$LiNO_3$–KNO_3	At the starting point	At the starting point	At the starting point	At the starting point
	$LiNO_3$–KNO_3 10% NH_4NO_3	At the starting point with comet	At the starting point	At the starting point with comet	At the starting point with comet
	$LiNO_3$–KNO_3 15% KCl	At the starting point with comet	$R_F = 0.5$	At the starting point	At the starting point
	$LiNO_3$–KNO_3 15% NH_4Cl	At front with comet	At the starting point with comet	At front with comet	At liquid front
Eu(III)	$LiNO_3$–KNO_3	At front with comet	At the starting point	At the starting point	At the starting point
	$LiNO_3$–KNO_3 10% NH_4NO_3	At liquid front	At the starting point with comet	At front with comet	At liquid front
	$LiNO_3$–KNO_3 15% KCl	$R_F = 0.6$	At the starting point	At the starting point	At the starting point
	$LiNO_3$–KNO_3 15% NH_4Cl	At liquid front	At the starting point with comet	At front with comet	At front with comet
Fe(III)	At the starting point under all conditions				
Th(IV)					

* Zirconium phosphate.
** Zirconium oxide.

RESULTS AND DISCUSSION

Table I presents results obtained by chromatography, on glass-fiber paper and glass-fiber paper impregnated with zirconium phosphate, zirconium oxide and γ-Al_2O_3, of various metal ions dissolved in the $LiNO_3$–KNO_3 eutectic. These results clearly indicate that the adsorption of Cs(I), Rb(I), Na(I), Tl(I), and Pb(II), on the ion exchangers tested, is very low. Ni(II), Co(II), UO_2(II) and Eu(III) are moderately adsorbed, whereas Fe(III) and Th(IV) are strongly adsorbed. Comparison of our results with the scale of the approximate solubilities of oxides in both the LiCl–KCl[15–17] eutectic and fused alkali fluorides[16] shows a marked analogy between adsorption and solubility of a given metal ion. This analogy is illustrated in Table II,

TABLE II

COMPARISON BETWEEN ADSORPTION, OXIDE SOLUBILITY AND IONIC POTENTIALS

Scale of charge/ ionic radius ratio		*Approximate scale of the adsorption (increasing downward) in $LiNO_3$–KNO_3*		*Approximate scale of oxide solubility (decreasing downward) in LiCl–KCl*[15]		*Approximate scale of oxide solubility (decreasing downward) in molten fluorides*[16]	
Cs(I)	0.60	Cs(I)	*	Cs_2O	+	Cs_2O	+
Rb(I)	0.68	Rb(I)		Rb_2O		—	
Tl(I)	0.68	Tl(I)		Tl_2O		—	
Na(I)	1.03	Na(I)		Na_2O		Na_2O	
Pb(II)	1.67	Pb(II)		PbO		—	
Zn(II)	2.70	—	**	ZnO	++	ZnO	++
Co(II)	2.78	Co(II)		CoO		—	
Ni(II)	2.90	Ni(II)		NiO		—	
Mg(II)	3.03	—		MgO		MgO	
—		UO_2(II)		UO_3		—	
Eu(III)	3.06	Eu(III)		Eu_2O_3		—	
—		—	***	—	+++	Al_2O_3	+++
—		—		—		UO_3	
Fe(III)	4.69	Fe(III)		Fe_2O_3		—	
Sn(IV)	5.63	—		SnO_2		SnO_2	
Al(III)	5.88	—		Al_2O_3		—	
Ti(IV)	5.88	—		TiO_2		TiO_2	
Si(IV)	9.52	—		SiO_2		SiO_2	

* Scarcely adsorbed.
** Moderately adsorbed.
*** Strongly adsorbed.

\+ Soluble.
++ Sparingly soluble.
+++ Insoluble.

which shows not only the approximate scales of the solubilities and adsorptions but also, for comparison, a scale of the ionic potentials (charge/ionic radius) of the cations under investigation. The correspondence between these scales indicates that as the value of the ionic potential increases, the chromatographic adsorption of a given cation on the tested materials increases, whereas its solubility decreases. At present the nature of these observations is only qualitative. A knowledge of the distribution coefficients and solubility products for a large number of metal ions dissolved in the same ionic melt is required to arrive at more quantitative conclusions.

In this way more exact scales, based on numerical values, would be obtained, and also some anomalies due to the different complexing power of the solvent employed could be avoided. The relation between the above-mentioned scales could be improved by replacing the values of ionic radii by other values involving the actual distances between the cations and the fixed ionic groups of the ion exchangers, *e.g.* taking into account the charge of the ions and also their polarizability.

Table I gives chromatographic results obtained employing as solvent a solution of NH_4Cl in the $LiNO_3$–KNO_3 eutectic. These results clearly show the high eluting power of NH_4Cl, already mentioned by GRUEN[1]. This author employed the same solvent to elute UO_2(II), Co(II), Cu(II) and Ni(II) previously adsorbed on γ-Al_2O_3. According to GRUEN the high eluting power of NH_4Cl is due to the formation of chlorocomplexes. The metal ions were eluted in the same order as mentioned above and confirm the results of spectrophotometry for the relative stabilities of the chlorocomplexes.

In order to derive more exact information concerning the elution mechanism of NH_4Cl, it was necessary to test the eluting power of NH_4^+ and Cl^- ions respectively. For this purpose, NH_4NO_3 and KCl were added separately to the $LiNO_3$–KNO_3 eutectic. As shown in Table I, all the ions eluted by NH_4Cl are also eluted by NH_4NO_3 whereas only UO_2(II) and Co(II) are displaced to any extent when KCl is added to the $LiNO_3$–KNO_3 eutectic. Thus the eluting power of NH_4Cl is due to both NH_4^+ and Cl^- ions. It follows that the results of chromatographic experiments employing solutions of NH_4Cl in the $LiNO_3$–KNO_3 eutectic cannot be used as evidence of chlorocomplex formation. Although elutions performed with eutectics containing KCl confirm the formation of chlorocomplexes of UO_2(II) and Co(II) already reported by GRUEN[1], our results for Ni(II) show that the tendency to form complexes of the type $NiCl_4^{2-}$ is very low, since Ni(II) is eluted by eutectics containing NH_4Cl or NH_4NO_3, but it is not displaced when KCl is dissolved in the molten nitrates. Furthermore we observed that Ni(II) does not form an anionic chlorocomplex in noticeable quantities even in the LiCl–KCl eutectic at 450°, since it behaves as a cation when electrophoresis is carried out in this solvent[11]. Co(II) and UO_2(II) under the same conditions behave as anions.

The chromatographic technique employing impregnated glass-fiber paper was also used to investigate the disagreement between results obtained by different authors. For example, GRUEN observed that Pr(III) and Nd(III), dissolved in molten alkali nitrates, were not absorbed on γ-Al_2O_3[1]. On the contrary LINDNER AND JOHNSON[3] reported that, under the same experimental conditions, rare earths were strongly adsorbed. Table I shows that Eu(III) is moderately adsorbed on γ-Al_2O_3, in agreement with both the value of the ionic potential and the low solubility of Eu_2O_3 in molten alkali nitrates. Since the difference between the ionic crystal radii of the rare earths is larger than the difference between their hydrated ionic radii, separations of the trivalent rare earth ions should be achieved in molten salts.

BENARIE[10] reported that Cu(II), Ni(II), Co(II) and Mn(II) dissolved in the LiCl–KCl eutectic, were adsorbed on glass powder. The adsorption decreased in the same order as mentioned above, with the exception of Cu(II), which can be partially reduced to Cu(I) in molten alkali chlorides in the presence of O^{2-} ions. It must be mentioned that the order of adsorption is in agreement with the charge of the ion/ionic radius ratios, which are respectively: Ni(II) 2.94, Co(II) 2.77 and Mn(II) 2.50.

GRUEN[1], on the other hand, reported that Co(II), Ni(II), Cu(II), dissolved in the LiCl–KCl eutectic, were not adsorbed on γ-Al_2O_3. This could be due to different experimental conditions, since GRUEN dehydrated the LiCl–KCl eutectic carefully with gaseous HCl, whereas BENARIE does not mention any special care in preparing the melt during the experiments. In a previous paper[11] we reported that the formation of insoluble oxides was observed when chromatography was carried out in non-dehydrated molten alkali chlorides, *e.g.* Ni(II) and Mn(II) adsorbed on glass-fiber paper in the presence of air did not move when a non-dehydrated eutectic was used. When pyridinium chloride at 130°, or the LiCl–KCl eutectic dehydrated with gaseous HCl were used as eluents, these ions moved with the front of the solvent.

Wettability of glass-fiber paper

As we reported elsewhere[11] the wettability of glass-fiber paper by fused salts depends upon the presence of traces of water or O^{2-} ions in the melt, *e.g.* glass-fiber paper is wetted by a non-dehydrated LiCl–KCl eutectic, whereas the wettability is greatly lowered when alkali chlorides are dehydrated by gaseous HCl. Furthermore when an atmosphere of gaseous HCl is maintained over the melt, the wettability decreases to such an extent that chromatographic experiments are no longer possible. In general, glass-fiber paper is wetted with difficulty by the $LiNO_3$–KNO_3 eutectic, so that chromatographic experiments are sometimes unsuccessful. By adding 1–2 % KOH to the molten nitrates, the wettability is greatly increased. Similar results are obtained with glass-fiber paper impregnated with zirconium phosphate in the hydrogen form. On the basis of these results and assuming that the affinity of the H^+ ion for the ion exchangers is high (since the non-hydrated H^+ ion is very small relative to other cations) the wetting mechanism could be explained as follows: glass-fiber paper is purified by dipping into an aqueous solution of HNO_3. The strips are therefore superficially converted into the hydrogen form. In perfectly anhydrous melts or at low concentrations of O^{2-} ions, the extent of exchange between the cations present in the solvent and the H^+ ions of the exchanger material is very low and a poor wettability will therefore result. At high concentrations of O^{2-} ions in the melt, the H^+ ion can be exchanged since it reacts to form H_2O. In non-dehydrated melts, also, the H^+ ion could be displaced by other cations, as the hydrated H^+ ion is markedly larger than the anhydrous proton. In such cases a wettability will be obtained.

We tried also to increase the wettability of glass-fiber paper by replacing the H^+ ion with a cation present in the solvent. For this purpose the strips, after purification with HNO_3, were dipped in concentrated solutions of KNO_3. These strips, dried at 160°, were perfectly wetted by the $LiNO_3$–KNO_3 eutectic. Further investigations clearly demonstrate the importance of traces of water or O^{2-} ions on the proton exchange in molten salts. A quantitative study of the affinity of the H^+ ion, in the presence of different amounts of water, seems to be of interest for the chemistry of the proton in molten salts.

CONCLUSIONS

It was shown that some problems connected with the adsorption of metal ions dissolved in fused salts can be investigated by a simple chromatographic technique employing glass-fiber paper impregnated with various inorganic ion exchangers.

The present results support the theory that metal ions are mainly adsorbed by ion exchange, although other mechanisms are possible. For example the water of hydration held by several ion exchangers, even at relatively high temperatures, could give rise to oxide precipitation. Several metal ions forming insoluble oxides could therefore be fixed in this way by the adsorption material. The precipitation of oxides is in agreement with both the analogy between chromatographic adsorption and oxide solubility, and the eluting power of NH_4^+ ions. It is well known that the NH_4^+ ion, behaving as an O^{2-} ion acceptor, dissolves oxides. Furthermore, in some cases, specific interactions with the fixed ionic groups of the ion exchanger may take place, *e.g.* metal ions forming insoluble phosphates can be selectively adsorbed by zirconium phosphate. It will be of interest to obtain more quantitative data (*e.g.* values of distribution coefficients and of exchange capacities, percentage of solvent invasion in the ion echanger, etc.) to improve the knowledge of ion adsorption in fused salts and also to confirm the differences predicted between the properties of ion exchangers in aqueous solutions and in ionic melts. An investigation of anion exchange in fused salts should also prove interesting.

SUMMARY

The chromatographic adsorption on γ-alumina and synthetic inorganic ion exchange materials of various metal cations dissolved in a fused lithium nitrate–potassium nitrate eutectic was studied. The results obtained seem to confirm that metal ions are adsorbed by ion exchange, although other mechanisms, *e.g.* oxide precipitation, or formation of insoluble phosphates, are possible. A relationship between the adsorption of the metal ions tested, the solubility of their oxides and the ionic potentials was found. Some conflicting results of other authors are discussed.

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CHELATBILDENDE AUSTAUSCHERHARZE

VI. PYRIDIN-2,6-DICARBONSÄURE-AUSTAUSCHER FÜR DIE TRENNUNG Ca^{2+}/Sr^{2+}

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Der Einbau von Komplexbildnern in Harzgerüste führt zu Chelataustauschern[1-5], die zur Lösung schwieriger anorganisch-analytischer Probleme eingesetzt werden können.

Die Eigenschaften eines Chelatharzes werden vom Aufbau der komplexbildenden Ankergruppe und somit durch die Art und Weise ihres Einbaus in das polymere Harzgerüst beeinflusst. Zur Untersuchung dieser Abhängigkeit bei Pyridin-2,6-dicarbonsäure-Harzen wurden als Modellsubstanzen eine Reihe in 4-Stellung substituierter Pyridin-2,6-dicarbonsäuren (PDCS) herangezogen und die Komplexstabilitäten ihrer Erdalkalichelate durch Aufnahme von Titrationskurven bestimmt[6]. Bei den 4-Amino- und bei der 4-Hydroxy-PDCS ergaben sich verhältnismässig hohe Unterschiede zwischen den Komplexstabilitäten des Ca^{2+}- bzw. Sr^{2+}-Chelats. Darstellung und Eigenschaften eines Kondensationsharzes aus 4-Hydroxy-PDCS, Resorcin und Formaldehyd für die chromatographische Trennung Ca^{2+}/Sr^{2+} wurden schon beschrieben[2].

Um einen Chelatbildner fest in einem Harzgerüst zu verankern, wird er, falls geeignet, häufig mit Formaldehyd und einem Vernetzer zu einem Harzgel kondensiert[1-3,5]. Wird der Austauscher durch Polymerisation des Chelatbildners hergestellt, so wächst seine Widerstandsfähigkeit gegenüber mechanischer und chemischer Beanspruchung. Die monomeren Verbindungen sind jedoch sehr schwierig darzustellen. Eine weitere Methode für den Aufbau von Ionenaustauschern besteht darin, dass zunächst ein Harzgerüst durch Polymerisation hergestellt wird und die aktiven Gruppen durch Umsetzungen am Polymeren eingeführt werden. Nach diesem Prinzip wird der grösste Teil der gebräuchlichen Kationen- und Anionenaustauscher hergestellt.

Die einzige PDCS, die für nachträgliche Substitutionsreaktionen an Polymeren geeignet erscheint, ist die 4-Chlor-PDCS. Als fester polymerer Harzgrundkörper kann chlormethyliertes Polystyrol[4,7,8] eingesetzt werden, das nach Aminierung nochmals chloraryliert wird. Auf ähnliche Weise wird unlösliches Poly-aminostyrol, das aus Poly-nitrostyrol[9-12] oder monomerem Aminostyrol[6,13,14] zugänglich ist, weiterverarbeitet.

Analoge Substitutionen sind auch an flüssigen Polymeren möglich. Gegenüber festen Polymeren ergibt sich der Vorzug, dass das in flüssiger Phase gewonnene

Produkt eine grössere Einheitlichkeit aufweist. Der Substitution muss eine Vernetzungsreaktion folgen. Als Ausgangssubstanzen sind unvernetztes Polystyrol und Polyäthylenimin[15] in gelöster Form zu nennen.

In der vorliegenden Arbeit werden ausgehend von drei verschiedenen Polymeren (Polystyrol, chlormethyliertes Polystyrol und Polyäthylenimin) Chelatharze hergestellt, an denen die Struktur von drei verschiedenen 4-Amino-PDCS, 4-(N-Phenyl)-amino-PDCS, 4-Methylamino-PDCS und 4-Dimethylamino-PDCS, weitgehend realisiert ist.

Im Anschluss an eine frühere Veröffentlichung[3] über die Hydrazide aliphatischer Dicarbonsäuren und die von ihnen abgeleiteten Chelataustauscher ist auch das Verhalten des PDCS-dihydrazids als Harzbaustein von Interesse.

Die Trennleistung der hergestellten Harze wird anhand chromatographischer Versuche überprüft.

HERSTELLUNG UND EIGENSCHAFTEN VON CHELATHARZEN AUF 4-AMINO-PYRIDIN-2,6-DICARBONSÄURE-BASIS

Im folgenden wird die Herstellung von Austauscherharzen mit PDCS-Gruppen durch Umsetzung von 4-Chlor-PDCS mit drei verschiedenen polymeren Aminen, die teils in unlöslicher, teils in löslicher Form vorliegen, beschrieben.

(*a*) *Gerüstkörper Poly-aminostyrol*

4-Chlor-pyridin und Anilin vereinigen sich beim Erhitzen in lebhafter Reaktion zu 4-Anilino-pyridin[16]. Die Umsetzung von 4-Chlor-PDCS mit Anilin[6] verläuft ohne Schwierigkeiten. Poly-aminostyrol reagiert ebenfalls verhältnismässig leicht mit 4-Chlor-PDCS.

20 g eines Styrol-Perlpolymerisats (PERMUTIT P 2, Vernetzung 2 %) werden 24 Stunden im Soxhlet mit Chloroform extrahiert. Die Bestandteile der Nitriersäure, 50 ml HNO_3 (Dichte 1.52) und 125 ml H_2SO_4 (Dichte 1.84) werden auf —10° gekühlt, und zunächst die Schwefelsäure auf die Perlen gegeben, die sich in einem 1 l-Becherglas befinden. Unter Rühren wird die Salpetersäure hinzugefügt, langsam innerhalb 4 Stunden auf 100° erhitzt und nach dem Abkühlen über Nacht stehen gelassen. Das Reaktionsgemisch wird auf Eis gegossen, die Perlen werden in einer Säule mit Wasser neutral gewaschen und im Soxhlet 24 Stunden mit Ammoniak-Lösung extrahiert.

Als Reduktionsmittel dient eine Lösung von 32 g Schwefel und 240 g $Na_2S \cdot 9H_2O$ in 500 ml Wasser. In einem 1 l-Becherglas werden die nitrierten Perlen mit der Polysulfid-Lösung im Autoklaven unter 50 atm N_2 auf 150° erhitzt. Nach 24 h Reaktionsdauer werden die Poly-aminostyrol-Perlen mit Wasser neutral gewaschen, dann je 24 h mit Dioxan bzw. CS_2 extrahiert. Das Harz erscheint nur wenig dunkler als das Nitroprodukt. Die Analysenwerte der beiden Herstellungsstufen finden sich in Tabelle I.

Für die Umsetzung mit 4-Chlor-PDCS[17] werden die Poly-aminostyrol-Perlen in 50 ml Dioxan aufgeschwemmt, dann anschliessend 13 g Zinkpulver, 25 g 4-Chlor-PDCS und soviel Wasser hinzugefügt, dass durch Umrühren ein dickflüssiger Brei entsteht. Die Reaktion wird im Autoklaven unter den ob en Bedingungen durchgeführt. Das mechanisch sehr stabile Endprodukt wird durch mehrfaches Umladen mit

TABELLE I

ANALYSENWERTE DES NITRIERTEN BZW. REDUZIERTEN POLYSTYROLS

	% *C*	% *H*	% *N*
NO_2-Harz	51.0	3.59	10.97
(Theoret.)	64.5	4.7	9.4
NH_2-Harz	60.3	6.24	13.43
(Theoret.)	80.6	7.6	11.75

2 *N* HCl und 2 *N* NaOH vorbereitet und aus der H^+-Form mit 10 l destilliertem Wasser neutral gewaschen. Bei Ausgang von 20 g roher Polystyrol-Perlen ergibt sich eine Ausbeute von *ca.* 45 g lufttrockener Chelatharz-Perlen. Die Kapazitätswerte finden sich in Tabelle II.

Aus dem Molekulargewicht von 284 der theoretischen Austauschereinheit 4-(N-Styryl)-amino-PDCS folgt eine theoretische Kapazität des Harzes von 7.04 mVal/g. Unter der Annahme, dass die theoretische Struktur synthetisiert wurde, ist demnach bei der dreistufigen Darstellung des Chelatharzes eine Gesamtausbeute von ca. 50 %, bezogen auf Styrol, erreicht worden.

TABELLE II

KAPAZITÄTEN VON PYRIDIN-2,6-DICARBONSÄURE-HARZEN

Gerüstkörper	*Form des AT*	*Ca^{2+}-Kap. (mVal/g)*	*Cu^{2+}-Kap. (mVal/g)*
Poly-amino-styrol	lufttrocken	2.90	3.04
	vakuumtrocken	3.58	3.76
Poly-(äthylendiamino-methyl)-styrol	lufttrocken	1.18	1.69
	vakuumtrocken	1.37	1.97
Polyäthylenimin	lufttrocken	3.13	3.67
	vakuumtrocken	4.18	4.91
PDCS-dihydrazid-Kondensationsharz	lufttrocken	1.19	3.55
	vakuumtrocken	1.52	4.50

(*b*) *Gerüstkörper Poly-(äthylendiaminomethyl)-styrol*

50 g chlormethyliertes Styrol-Perlpolymerisat (PERMUTIT 446, Vernetzung 2 %) werden mit Äthylendiamin umgesetzt[4], danach in Dioxan vorgequollen, und 25 g 4-Chlor-PDCS untergerührt. Das Gemisch wird 48 h im Autoklaven unter den beschriebenen Bedingungen umgesetzt. Der Anteil der zerstörten Harzkugeln im Produkt ist ungewöhnlich hoch, seine mechanische Widerstandsfähigkeit weitaus geringer als die der Harze aus Poly-aminostyrol. Auch die Kapazität (Tabelle II) ist vergleichsweise gering.

(*c*) *Gerüstkörper Polyäthylenimin*

Zur Ermittlung der optimalen Herstellungsbedingungen wurden Poly-äthylenimin und 4-Chlor-PDCS in wechselnden molaren Verhältnissen zur Reaktion gebracht. Eine vollständige Umsetzung der 4-Chlor-PDCS ist erst bei einem 3,5-fachen Überschuss des polymeren Amins zu erreichen.

In einem 250 ml-Becherglas werden 25.3 g (0.125 Mol) 4-Chlor-PDCS, 37.8 g

(0.438 Mol) 50 %-ige wässrige Polyäthylenimin-Lösung (Molekulargewicht 30–40,000) und 5 g NaOH mit ca. 100 ml Wasser unter Erwärmen zu einer klaren Flüssigkeit verrührt, die im Autoklaven 24 h unter den oben genannten Bedingungen auf 150° erhitzt wird. Zur Vernetzung wird das aus einer bräunlichen klaren zähen Lösung bestehende Produkt in einer Porzellanschale mit 50 ml Methanol und 30 ml (0.38 Mol) Epichlorhydrin versetzt und unter Rühren auf 60° erhitzt. Nach ca. 30 min. klärt sich das Zweischichtengemisch zu einer einheitlichen Lösung, die nach weiteren 30 min langsam zäher wird. Die viskose Masse härtet in 10 Tagen bei 60° im Trockenschrank aus. Die Rohausbeute beträgt 71 g. Das Harz wird durch Mahlen und Sieben auf eine Korngrösse von 0.3–0.5 mm Durchmesser gebracht, und dann in einer Säule im Durchlauf mit Leitungswasser behandelt, mehrfach mit 2 *N* HCl und 2 *N* NaOH umgeladen und mit destilliertem Wasser neutral gewaschen.

Das Chelatharz besteht aus hellgelben, sehr quellfähigen, aber mechanisch stabilen Körnern und besitzt eine hohe Kapazität (Tabelle II). Die um 17 % über dem Ca^{2+}-Wert liegende Cu^{2+}-Kapazität des Harzes deutet darauf hin, dass die Polyäthyleniminkette zusätzlich als Chelatbildner wirken kann. Die Cu^{2+}-Form des Harzes ist von tiefdunkelblauer Farbe.

Legt man für die Berechnung der theoretischen Kapazität des Chelatharzes die Herstellungsvorschrift zugrunde, nach der auf eine PDCS-Gruppe 3.5 Äthylenimin-Einheiten des Polymeren und ein bis zwei Vernetzermoleküle entfallen, so ergeben sich aus den theoretischen Molekulargewichten von 373 bzw. 431 die Kapazitäten von 5.36 bzw. 4.64 mVal/g.

HERSTELLUNG UND EIGENSCHAFTEN EINES PYRIDIN-2,6-DICARBONSÄURE-DIHYDRAZID-KONDENSATIONSHARZES

Ein Austauscher auf PDCS-dihydrazid-Basis sollte den Eigenschaften seines Grundbausteins entsprechend[6] eine ausgeprägte Selektivität für Kationen der Nebengruppenelemente aufweisen. Da PDCS-dihydrazid keinerlei chemische Gruppierungen besitzt, die den Aufbau eines Harzgerüstes nach einer der oben angewendeten Methoden ermöglichen, muss das Harz durch Kondensation hergestellt werden.

Eine Mischung von 16 g (0.08 Mol) PDCS-dihydrazid[18], 17 g (0.16 Mol) Resorcin, 5 g NaOH in 100 ml Wasser und 60 ml 35 %-iger Formaldehyd-Lösung wird 15 h in einer Porzellanschale auf dem Wasserbad erhitzt. Das entstandene rote durchsichtige Harz härtet in weiteren 24 h im Trockenschrank bei 80° aus. Die Rohausbeute beträgt 54 g.

Das Harz wird zu einer Körnung von 0.3–0.5 mm Durchmesser gebrochen, in einer Säule zunächst mit 2 *N* HCl behandelt, dann mehrfach mit 2 *N* NaOH und 2 *N* HCl umgeladen und schliesslich mit destilliertem Wasser neutral gewaschen. Das rotgefärbte Harz ist empfindlich gegen konzentrierte Laugen, sonst aber mechanisch und chemisch beständig. Die Cu^{2+}-Kapazität des Harzes (Tabelle II) beträgt 300 % des Ca^{2+}-Wertes.

CHROMATOGRAPHISCHE TRENNUNGEN

An den hergestellten Chelataustauschern wurden chromatographische Modell-Trennungen durchgeführt. Nicht näher geprüft wegen seiner geringen Kapazität und

mechanischen Instabilität wurde das Chelatharz mit dem Gerüstkörper Poly-(äthylendiaminomethyl)-styrol.

Für die Versuche wurden jeweils ca. 60 ml (V_B) des Chelatharzes in einer 50-ml-Bürette unter den bereits beschriebenen Bedingungen[2,5] eingesetzt. Die Durchlaufgeschwindigkeit des Elutionsmittels betrug ca. 1.0 ml/min · cm².

(a) Gerüstkörper Poly-aminostyrol

Elutionsversuche mit Ca^{2+} und Sr^{2+} am PDCS-Harz mit dem Gerüstkörper Polyaminostyrol ergaben, dass eine Trennung dieser Ionen am Harz nicht möglich ist, Erdalkali- von Alkaliionen sich jedoch schnell und sicher abtrennen lassen. Als Modellanalyse wird eine $^{134}Cs^{+}/Ba^{2+}$-Trennung (Fig. 1, Tabelle III) beschrieben.

TABELLE III

VERSUCHSBEDINGUNGEN DER TRENNUNG $^{134}Cs^{+}/Ba^{2+}$

Harzgerüst	*Poly-aminostyrol*
Aufgabe	1 μC $^{134}Cs^{+}$ + 1 mg Träger 2 mVal Ba^{2+} (als Nitrat) in 2 ml
Cs-Elutionsmittel (V_E)	200 ml 0.2 *M* Ammonacetat pH 4.0
Ba-Elutionsmittel (V_E)	150 ml 1 *N* HNO_3

Nach Aufgabe des Ionengemisches wird $^{134}Cs^{+}$ schnell mit 0.2 *M* Ammonacetat-Lösung von der Säule gelöst. Ba^{2+} verbleibt auf dem Harz und wird schliesslich mit 1 *N* HNO_3 eluiert. Der Zeitbedarf der Trennung einschliesslich Bestimmung beträgt nicht mehr als 2 h. Nach dem Neutralwaschen mit destilliertem Wasser und Vorlauf von Elutionsmittel ist die Säule erneut gebrauchsfähig.

(b) Gerüstkörper Polyäthylenimin

Die Untersuchung des Chelatharzes mit dem Gerüstkörper Polyäthylenimin zeigt, dass an diesem Harz die Ionen Ca^{2+} und Sr^{2+} — auch wenn ein grosser Ca^{2+}-Überschuss vorliegt — getrennt werden können (Fig. 2, Tabelle IV). Die Güte der

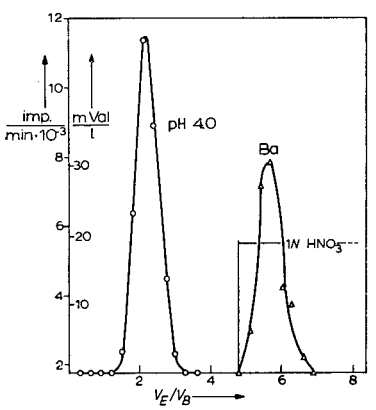

Fig. 1. Trennung 1 μC ^{134}Cs/2 mVal Ba^{2+}.

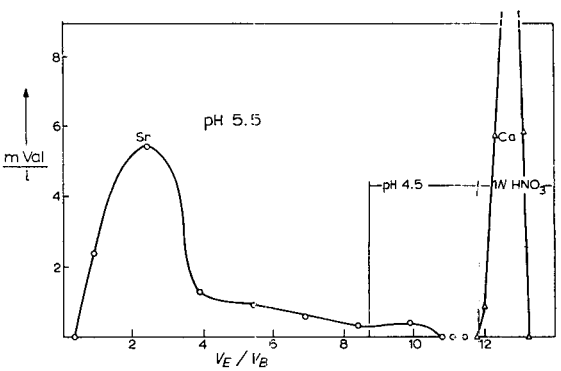

Fig. 2. Trennung von je 1 mVal Ca^{2+}/Sr^{2+}.

Trennung ist stark pH-abhängig. Die optimale Trennschärfe wird bei pH 5 erreicht. Um das Nachschleppen des Sr^{2+} abzukürzen, werden die letzten Sr^{2+}-Reste bei pH 4.5 von der Säule gewaschen.

TABELLE IV

VERSUCHSBEDINGUNGEN DER TRENNUNG VON JE 1 mVal Ca^{2+}/Sr^{2+}

Harzgerüst	*Polyäthylenimin*
Aufgabe (Fig. 2)	1 mVal Ca^{2+} + 1 mVal Sr^{2+} in 2 ml
Sr-Elutionsmittel (V_E)	530 ml 0.2 *M* Ammonacetat pH 5.5
Ca-Elutionsmittel (V_E)	130 ml 0.2 *M* Ammonacetat pH 4.5 100 ml 1 *N* HNO_3

Da die Trennung Ca^{2+}/Sr^{2+} für die quantitative Bestimmung von ^{90}Sr in natürlichen und technischen Wässern von erheblicher Bedeutung ist, wurde zur weiteren Überprüfung der Leistungsfähigkeit des Polyäthylenimin-Harzes ein Gemisch von $^{90}Sr^{2+}$ mit einem grossen Ca^{2+}-Überschuss eingesetzt (Fig. 3, Tabelle V).

TABELLE V

VERSUCHSBEDINGUNGEN DER TRENNUNG, BZW. ANREICHERUNG $^{90}Sr^{2+}/Ca^{2+}$

Harzgerüst	*Polyäthylenimin*
(*a*) *Anreicherung (Fig. 3a)*	
Aufgabe	10^{-3} μC $^{90}Sr^{2+}$ + 2 mg Träger + 10 mVal Ca^{2+} (als Nitrate) in 10 ml
Elutionsmittel (V_E)	570 ml 0.2 *M* Ammonacetat pH 5.5 180 ml 1 *N* HNO_3
(*b*) *Trennung (Fig. 3b)*	
Aufgabe	10^{-3} μC $^{90}Sr^{2+}$ + 2 mg Träger + 4 mVal Ca^{2+} (als Nitrate) in 4 ml
Sr-Elutionsmittel (V_E)	800 ml 0.2 *M* Ammonacetat pH 5.5
Ca-Elutionsmittel (V_E)	180 ml 1 *N* HNO_3

10^{-3} μC $^{90}Sr^{2+}$ und 10 mVal Ca^{2+} werden auf den Säulenkopf gegeben und bei pH 5.5 eluiert. Die Harzpackung wird durch den Ca^{2+}-Überschuss überlastet. In den ersten Fraktionen erscheinen Ca^{2+} und Sr^{2+} gemeinsam. Nachdem das gesamte Sr^{2+} eluiert ist, verbleiben 64 % des aufgegebenen Ca^{2+} auf der Säule und können so von den Sr^{2+}-Spuren abgetrennt werden (Fig. 3a).

Die vollständige Trennung gelingt an der 60-ml-Säule, wenn die aufgegebene Ca^{2+}-Menge auf 4 mVal verringert wird. Wie Fig. 3b zeigt, wird $^{90}Sr^{2+}$ in ca. 800 ml Puffer von der Säule gewaschen, ohne dass Ca^{2+} durchbricht. Aufgrund der hohen Ca^{2+}-Belastung der Säule ist die Sr^{2+}-Elution in beiden Fällen schneller als bei pH 5.5 erwartet beendet.

Mit Hilfe der beschriebenen Anordnung ist es somit möglich, Sr^{2+}-Spuren aus einem grossen Ca^{2+}-Überschuss aufzukonzentrieren, indem man zwei Elutionen hintereinander ausführt. Nach der ersten Trennoperation werden die ersten 800 ml

des Eluats eingedampft und, nachdem das abgetrennte Ca^{2+} mit Säure abgelöst worden ist, wieder auf die Säule gegeben. Da das Elutionsmittel nur wenig Ammoniumacetat und eine flüchtige Säure enthält, hinterbleiben beim Eindampfen nur geringe Rückstände. Es entfallen die Nachteile der üblichen chromatographischen Verfahren für die Ca^{2+}/Sr^{2+}-Trennung, bei denen der in der flüssigen Phase befindliche Komplexbildner vor der Bestimmung der Ionen zerstört werden muss.

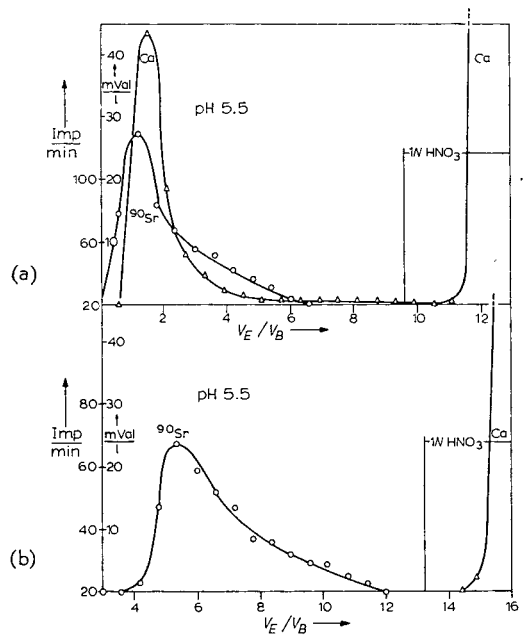

Fig. 3. (a) Anreicherung 10^{-3} μC ^{90}Sr/10 mVal Ca^{2+}; (b) Trennung 10^{-3} μC ^{90}Sr/4 mVal Ca^{2+}.

Entsprechend den Eigenschaften seines chelatbildenden Grundkörpers kann das PDCS-dihydrazid-Harz für die Trennung von Me^{2+}-Ionen der Nebengruppen, z.B. Co^{2+}/Ni^{2+}, eingesetzt werden.

DISKUSSION

Beim Vergleich des Verhaltens der hergestellten Chelataustauscher mit den Eigenschaften des den aktiven Gruppen des Harzes zugrundeliegenden Komplexbildners erweist sich, dass eine weitgehende Übereinstimmung des Komplexbildungsverhaltens von Chelatharz und monomolekularem Komplexbildner nur auftritt, wenn die Harzsynthese ohne Nebenreaktionen, die nichterwünschte Gruppen in das Harz einführen, verläuft.

Anomales Verhalten zeigt das Harz mit dem Grundkörper 4-(N-Phenyl)-amino-PDCS. Entgegen den Erwartungen ermöglicht dieses Harz keine Ca^{2+}/Sr^{2+}-Trennung und bindet die Erdalkaliionen fester als erwartet. Die Gründe für das von der Theorie abweichende Verhalten liegen in der Herstellung des Harzes. Bedingt durch den hochpolymeren Reaktionspartner kann während der Substitution durch

4-Chlor-PDCS eine unerwünschte Nebenreaktion eintreten: Anstelle des Chlor-Atoms in 4-Stellung reagiert eine der Carboxylgruppen mit der NH_2-Gruppe des Harzes zum Anilid, das ebenfalls einen Chelatbildner darstellt.

Die Substitution des flüssigen Polymeren Polyäthylenimin mit 4-Chlor-PDCS liefert ein Chelatharz, das in seinen Eigenschaften den theoretischen Erwartungen am nächsten kommt. Das Harzprodukt ist einheitlich aufgebaut und entspricht in seinem Komplexbildungsverhalten dem theoretischen Grundkörper 4-Dimethylamino-PDCS.

Das chromatographische Verhalten des PDCS-dihydrazid-Kondensationsharzes beweist, dass die Nebengruppenionen-Selektivität des Monomeren durch die Kondensationsreaktion nicht unterdrückt wird.

Die Kapazität eines Chelatharzes bestimmt die Aufgabemenge an Zweikomponentengemisch, die auf einer Säule von vorgegebenen Dimensionen noch einwandfrei getrennt werden kann. Gegenüber dem 4-Hydroxy-PDCS-Kondensationsharz[2] weist das Polyäthylenimin-Harz (Grundkörper 4-Dimethylamino-PDCS) eine erheblich gesteigerte Ca^{2+}-Kapazität auf (Tabelle VI). Entsprechend erhöht sich auch die Belastbarkeit der in der 50-ml-Bürette befindlichen Harzpackung.

TABELLE VI

KAPAZITÄTEN (VAKUUMTROCKEN) UND TRENNVERMÖGEN (60-ml-SÄULE) VON PYRIDIN-2,6-DICARBONSÄUREHARZEN

Harzgrundkörper	*mVal Ca^{2+}/g*	*1:1-Trennung*	*Überschuss bei Spurenabtr.*
4-(N-Phenyl)-amino-PDCS	3.58	—	2 mVal $Ba^{2+}/(^{134}Cs^{+})$
4-Dimethyl-amino-PDCS	4.18	je 1 mVal Ca^{2+}/Sr^{2+}	4 mVal $Ca^{2+}/(^{90}Sr^{2+})$
4-Hydroxy-PDCS	2.5	je 0.5 mVal Ca^{2+}/Sr^{2+}	2.5 mVal $Ca^{2+}/(^{90}Sr^{2+})$

Eine weitere wesentliche Erhöhung der Trennkapazität für Ca^{2+}/Sr^{2+} an Chelataustauschern dürfte nur zu erreichen sein, falls es gelingt, einen makromolekularen Komplexbildner zu synthetisieren, bei dem erhöhte Unterschiede in den Komplexstabilitäten zwischen Ca^{2+} und Sr^{2+} ($\Delta pK_{MeL} > 1.2$) auftreten[6].

DANK

Die vorliegende Arbeit wurde im Anorganisch-Chemischen Institut der Technischen Universität Berlin durchgeführt. Für die Bereitstellung von Mitteln und Apparaturen danken wir dem Bundesministerium für Wissenschaftliche Forschung, der Verwaltungsstelle für ERP-Vermögen und der Deutschen Forschungsgemeinschaft.

ZUSAMMENFASSUNG

Durch Umsetzung von Poly-aminostyrol, Polyäthylenimin und Poly-(äthylendiaminomethyl)-styrol mit 4-Chlor-pyridin-2,6-dicarbonsäure konnten drei chelatbildende Austauscherharze dargestellt werden, die im Aufbau ihres austauschaktiven

Grundkörpers angenähert der 4-(N-Phenyl)-amino-, 4-Dimethylamino-, bzw. der 4-Methylamino-pyridin-2,6-dicarbonsäure entsprechen. Als viertes Chelatharz wurde ein Mischkondensat aus Pyridin-2,6-dicarbonsäure-dihydrazid, Resorcin und Formaldehyd hergestellt.

Mit einer Ausnahme [Gerüstkörper Poly-(äthylendiamino-methyl)-styrol] besitzen sämtliche beschriebenen Chelatharze gute mechanische und chemische Eigenschaften sowie eine hohe Kapazität.

An den Pyridin-2,6-dicarbonsäure-Harzen auf Poly-aminostyrol- und Polyäthylenimin-Basis wurden chromatographische Trennungen durchgeführt. Vor allem das Polyäthylenimin-Harz ist für die Ca^{2+}/Sr^{2+}-Trennung gut geeignet. Es hat eine beachtliche Ca^{2+}-Kapazität (4.2 mVal/g) und kann daher zur Trennung grösserer Mengen eingesetzt werden.

SUMMARY

Three chelating resins were produced by reaction of poly-aminostyrene, polyethyleneimine and poly-(ethylenediaminomethyl)-styrene with 4-chloropyridine-2,6-dicarboxylic acid. The structures of the exchanging units of these resins approximate the structures of 4-(N-phenyl)-amino-, 4-dimethylamino- and 4-methylamino-pyridine-2,6-dicarboxylic acid. A fourth resin was prepared by mixed condensation of pyridine-2,6-dicarboxylic acid dihydrazide, resorcinol and formaldehyde. With one exception [matrix poly-(ethylenediaminomethyl)-styrene] the chelating resins described are of good mechanical and chemical stability and possess high capacity.

Chromatographic separations were performed in columns containing pyridine-2,6-dicarboxylic acid resins with the matrixes poly-aminostyrene and polyethyleneimine respectively. The polyethyleneimine resin especially is well suited for the chromatographic separation of Ca^{2+}/Sr^{2+}. Its Ca^{2+}-capacity is remarkably high (4.2 mequiv./g), so that it can be used for the separation of fairly large quantities.

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Notes

Gas chromatography of derivatives of maleic hydrazide

I. Alkyl carbonates

Maleic hydrazide is a growth regulant or retardant used to arrest sprouting in stored root crops (*e.g.*, potato, carrot, onion and beet) as well as for its extensive utility in the control of sucker growth in tobacco.

The analytical determination of maleic hydrazide may be accomplished by spectrophotometric means[1-3] via coupling *p*-dimethylaminobenzaldehyde with hydrazide, the hydrolytic product of maleic hydrazide. In addition, a semiquantitative paper chromatographic method for the estimation of maleic hydrazide has been developed by ANDREAE[4].

Diverse toxicological studies with maleic hydrazide in our laboratory have dictated the desirability of developing gas-chromatographic procedures for maleic hydrazide with ultimate extension of the possible application of this technique to residue samples of biological origin. Initial efforts to measure maleic hydrazide *per se* by gas chromatography have proven difficult due to its general insolubility in most organic solvents and to its very high melting point (296°–298°); hence, possible chromatographable derivatives were investigated.

Experimental

The alkyl carbonate derivatives of maleic hydrazide (1,2-dihydro-3-oxo-6-pyridazinyl) carbonates were prepared by treating equimolar quantities of maleic hydrazide and the respective alkyl chloroformate in aqueous potassium hydroxide at 0° to +5° according to the procedure of STEFANYL AND HOWARD[5].

The analyses were carried out on chlorophenyl silicone (Versilube F-50) and cyanoethylmethyl silicone (XE-60) columns. Both columns were $^1/_8$ in. O.D. by 4 ft. copper tubing packed with 60–80 mesh HMDS-treated Chromosorb W with 15 % substrate coating and housed in an F & M model 720 oven containing a hot-wire detector. The oven was coupled to an F & M model 500 gas chromatography unit. Specific operating conditions for the columns are given in the foot-notes of Table I.

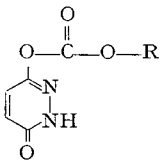
Results and discussion

Maleic hydrazide has been reported[6-8] to exist in the following tautomeric states:

(I) (II)

TABLE I

GAS CHROMATOGRAPHY OF ALKYL CARBONATE DERIVATIVES



R	Lit. m.p. (°C)	Found m.p.[b] (°C)	Relative elution[a] Versilube F-50[c]	XE-60[c]
Methyl	105–107[d]	121–122	0.60	0.47
Ethyl	106–108[e], 98–105[d]	101–103	0.69	0.67
Propyl	79–81[e]	79–81	1.0	1.0
Butyl	84–85[e]	83.5–85	1.6	1.4
Amyl	49–51[e]	48–50	2.5	2.0
3-Chloropropyl	99–100[e]	98–99	11.75[f]	11.1[f]

[a] Relative to the propyl carbonate derivative (propyl-2,3-dihydro-3-oxo-6-pyridazinyl carbonate) as 1.0. Elution of the propyl carbonate derivative was 7.2 min on Versilube F-50 and 4.7 min on XE-60.

[b] Maleic hydrazide melted at 296–298°.

[c] 15% w/w on 60–80 mesh Chromosorb-W (HMDS pretreated), 4 ft. by 0.125 in. O.D. copper column. Operating conditions: column 175°; injection port 270°; detector 200°; filament current 150 mA; hot-wire detector; helium 1.7 ml/min for Versilube F-50; helium 16.4 ml/min for XE-60.

[d] Ref. 10.

[e] Ref. 5.

[f] Determined at a column temperature of 205°.

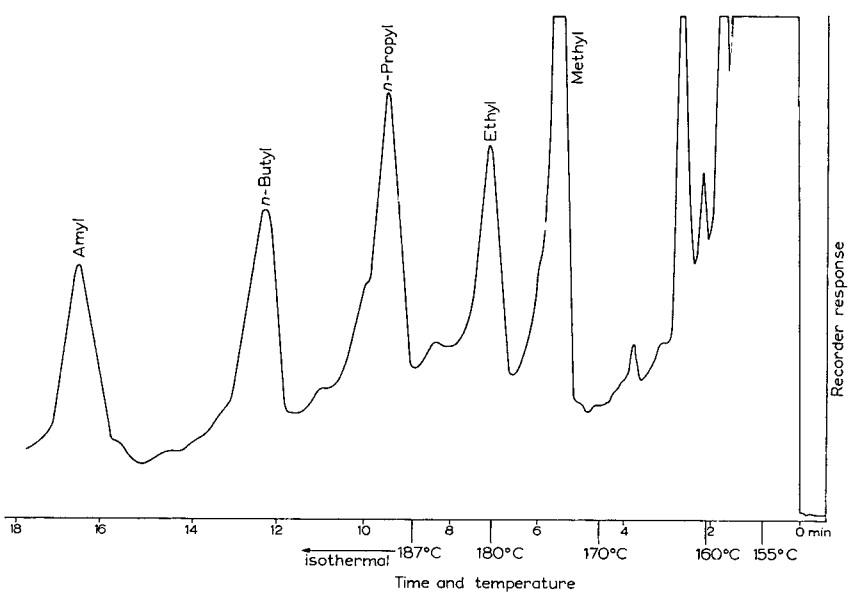


Fig. 1. Composite chromatogram of maleic hydrazide alkyl carbonates. Column: 4 ft. by 0.125 in. O.D. copper, packed with 15% Versilube F-50 coated on 60–80 mesh HMDS-treated Chromosorb W. Conditions: Column programmed at 4°/min, 155–187°; detector 250°; injection port 265°; hot-wire detector; filament current 170 mA; helium 16 ml/min.

The monoenolic properties of maleic hydrazide (II) have been reported to afford facile acylation[5,9–12] yielding exclusively O-substituted products, *e.g.*, alkyl carbonates[5,9,10], benzene sulfonates[10,12] and simple esters[11]. The present study covers the gas-chromatographic behavior of alkyl carbonate derivatives of maleic hydrazide. The latter appear to possess higher solubilities in the commonly employed solvents and have sharply lowered melting points.

The effect of acylation on the lowering of the melting point of maleic hydrazide may be seen in Table I. This fact greatly enhances the feasibility of gas-chromatographic analysis for maleic hydrazide via the route of an appropriate derivative.

The gas-chromatographic data are included in Table I. Facile separations were observed for the methyl through amyl carbonate derivatives, a composite chromatogram of which is presented in Fig. 1.

A graph of the log retention time *vs.* alkyl chain length of the alkyl carbonate moiety appears in Fig. 2. It is apparent from these results that alkyl carbonate derivatives of maleic hydrazide are quite amenable to gas-chromatographic analysis.

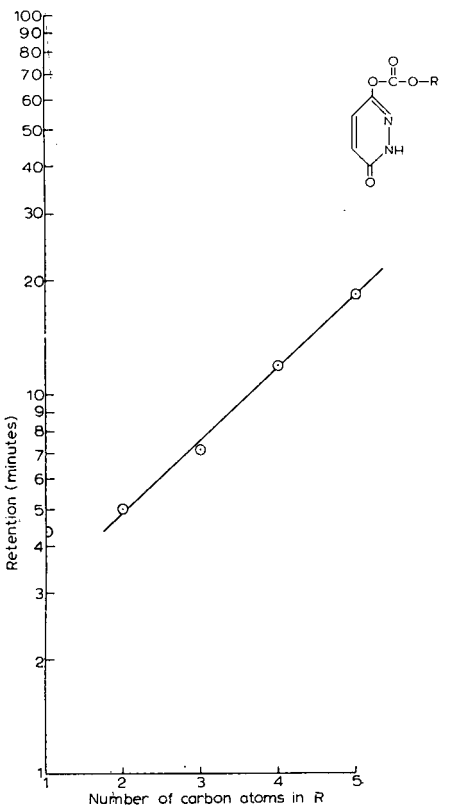


Fig. 2. Retention *versus* *n*-alkyl chain length for *n*-alkyl carbonate derivatives of maleic hydrazide.

The potential utility of this approach to the analysis of maleic hydrazide residues is obvious. This work is being extended to other classes of derivatives (*e.g.* halogenated esters and benzoates) in order that their susceptibility to gas-chromatographic

analysis may be determined. Several chloroalkyl carbonate derivatives have thus been investigated. The elution for the 3-chloropropyl carbonate derivative is given in Table I. It would not be unreasonable to except that such halogenated derivatives would be amenable to gas-chromatographic analysis employing an electron capture detector and this course of investigation for maleic hydrazide residues is presently being investigated.

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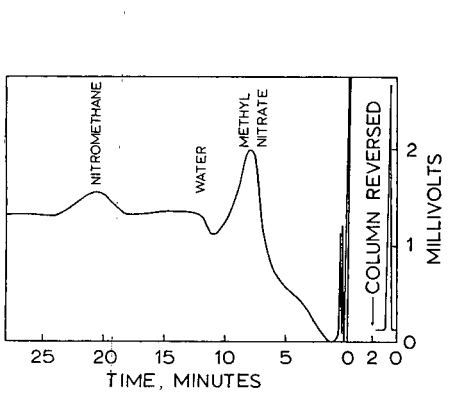
Gas chromatographic estimation of nitromethane in large excesses of nitrogen dioxide

In recent experiments on the reactions of methyl radicals with nitrogen dioxide[1] it was required to analyse up to 7 μmoles nitromethane in the presence of up to 70 μmoles nitrogen dioxide using a Perkin Elmer Model No. 116 Fraktometer. When the nitrogen dioxide concentration was low, say less than 15 μmoles, it was possible to introduce the sample directly into the chromatograph. The retention times of nitrogen dioxide and nitromethane were sufficiently different to have a complete separation. However, when the nitrogen dioxide concentration was large, nitromethane estimation was impossible because of severe tailing of the nitrogen dioxide. The tailing was perhaps due to reaction of nitrogen dioxide with the column packing (2 m Perkin Elmer F, tetraethylene glycol monomethyl ether on Embacel at 64°). It was attempted to absorb preferentially the nitrogen dioxide on a column of resorcinol but this was unsuccessful.

The problem was overcome using a column reversal technique. The mixture of nitromethane, nitrogen dioxide and other reaction products such as nitric oxide,

neopentane, methyl nitrate and water was passed through a 0.5 m column of Perkin Elmer S, silica gel poisoned with hexamethyl sebacate. The carrier gas was hydrogen, inlet pressure 0.5 kg/cm², flow rate 230 cc/min, column temperature 64°. After 2 min, some of the products such as nitric oxide, nitrogen dioxide and neopentane had eluted as a single peak (see Fig. 1). The other products, including nitromethane, were still adsorbed on the column. The column was then reversed and coupled in series with the 2 m tetraethylene glycol monomethyl ether in the oven at 64°. With the inlet pressure still at 0.5 kg/cm² the flow rate fell to 75 cc/min. The products were eluted from the poisoned silica gel on to the separating column. The base line drifted considerably during the first 15 min when the methyl nitrate and water were eluted but had stabilised by the time the nitromethane peak appeared.

A good straight-line calibration graph of μmoles nitromethane plotted against



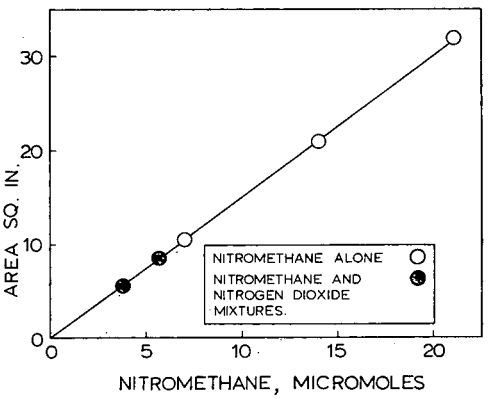


Fig. 1. A chromatogram showing separation of nitromethane from other reaction products using the column reversal technique.

Fig. 2. A calibration of nitromethane concentration *vs.* peak area for nitromethane alone and mixtures of nitromethane and nitrogen dioxide.

peak area was obtained using freshly distilled nitromethane (British Drug Houses) (see Fig. 2). Known synthetic mixtures of nitromethane in a ten-fold excess of nitrogen dioxide were analysed using the column reversal technique. The results (see Fig. 2) were in good agreement with the calibration.

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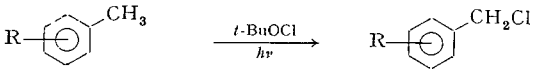
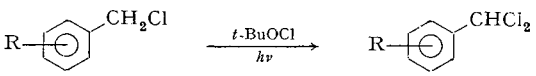
Photochlorinations of substituted toluenes: structure assignments on the basis of gas chromatographic retention times

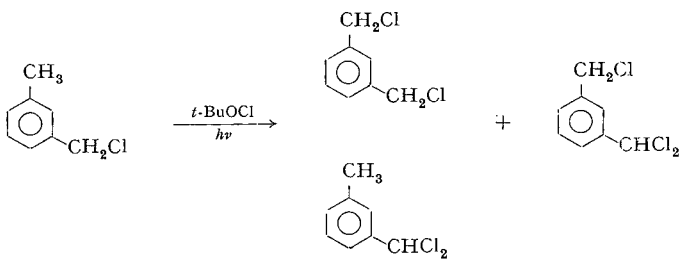
In the course of light-catalyzed free radical chlorinations of substituted toluenes by means of *tert.*-butyl hypochlorite[1,2] to give the corresponding benzyl chlorides,



the occurrence of multiple substitution in the side-chain, to give the corresponding benzal chlorides, is prevented by the use of a large excess (approximately three molar



equivalents) of the toluene. This technique works well in simple cases, where only one product is formed; however, in more complex cases, *e.g.* where the presence of both methyl and methylene groups presents alternate sites of chlorination, as in *m*-methylbenzyl chloride, at least two products are obtained and the identity of the peaks in the



chromatogram is no longer obvious. The boiling points of the isomeric products are, at best, very close (*cf.* the well-known b.p.'s of benzal chloride and *p*-chlorobenzyl chloride) and no useful correlation exists between infrared absorption spectra and the grouping $ArCH_2Cl$. Lacking nuclear magnetic resonance instrumentation, the development of a rapid gas chromatographic technique for the analysis of reaction mixtures and the assignment of structures of the individual components of the chromatogram seemed imperative.

Experimental

The reactive nature of the benzyl chlorides led to the selection of the inert, thermally stable liquid phase QF-1 (FS-1265) coated on Chromosorb W (60–80 mesh) which had been treated with hexamethyldisilazane; the coiled columns were made of aluminum. Two basic column-sizes were employed: a 610 cm × 0.95 cm preparative column used for the more volatile samples as well as the collection of samples for infrared analysis, and a 150 cm × 3 mm column to perform rapid-scan evaluations as well as analyses of the higher-boiling products. The concentrations of liquid phase were 18% and 20%, as noted. The carrier-gas was helium, at inlet pressure of 2 atm,

used at 120 ml/min for the preparative column, and 27 ml/min in the analytic column. The respective column temperatures were 193° and 175°. The thermal conductivity detector was operated at 298° throughout, the strip-chart recorder at a speed of 2.54 cm/min.

The instrument used for the preparative as well as for the analytical runs was an Aerograph Autoprep A-700, manufactured by Wilkens Instruments.

Discussion

The data presented in Tables I–III show a steady upward progression of retention time ratios, the upward progression being roughly proportional to increasing molecular weight and increasing polarity. Although no attempt was made to meet KOVÁTS'[3] stringent requirements of exact reproducibility of temperature, gas flow, etc., the precautions taken being of a routine, everyday nature, the data nevertheless exhibit a remarkable degree of constancy, predictability, and usefulness.

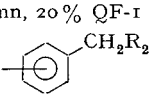
The problem of establishing the structures of several products arising from photochlorination with *tert.*-butyl hypochlorite first arose in the analysis of the products obtained from the photochlorination of *m*-xylene. The reaction mixture contained, besides unreacted *m*-xylene, peaks at 7.9 min, 11.0 min and 20 min. The first, or 7.9 min peak could safely be assigned to 3-methylbenzyl chloride; this assignment was in agreement with the expected retention time and confirmed by injecting a known sample of 3-methylbenzyl chloride.

Of the remaining two peaks, chemical intuition as well as retention time ratios

TABLE I

RETENTION TIMES OF SUBSTITUTED TOLUENES AND BENZYL CHLORIDES

610 cm × 0.95 cm column, 20% QF-1

Substituted toluenes 

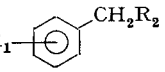
R_1	*Retention times (min)*		
	$R_2 = H$	$R_2 = Cl$	*Ratio*
H	3.6	6.7	1.9
3-Methyl	3.9	7.9	2.0
2,4-Dichloro	8.0	16.8	2.1
4-Chloro	5.4	11.5	2.1
3-Chloro	5.3	11.6	2.2
3-Bromo	6.6	14.8	2.2
3,4-Dichloro	9.3	21.1	2.3
2,5-Dichloro	7.3	16.6	2.3
4-Isopropyl	5.2	11.9[a]	2.3
2,6-Dichloro	7.7	17.7	2.3
2-Cyano	13.3	31.3	2.35
1-Methylnaphthalene	16.3	38.1	2.34

[a] Sole product isolable under gas chromatographic conditions. In the photochlorination of *p*-cymene one would expect attack on the tertiary carbon of the isopropyl group as well as on the methyl group; that this actually happens was indicated by the infrared spectrum of the chlorination product, which contained several peaks not assignable to *p*-isopropylbenzyl chloride. This second chlorination product, however, appears to have undergone dehydrochlorination on the column, presumably followed by polymerization of the thus obtained methylstyrene.

TABLE II

RETENTION TIMES OF SUBSTITUTED TOLUENES AND BENZYL CHLORIDES

610 cm × 0.95 cm column, 18% QF-1

Substituted toluenes 

R_1	*Retention times (min)*		
	$R_2 = H$	$R_2 = Cl$	*Ratio*
H	3.3	6.0	1.8
4-Fluoro	3.4	6.4	1.9
3-Methyl	3.8	7.8	2.1
2,4-Dichloro	7.2	15.3	2.1
2,5-Dichloro	7.2	15.4	2.1
4-Chloro	4.8	10.5	2.2
3,5-Dimethyl	4.0	8.8	2.2
3-Chloro	4.8	10.6	2.2
3,4-Dichloro	8.5	19.1	2.3
3-Bromo	6.1	13.9	2.3
4-Isopropyl	4.7	10.8[a]	2.3
4-Bromo	6.0	13.9	2.3
2-Carbethoxy	13.2	32.0	2.42
3-Chloromethyl	7.8	19.6	2.5
2-Chloro-4-methyl	7.8	20.1[b]	2.6
1-Methylnaphthalene	14.6	37.4	2.56

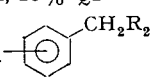
[a] See footnote a, Table I.

[b] Presumably unresolved peak; sum of 2-chloro-4-methylbenzyl chloride and 3-chloro-4-methylbenzyl chloride.

TABLE III

RETENTION TIMES OF SUBSTITUTED TOLUENES AND BENZYL CHLORIDES

150 cm × 3 mm column, 20% QF-1

Substituted toluenes 

R_1	*Retention times (min)*		
	$R_2 = H$	$R_2 = Cl$	*Ratio*
4-Fluoro	0.39	0.76	1.9
3-Methyl	0.43	1.00	2.3
2-Nitro	2.00	4.66[a]	2.33
2,4-Dichloro	0.86	2.11	2.5
3,4-Dichloro	1.06	2.63	2.48
2,6-Dichloro	0.85	2.15	2.5
3,5-Dimethyl	0.48	1.21	2.5
4-Isopropyl	0.53	1.37[b]	2.6
2-Chloro-4-methyl	0.98	2.54[c]	2.6
2-Carbethoxy	1.84	4.89	2.66
3-Bromo	0.70	1.89	2.7
4-Chloro	0.52	1.42	2.7
3-Chloromethyl	1.00	3.00	3.00
1-Methylnaphthalene	2.00	5.30	2.65

[a] Not obtained by photochlorination, but by purchase.

[b] See footnote a, Table I.

[c] See footnote b, Table II.

dictated the assignment of the 3-methylbenzal chloride structure to the 11 min peak, and α,α′-xylidine dichloride to the 20 min peak. Confirmation was obtained by isolating the 11 min peak by preparative scale gas chromatography and comparing the infrared spectrum and gas chromatographic retention time of the compound thus obtained with the corresponding data for 3-methylbenzal chloride synthesized from *m*-tolualdehyde by treatment with phosphorus pentachloride in ether.

The data obtained for the 3-methylbenzyl chloride/3-methylbenzal chloride system are further strengthened by data for 4-isopropylbenzal chloride, obtained in the standard manner. The compound has a retention time of 15.7 min under conditions which give a 10.8 min retention time for 4-isopropylbenzyl chloride. Thus, the ratio of the retention times is 1.45, far too low a value to permit the assignment of a benzyl chloride-type structure.

Without underestimating the importance of retention indices as proposed by KOVÁTS[4–6] and recently defended by GUIOCHON[7], it should be pointed out that retention indices are not necessary for the solution of the particular problem at hand. The products arising from individual photochlorinations are few in number, and their structures may be assigned by the use of simpler methods. The retention indices, however, are being worked on in this laboratory, and will be the subject of a future communication. The work relating to the photochlorinations which served to prepare the compounds described above will be published elsewhere.

Acknowledgement

The author thanks Dr. R. W. HIGGINS for the use of his gas chromatograph.

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A pressure control valve as an aid in column chromatography

Column chromatography is often problematic and requires careful attention during relatively long periods of time. When using columns such as silicic acid, the eluant is forced through the column, under pressure, by an inert gas such as nitrogen, thereby reducing the elution time. Frequently the flexible hose used to connect the nitrogen gas lead to the column is forced off the column by a build-up in pressure, and

necessitates constant watching and replacing the hose. This makes it inconvenient to operate more than one column at a time.

A simple pressure control valve was designed to eliminate this pressure build-up, and to allow simultaneous use of a series of columns (silicic acid) from one gas (nitrogen) source. The valve can be made from supplies available in most laboratories as follows: T-tube connector, rubber hose, glass tube, small spring, and a rubber gasket (Fig. 1).

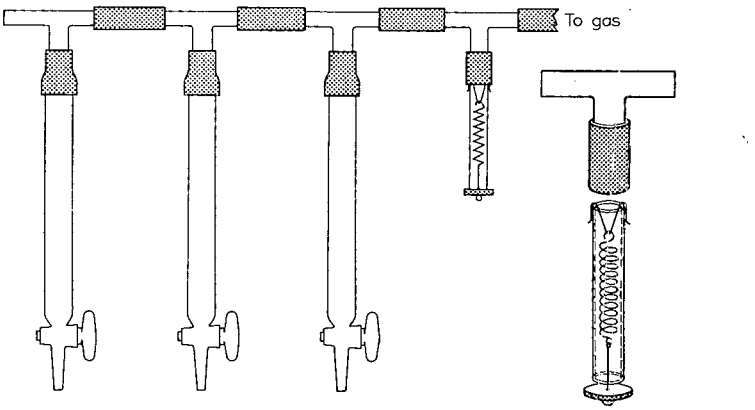

Fig. 1. (a) Series of columns with control valve. (b) Valve detail.

The spring tension can be adjusted during assembly, and if necessary, other methods of tension control can be devised. After the tension has been adjusted, variable gas flows can be introduced into the system to force the eluant through the column at a rate which may vary from a drop at a time to a continuous flow, without forcing off the rubber hose from the column. Excess gas will escape through the valve. Care should be exercised in adjusting the rate of gas flow to prevent excessive loss through the pressure control valve.

When using this valve, it is possible to attach several columns in a series to one nitrogen gas source and operate them at the same time. In this system, all of the connections will remain intact even if all stopcocks are turned off. Clamps may be placed between the columns and the T-tube connectors to enable refilling of an individual column without loss of pressure to the other columns. However, if the columns are uniformly packed, the eluant will flow through each at an even rate, and all columns may be refilled at the same time. One person can conveniently operate several columns at a time with precision, thus saving considerable time.

A procedure using silicic acid columns equipped with the above described valve has been used successfully for the separation of neutral and phospholipids.

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Powdered glass as an adsorbent applied to thin-layer chromatography

Several different adsorbents have been used for thin-layer chromatography (TLC), most of them being adsorbents used previously for column or gas chromatography. Amongst all of them, silica gel and aluminium oxide are most often used; both are specially prepared for TLC by G. Merck, Darmstadt. In addition to these two the early papers on TLC mention silicic acid, silicates and diatomaceous earth[1]. Recently many other adsorbents have been used, such as cellulose for dyes[2], Celite 545 for steroids[3], and calcium sulphate for the separation of fatty acids[4]. DAVIDEK AND POKORNÝ used polyamide powder[5], and KNAPPE AND PETERI employed polyethylene glycol mixed with diatomaceous earth or silica gel[6]. Nearly all of these adsorbents can be used with the addition of starch or plaster of Paris as a binder. The selection of the adsorbent, according to its properties, as well as the method of coating the plates, depends on the properties of the substance to be separated. Silica gel and aluminium oxide have an all-purpose application.

In this paper the use of glass powder prepared from broken Jena glassware as a universal adsorbent is proposed. The glass powder can be used either for the coating of plates using a STAHL applicator (adsorbent suspended in water) or for producing non-bounded layers with dry powder.

Experimental

Preparation of glass powder. Broken glass from flasks, beakers or other glassware produced by Schott u. Gen, Jena, were crushed in a mortar and then ground in a ball mill to a homogeneous, white, floury looking powder. The powder was sieved and the fraction with a particle size from 0.102 to 0.12 mm was collected.

This fraction was next divided into two using a sedimentation method. Both the precipitated powder and the one obtained from the suspension were dried at 110° and crushed into homogeneous powders. The powder obtained from the precipitate was called S40 adsorbent and its particles were about 0.04 mm in diameter. The powder obtained from the suspension had particles from 0.008 to 0.010 mm in diameter and was called S10 adsorbent.

Application and testing of adsorbent quality. The S40 adsorbent was used for the dry coating of plates using the method described previously[7–9]. The thickness of the layers obtained in this way was from 0.3 to 0.4 mm andt heir properties were examined with a test mixture containing 50 mg Victoria Blue, 50 mg bromothymol blue,

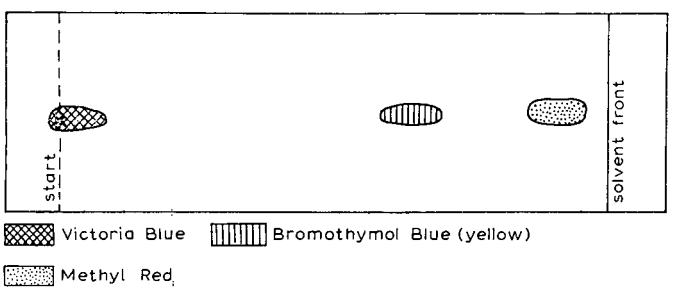

Fig. 1. Separation of a test mixture of dyes on S40 adsorbent. Solvent: ethyl acetate–benzene (7:3, v/v).

and 20 mg methyl red in 50 ml of acetone. A mixture of ethyl acetate and benzene (7:3, v/v) was used as eluent (see Fig. 1). For comparison of S40 adsorbent with other adsorbents, the dyes used in the above test mixture were separated on layers prepared in the same way from silica gel (particle size about 0.05 mm) and aluminium oxide (particle size about 0.04 mm). A mixture of diethyl ether and glacial acetic acid (9:1, v/v) was used as solvent. The resulting chromatograms are shown in Fig. 2. The length of the run was 15 cm; the time of development was about 8 min for S40 adsorbent and silica gel and 10 min for aluminium oxide.

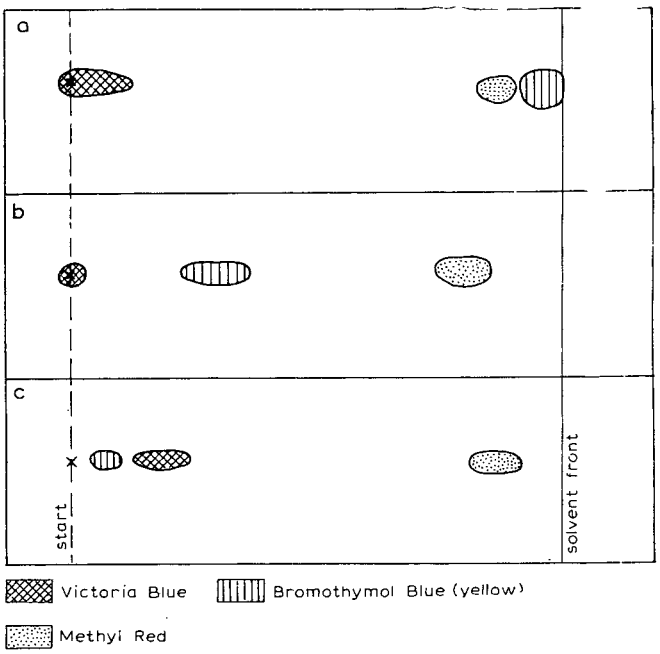

Fig. 2. Separation of a test mixture of dyes on different adsorbents. (a) S40 adsorbent; (b) silica gel; (c) aluminium oxide. Solvent: diethyl ether–glacial acetic acid (9:1, v/v).

The S10 adsorbent was suspended in water and spread on plates with the STAHL applicator (Desaga, Heidelberg). The layers were dried in air at room temperature and activated by heating at 110° for 30 min. The efficacy of the adsorbent was tested with the STAHL test mixture. Chromatograms were developed using the method described by STAHL, with benzene as eluent. Such a chromatogram is shown in Fig. 3a. The time of development was 45 min; the length of run was 8 cm. Spots of the STAHL test mixture as obtained by STAHL on Silicagel G from Merck, Darmstadt, are indicated by the small spots at the top. Fig. 3b illustrates the separation of our test mixture on S10 adsorbent using ethyl acetate–benzene (9:1, v/v) as eluent.

Regeneration of adsorbents. Both S10 and S40 adsorbents were regenerated by washing with a saturated solution of potassium chromate in sulphuric acid and rinsing with distilled water until the filtrate was neutral. The precipitate was dried

for 2 h at 110°. It was found that the regenerated adsorbent has the same activity as that freshly prepared.

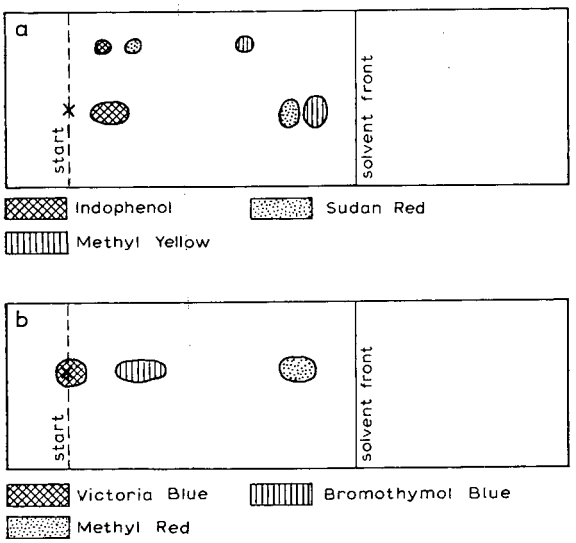

Fig. 3. Separation of dyes on S10 adsorbent. (a) STAHL test mixture. Solvent: benzene. The small spots at the top indicate the separation of this mixture on Silica Gel G (Merck). (b) Test mixture. Solvent: ethyl acetate–benzene (9:1, v/v).

Conclusion

As was shown above, both S10 and S40 adsorbents can easily replace silica gel or aluminium oxide. The glass powder may be prepared from waste glass, and because it can easily be regenerated, it is very economical. Adsorbents S40 and especially S10 can be used with the addition of binders like starch and plaster of Paris but this makes regeneration very difficult. The S40 adsorbent is also suggested for use in column chromatography. The possibility of using different kinds of glass powders as supports for gas chromatography has previously been suggested by YASCHIN *et al.*[10].

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A simple device for spotting preparative thin-layer chromatography plates

Preparative thin-layer chromatography has proved itself superior to conventional column chromatography in cases where small amounts of material have to be isolated from complex mixtures.

The laborious means of applying the sample effectively to a preparative plate, however, has made this method unattractive to many chemists. Although several types of commercial apparatus for the application of the sample to the plate are available, they are expensive and elaborate to handle. A simple device has been successfully used in our laboratory for the same purpose; it can easily be made by an amateur glassblower and the dimensions are not very critical (Fig. 1). When the

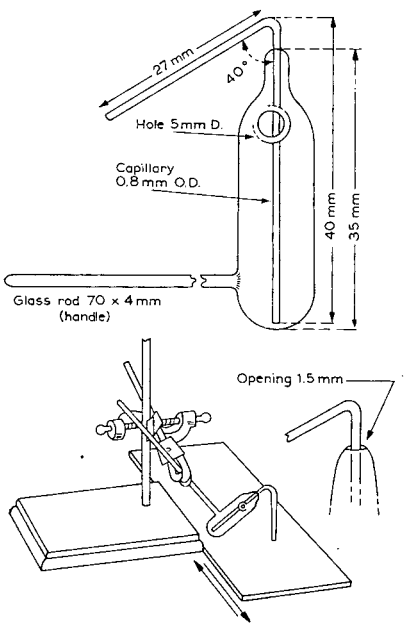

Fig. 1.

device is clamped by its handle in such a manner that the capillary (which moves freely inside the reservoir) feeding the solution will just touch the plate at right angles, the spotting can be performed conveniently by sliding the plate back and forth in a straight line—this can be achieved by placing the stand clamping the applicator on top of a thick glass plate against the edge of which the preparative plate slides. In this way, one can easily obtain narrow lines of the applied sample without any danger of making holes or grooves on the surface of the plate.

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Continuous flow thin-layer chromatography

The desire to combine the advantages of a continuously running solvent as found in descending paper chromatography with the well-known advantages of the thin-layer chromatographic technique (TLC) has led to the development of many ingenious assemblies for continuously flowing thin-layer chromatography[1–6]. The use of the term "continuous" in thin-layer chromatography has become somewhat confused in the literature, possibly because of semantics involved in translations. Several workers[1,3,9,11] use the terms "continuous development" or "continuous flow" when referring to a continuously flowing solvent acting in a manner similar to that of descending paper chromatography. Other authors[5,13] use the term "through-flow" chromatography and refer to the "overflow" of solvent. The term "continuous chromatography" is reserved by some workers[10] to describe those processes wherein both the solvent flow is continuous and the sample is continually fed to the system. In our work we will use the terms "continuous flow" or "continuous development" in the sense of a continuously flowing solvent with a single application of sample.

We have devised two methods which are as effective as any reported and are simple to assemble. The materials and dimensions described are offered as a guide since the individual worker may have other equipment which can be readily adapted to the same principles.

The first method was based on the continuously flowing ascending paper chromatographic technique described by FISCHBACH AND LEVINE[7] and is similar to ones described recently by BOBBITT[1] and TRUTER[9]. In this method a 15 × 20 cm TLC plate, prepared by the rod and tape procedure of LEES AND DEMURIA[8], is placed in a cylindrical jar (diam. 17.5 cm, height 23 cm) and supported from below so that about 2 cm of the coated plate extends above the top of the jar. Solvent is placed in the jar so that about 1 cm of the lower edge of the plate is contacted by the solvent, the samples previously having been applied about 3 cm from the bottom edge of the plate. While carefully holding the coated plate vertically in the jar, two plain glass plates are placed on top of the jar leaving a narrow slot through which the TLC plate extends. These cover plates are then slid against the coated plate, taking care not to break the coating. The openings on either side of the coated plate are sealed with adhesive tape, thus leaving about 2 cm or more of the coated plate exposed to the atmosphere. Two plates can be developed simultaneously if they are placed back-to-back. To reduce the traveling distance of the solvent, spots may be placed further from the bottom edge and more solvent added to the jar. Other methods that have been used to reduce the solvent traveling distance were to employ shallower solvent jars or to place the solvent in a small trough which could be raised or lowered to regulate the solvent traveling distance. The shorter this distance the greater the flow rate.

The second method devised by us was a horizontal one in which a TLC plate is used as the lid (coated side down) of a shallow glass tray serving as the solvent chamber. The plates (15 × 20 cm) were coated with silica gel or other adsorbent by the rod and tape method described previously. Some of the coating of the TLC plate is scraped from three edges of the plate so as to restrict the solvent flow to one direction (Fig. 1). Method B gives a somewhat better vapor seal than method A, but in either case solvent vapor leakage is not too great and saturation conditions are easily

maintained because of the close proximity of the solvent layer and the TLC plate.

Samples were applied about 2 cm in from the edge of the coating on the side opposite the unscraped end of the plate (*cf.* Fig. 1). The developing chamber is simply a glass photographic tray approximately 18 × 13 × 3 cm, the edges of which have been ground flat so as to form a tight seal with a plate glass lid. These trays are usually made of soft glass and are easily ground down with a carborundum paste.

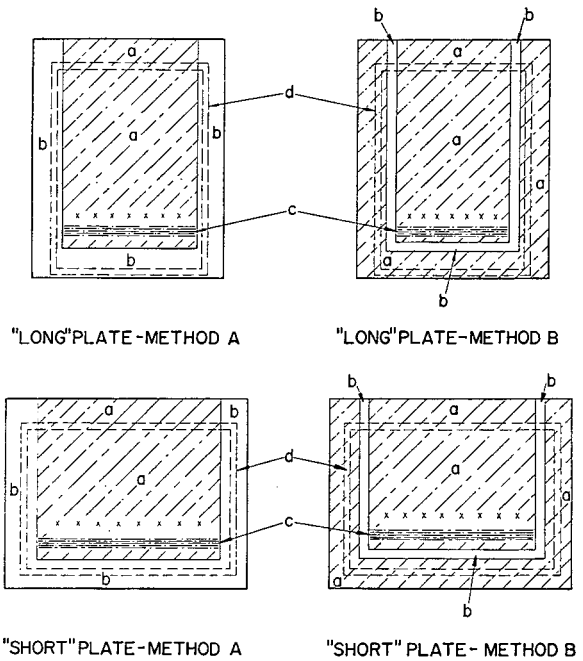

Fig. 1. Preparing horizontal thin-layer plates. (a) Silica gel coating; (b) area from which silica gel is removed; (c) area of silica gel contacted by solvent wick; (d) section of TLC plate contacted by edge of solvent tray; (x) sample application points.

Larger trays have also been used with correspondingly larger TLC plates. About 200 ml of the developing solvent is placed in the tray and a rolled paper wick either 15 cm wide for the "short" plates or 10 cm wide for the "long" plates is placed in the proper end of the chamber (Fig. 2). The paper wick is made by loosely rolling a 10 cm long piece of chromatographic grade paper such as Whatman No. 3MM of the proper width into a roll approximately 2 cm in diameter. A piece of heavy glass rod (12 mm diam.) is inserted into the center of the roll to anchor the wick in place. The upper side of the rolled wick should contact the silica gel layer just before the point where the samples are added and care must be taken so as not to have the roll so bulky that it touches the applied samples or so tightly rolled that it does not make good contact. Cellulose sponge can be cut to form a wick and works quite well for many solvents except that unwanted materials were found to be extracted from the sponge itself in several cases. Once the TLC plate is prepared and the samples applied to it, the plate is inverted over the chamber so as to serve as a lid; the unscraped edge of the plate is exposed to the atmosphere for at least 2 cm.

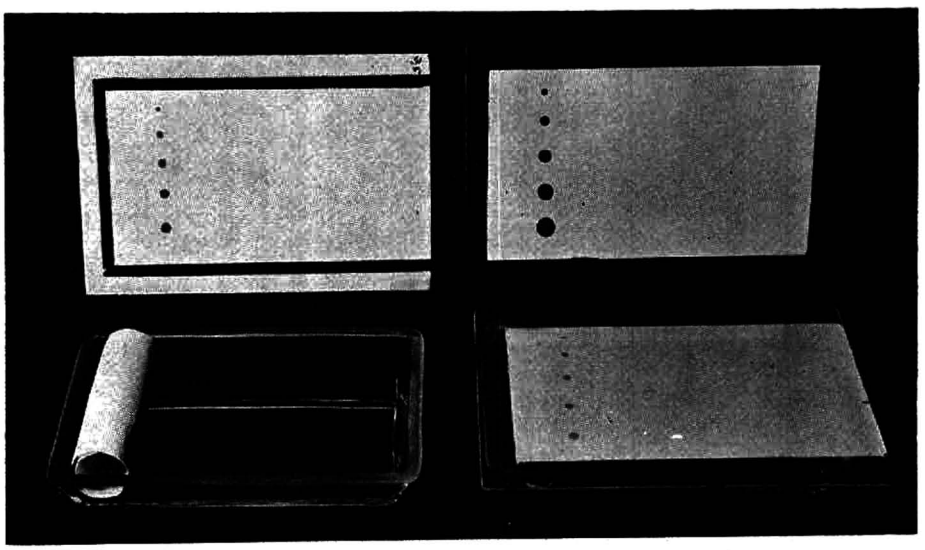

Fig. 2. Apparatus for horizontal continuous flow thin-layer chromatography.

Continuous development by the horizontal or ascending methods depends on the constant evaporation of solvent as it reaches the exposed end of the coating layer. The rate of the solvent migration through the coating varies inversely with the distance to be traveled and directly with the volatility of the solvents used. Short plates, while permitting a large number of solvent passes, restrict the actual distance in which the separation is to be achieved.

The vertical and later the horizontal methods were first applied to the separation of cholesterol and desmosterol. More recently the separation of these compounds as the alcohols has been described by WOLFMAN AND SACHS[12] using a reversed-phase TLC technique. Fig. 3 shows the separation obtained for these compounds as the acetates* when a silica gel G plate was developed in the horizontal manner for one hour using benzene–hexane (1:3). This time is equivalent to about three "passes"; one "pass" being the time required for the solvent front to reach the far edge of the plate. The spots were visualized by spraying the plate with concentrated sulfuric acid, heating, and then observing the plate under white or long-wave ultraviolet light.

Fig. 4 illustrates the separation of cholesterol and desmosterol, as the free alcohols, by the horizontal technique using a silver nitrate impregnated silica gel G plate. The developing solvent was benzene–ethyl acetate (95:5) and the separation took about 6 h. Sulfuric acid was used to visualize the spots. AVIGAN *et al.*[14] used silver nitrate impregnated plates for separating these compounds, but found it necessary to use the acetate forms. We found that by dipping a previously prepared silica gel G plate in a 5% solution of silver nitrate in acetonitrile, followed by air drying to remove the acetonitrile, we were able to obtain much less background discoloration than when the method of preparation described by AVIGAN was used.

* Samples of the alcohol and acetate forms were kindly supplied by Dr. S. K. FIGDOR of Chas. Pfizer & Co., Inc.

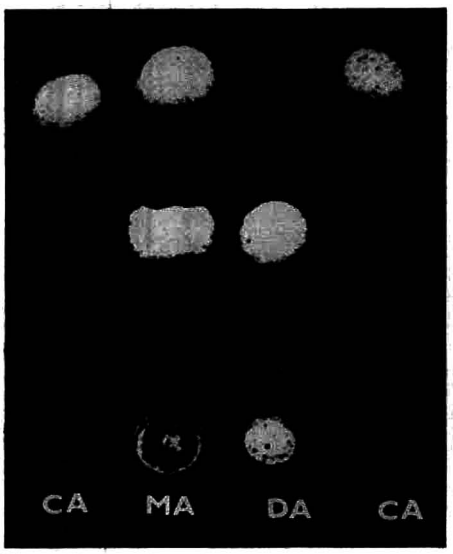

Fig. 3. Separation of cholesterol and desmosterol acetates. CA = cholesterol acetate; DA = desmosterol acetate (impure); MA = mixture of the two acetates. Photographed by ultraviolet light.

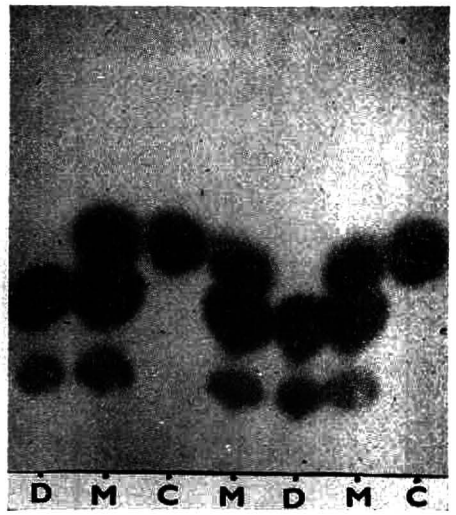

Fig. 4. Separation of cholesterol and desmosterol. C = cholesterol; D = desmosterol (impure); M = mixture of the two alcohols. Photographed by white light.

The techniques described can be adapted to many shapes and sizes of vessels, and, besides affording good separations, have the attributes of simplicity and inexpensiveness. These methods have been applied to the separation of cholesterol and desmosterol and can be applied to other difficultly separable compounds.

We wish to acknowledge the assistance of Mr. I. CHMURA in preparing Fig. 1.

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Degradation of cholesterol during transesterification of cholesterol stearate

In the analysis of the components of the lipid fractions of serum and tissues, it is tempting to analyze both moieties of steryl esters by transesterification followed by gas–liquid chromatography of the fatty acid methyl esters and then either colorimetric or chromatographic assay of the sterol. We have investigated the steroid products obtained after cholesteryl stearate has been subjected to methanolysis. Analysis by a combination of thin-layer and gas-chromatographic methods indicates that, under the conditions used, several degradation products and derivatives of cholesterol are formed.

Materials and methods

All solvents used were of reagent quality. All the steroids used were purified by chromatography prior to use. Cholesteryl methyl ether and bis-cholesteryl ether (bis-(5-cholesten-3β-yl) ether) were purchased from Chemed, Inc., Odenton, Maryland. Cholesteryl stearate and 3,5-cholestadiene were purchased from Steraloids, Inc., Pawling, New York. Cholesterol-4-^{14}C stearate was purchased from New England Nuclear Corp., Boston, Massachusetts. Cholesterol was also purified via the dibromide.

Thin-layer chromatography was carried out on 20 × 20 cm plates using Silica Gel G (Brinkmann). Plates were activated immediately prior to use by heating at 110° for 50 min. Steroids were separated using one of the three solvent systems listed in Table I and were visualized by spraying with concentrated H_2SO_4. With this reagent 3,5-cholestadiene gave an immediate magenta color and the other compounds gave a blue color after heating at 130° for 10–15 min.

TABLE I

R_F VALUES OF VARIOUS STEROIDS ON SILICA GEL G IN THREE THIN-LAYER SOLVENT SYSTEMS

Steroid	*Solvent system*		
	Petroleum ether–diethyl ether–acetic acid (I) (90:10:1)	*Benzene–hexane (II)* (1:4)	*Hexane (III)*
Cholesterol	0.06	0.00	0.00
Cholesteryl methyl ether	0.62	0.07	0.00
3,5-Cholestadiene	0.88	0.78	0.68
Cholesteryl stearate	0.85	0.20	0.00
Bis-cholesteryl ether	0.84	0.07	0.00

Gas–liquid chromatography was carried out on 6 ft. coiled glass columns packed with 3.85 % SE-52 adsorbed on Gas Chrom P, 80–100 mesh. The instrument used was an E.I.R. Clinigraph operated under the following conditions: flash heater, 295°, column 246°, argon inlet pressure, 42 p.s.i., detector cell, 800 V. All the compounds were applied as acetone solutions. Detector response was linear for each compound over a 3 μg range. Semi-quantitative estimations of the steroids were obtained by preparing standard curves for each compound. Retention times relative to cholesterol (1.00) (28.5 min under our conditions) were: cholesteryl methyl ether 0.86, 3,5-cholestadiene 0.55, bis-cholesteryl ether 0.56.

Transesterification of cholesteryl stearate was carried out by a slight modification of the method of LORETTE AND BROWN[1]. In a representative experiment, 5–10 mg of material were added to a test tube equipped with a Teflon-lined, screw-on cap, 5 ml of 5 % H_2SO_4 in methanol, 0.3 ml of dimethoxypropane were added and hydrolysis was allowed to proceed for 4 h at 70°. The reaction mixture was diluted with an equal volume of water and extracted three times with 4 ml portions of petroleum ether. The combined petroleum ether extracts were dried over anhydrous Na_2SO_4 and used for further studies.

When radioactive substrates were used, radioactivity of the reaction products was determined by liquid scintillation spectrometry in a Packard Tri-Carb liquid scintillation spectrometer in toluene containing 0.6 % 2,5-diphenyloxazole (PPO) and 0.02 % dimethyl 1,4-bis-2-(5-phenyloxazolyl) benzene (dimethyl POPOP). Samples were counted for sufficient lengths of time to insure less than 1 % counting error.

Colorimetric analyses of cholesterol were carried out using the method of MANN[2].

Results and discussion

The products of the transesterification reaction were initially subjected to thin-layer chromatography on Silica Gel G in solvent system I. This system afforded separation of the free cholesterol and the cholesteryl methyl ether from the other products of the reaction. The amount of material in each zone was estimated by scraping the silicic acid from the plate, elution of the steroid with acetone and estimation by gas–liquid chromatography. The three products (3,5-cholestadiene, cholesteryl stearate, bis-cholesteryl ether) which are virtually inseparable in system I can be easily separated in either of the other two thin-layer solvent systems shown in Table I.

On the basis of several analyses of this type, the products of the transmethylation of cholesteryl stearate were assessed as being cholesterol, cholesteryl methyl ether, 3,5-cholestadiene, bis-cholesteryl ether and a small quantity of unreacted cholesteryl stearate. When pure cholesterol was subjected to treatment with 5 % H_2SO_4 in methanol and dimethoxypropane, about 75 % of the sterol was recovered unchanged, 15 % was converted to cholesteryl methyl ether and the remaining 10 % was composed equally of bis-cholesteryl ether and 3,5-cholestadiene. The latter compound was further characterized by its characteristic U.V. absorption spectrum.

In order to quantitate the amount of each product formed during the reaction cholesterol-4-^{14}C stearate was subjected to transmethylation and one aliquot of the products was separated by thin-layer chromatography in solvent system I (petroleum ether–ethyl ether–acetic acid, 90:10:1). The plate was ruled into one-cm strips and each was eluted and individually assayed for radioactivity. Further thin-layer separations in the other systems and elution of bands from a plate developed in one solvent system and re-run in another yielded the following pattern of products, based upon recovery of radioactivity: cholesterol, 50 %; cholesteryl methyl ether, 2 %; cholesteryl stearate 1 %; bis-cholesteryl ether, 9 %; 3,5-cholestadiene, 25 %; and an unknown compound which trails immediately behind 3,5-cholestadiene when subjected to thin-layer chromatography in hexane, 13 %. We believe the unknown compound may be bis-cholestadiene. While this manuscript was in preparation, MORRISON AND SMITH[3] reported finding a similar spectrum of compounds when cholesteryl stearate was subjected to methanolysis in BF_3–methanol. No quantitation of products was attempted.

Colorimetric analysis for cholesterol of the transmethylation mixture based on the assumption that all of the ester had been converted to free cholesterol yielded values for mg/100 ml of cholesterol which compared closely with calculated values. Treatment of equal amounts (by weight) of cholesteryl methyl ether, 3,5-cholestadiene and cholesterol with the color reagent ($FeCl_3$–HOAc–H_2SO_4) showed that the ether gave about 20% more color than did the free sterol and the diene gave 5% less color.

TABLE II

COLOR YIELDS OF STEROID MIXTURES COMPARED WITH CHOLESTEROL*

Steroid	*Percentage in mixture*			
	I	*II*	*III*	*IV*
Cholesterol	100	55	50	40
3,5-Cholestadiene	—	40	40	40
Cholesteryl methyl ether	—	10	10	10
Cholesteryl stearate	—	—	—	10**
mg% as cholesterol	100	106	103	97

* Using $FeCl_3$–HOAc–H_2SO_4.
** This corresponds to 6 mg of cholesterol. Thus expected value for total would be 96 mg%.

Several synthetic mixtures were prepared and the color yields were close to those expected from equal weights of cholesterol. As Table II shows, the differences are within the experimental error experienced in routine analysis for cholesterol. This may be explained by the fact that several of the principal products are compounds which are known to be formed in the course of the LIEBERMANN-BURCHARD or SALKOWSKI reactions[4,5].

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Preparative thin-layer chromatography of anthocyanins

Paper chromatography has been an indispensable tool for separating and analyzing anthocyanins[1]. More recently, however, HESS AND MEYER[2], TANNER AND RENTSCHLER[3], and NYBOM[4] used thin-layer chromatography (TLC) as an analytical method. The advantages were shorter developing times, better resolution, and greater sensitivity. Preparative chromatography of anthocyanins has been performed almost exclusively by paper chromatography, although in some cases column chromatography has been used. The disadvantages of these procedures is that they are time-consuming. In the present communication, a simple and rapid method is reported for the isolation of milligram amounts of anthocyanins by preparative thin-layer chromatography (PTLC).

PTLC was carried out on 20 × 20 cm chromatoplates with 1 mm layers of a mixture of 2/3 silica gel (adsorbosil-2) and 1/3 cellulose powder (MN-300, gypsum free). For good separation the adsorbents should be as free of iron as possible. To prepare two 20 × 20 cm chromatoplates, 30 g of adsorbosil-2 and 15 g of cellulose powder (MN-300, gypsum free) were added to 110 ml of water and mechanically agitated for one minute. The plates were air dried and were then activated at 110° for 30 min. One mm layers containing more than 1/3 cellulose tended to crack when dried.

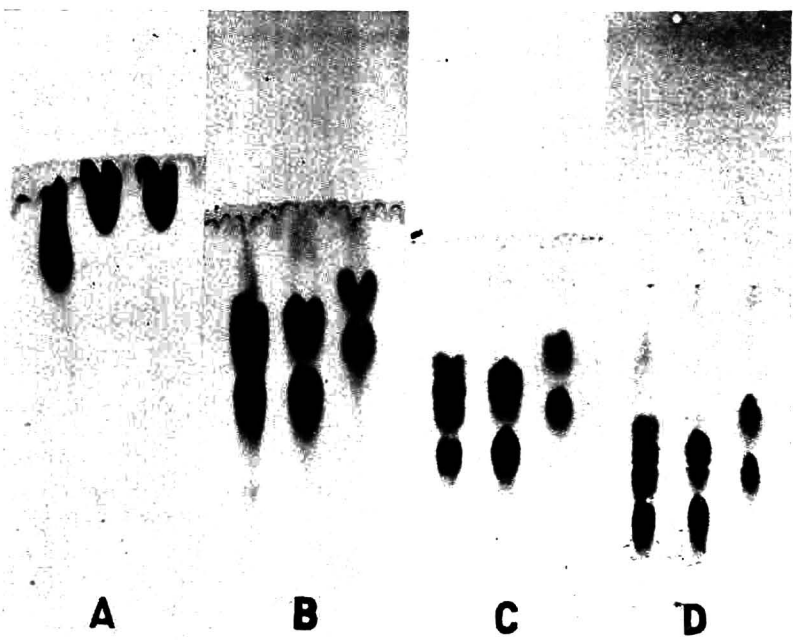

Fig. 1. Separation of anthocyanins from bracts of *Euphorbia pulcherrima* on 250 μ layers of silica gel without a binder (adsorbosil-2), cellulose powder without a binder (MN-300), and mixtures of both. Solvent: acetone–0.5 *N* HCl (1:3, v/v). Development time: 30 min. A = silica gel; B = 75% silica gel + 25% cellulose powder; C = 50% silica gel + 50% cellulose powder; D = cellulose powder. Spots on each chromatoplate from left to right: 3 glucoside + 3 rhamnoglucoside of cyanidin and pelargonidin; 3 glucoside + 3 rhamnoglucoside of cyanidin; 3 glucoside + 3 rhamnoglucoside of pelargonidin.

The advantages of using chromatoplates made of silica gel and cellulose powder were shown by VAN SUMERE *et al.*[5] for phenolic acids, coumarins, and related substances. They were more stable than silica gel chromatoplates, and offered better resolution. The resolution obtained with the 3-glucoside and the 3-rhamnoglucoside of cyanidin and pelargonidin (anthocyanins from the bracts of *Euphorbia pulcherrima*[6]) is shown in Fig. 1. No separation was obtained with silica gel but the addition of cellulose powder resolved the individual glucosides of pelargonidin and cyanidin. The more cellulose in the mixture, the tighter were the spots and the better was the resolution. Cellulose layers, although they gave the best separation, had the disadvantage of being difficult to remove from the glass plates, whereas mixtures of silica gel and cellulose powder were removed with ease.

One ml of the concentrated anthocyanin extract (methanol containing 1 % HCl) was carefully applied to each chromatoplate as a narrow band with a Desaga micro spray pistol. This was approximately equivalent to 6 mg of anthocyanins. Other controlled sample applicators[7] are available for this purpose.

Solvents normally used for the separation of anthocyanins on filter paper can also be used with chromatoplates having a layer of a mixture of silica gel and cellulose powder. The chromatoplates were first developed with the upper layer of the mixture 1-butanol–2 *N* HCl (1:1, v/v). This solvent was aged for 24 h before being used and it separated the cyanidin glucosides from the pelargonidin glucosides. Development time was 4 h. Individual glucosides of cyanidin and pelargonidin were then separated with water–HCl–formic acid (8:4:1, v/v), and further purified with 1 % HCl, and acetone–0.5 *N* HCl (1:3, v/v). Development time was 30 min for each solvent. The resolved anthocyanin bands were removed with vacuum into an extraction thimble. They were then removed from the extraction thimble and placed in a chromatographic column. The anthocyanins were then eluted under pressure with methanol containing 1 % HCl.

Thus the purification of anthocyanins from plant tissues in milligram amounts could be accomplished in a day or two by PTLC as compared to a week or longer when paper chromatography is used.

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An improved method for the detection of flavanones on paper chromatograms

The reaction of flavanones[1,2] and flavanonols[3] with sodium borohydride and hydrochloric acid is well known. In the detection of these compounds on paper chromatograms, the chromatogram is first sprayed with a freshly prepared solution of 1% (w/v) sodium borohydride in propan-2-ol[1] (or a 2% (w/v) methanolic solution of sodium borohydride[2]) and is then subjected to hydrochloric acid vapours. The sensitivity of the reaction depends largely on the extent to which the sodium borohydride treated chromatogram can be subjected to hydrogen chloride gas, attempts to spray the chromatogram with aqueous hydrochloric acid being unsatisfactory[2]. While the techniques described[1,2] are suitable for strip chromatograms and for small-scale work, they are not readily adaptable to larger paper sheets such as are often used in two-dimensional chromatography or where it is desirable to compare a large number of compounds on the same chromatogram.

In this laboratory the need for subjecting sodium borohydride treated chromatograms to a hydrogen chloride atmosphere is obviated by spraying the papers with 5% (w/v) alcoholic aluminium chloride and heating at 120° for 1.5 min. Under these conditions flavanones are revealed as blue to purple spots.

Direct comparison of the $NaBH_4$–HCl and $NaBH_4$–$AlCl_3$ techniques under optimum conditions has revealed that the latter is more sensitive. As little as 0.5 μg flavanone could be detected by this modified procedure, indicating that it is one of the most sensitive of chromogenic spray techniques for flavonoid compounds.

Comparison of $NaBH_4$–$AlCl_3$ treated chromatograms with duplicate chromatograms heated at 120° for 1.5 min after spraying with 1% (w/v) alcoholic aluminium chloride[4] provides valuable information on the types of compounds present in flavonoid extracts. Thus *peri*-hydroxycarbonyl flavonoids which have phloroglucinol-derived A-rings and which possess a six-membered heterocyclic ring or an acyclic structure form aluminium chelates which fluoresce a bright orange, yellow or green under ultraviolet light. The loss of such fluorescence on $NaBH_4$–$AlCl_3$-treated chromatograms is observed in the case of flavanonols (*e.g.* taxifolin) and dihydrochalcones (*e.g.* phloretin), indicating that, as with flavanones, the carbonyl group in these compounds is reduced by the sodium borohydride treatment. Unlike flavanones, however, the products remain colourless on subsequent treatment with aluminium chloride and heat under the conditions described above. Prolonged heating will, however, reveal the presence of flavanonols as red-brown spots.

The duplicate chromatograms, treated with aluminium chloride and heat alone, will reveal directly the presence of flavan-4-ols as red to violet spots whereas flavanones will only appear as such after prior treatment with sodium borohydride. Prolonged heating of the chromatograms will also reveal the presence of phloroglucinol-derived leuco-anthocyanidins and flavan-3,4-diols as red-brown spots and in this respect the aluminium chloride reagent is similar to, but somewhat less effective than, toluene-*p*-sulphonic acid[5]. The $NaBH_4$–$AlCl_3$ test may therefore be considered specific for flavanones when dealing with phloroglucinol-derived flavonoid compounds.

With 5-deoxyflavonoids, however, both flavanones (*e.g.* butin) and flavanonols (*e.g.* dihydrorobinetin) give positive reactions and duplicate chromatograms treated

with aluminium chloride and heat alone will reveal directly the presence of leuco-anthocyanidins, flavan-4-ols and flavan-3,4-diols as blue to violet spots.

The failure of phloroglucinol-derived flavanonols such as taxifolin to give a positive $NaBH_4$–$AlCl_3$ reaction is due, therefore, not to the failure of sodium borohydride to reduce the compounds to the corresponding flavan-3,4-diols (leuco-cyanidin has, in fact, been prepared by sodium borohydride reduction of taxifolin[6]), but rather to the inability of the acid reagent to convert the flavan-3,4-diols to deeply coloured products. This conclusion is supported by similar observations for the toluene-*p*-sulphonic acid reagent, which has been found to be a most effective chromogenic spray for 5-deoxyleuco-anthocyanidins but considerably less effective for 5-hydroxyleuco-anthocyanidins such as leuco-cyanidin and leuco-delphinidin[5].

In brief, the advantages of the $NaBH_4$–$AlCl_3$ technique over the $NaBH_4$–HCl method are: (a) it is more easily and conveniently employed, (b) it is more sensitive and (c) it provides additional information about other flavonoids present.

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Separation of imidazoles by thin-layer chromatography

A number of workers have separated and identified imidazolic compounds by paper chromatography[1–3]. However, during a study of the reaction between carbohydrates and ammonia it was found that the imidazoles formed were more rapidly and efficiently analysed during separations by thin-layer chromatography (TLC). The complex mixtures of imidazoles were effectively separated on silica gel G* and aluminium oxide G* layers using relatively basic solvent systems.

Materials and methods

Following the method of STAHL[4] silica gel G and aluminium oxide G were applied to 20 × 20 cm glass plates to a thickness of 250 μ using a Desaga apparatus.

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TABLE I

R_F AND R_{Im} VALUES FOR SEVENTEEN IMIDAZOLES IN FIVE SOLVENT SYSTEMS*

Compound	*Silica gel G layer (activated)*		*Aluminium oxide G layer (unactivated)*								*Published pKa values*	*Colour of dye with diazotized sulphanilic acid spray*
	Solvent A		*Solvent B*		*Solvent C*		*Solvent D*		*Solvent E*			
	R_F	R_{Im}	R_F	R_{Im}	R_F	R_{Im}	R_F	R_{Im}	R_F	R_{Im}		
2-Acetyl-4(or 5)-methylimidazole	0.84	1.50	0.56	2.55	0.78	1.97	0.69	1.13	0.77	1.14	—	yellow-orange
2-Ethyl-4(or 5)-methylimidazole	0.50	0.90	0.42	1.91	0.70	1.67	0.65	1.09	0.66	1.18	—	yellow
2,4(or 5)-Dimethylimidazole	0.36	0.64	0.32	1.45	0.54	1.33	0.62	1.02	0.75	1.09	8.36	lemon-yellow
4,5-Dimethylimidazole	0.56	0.88	0.28	1.21	0.51	1.23	0.62	1.03	0.68	1.00	—	yellow
2-Methylimidazole	0.40	0.71	0.24	1.09	0.61	1.45	0.63	1.05	0.76	1.09	7.86	lemon-yellow
4(or 5)-Methylimidazole	0.52	0.93	0.24	1.09	0.46	1.04	0.62	1.03	0.70	1.00	7.52	red
Imidazole	0.56	1.00	0.22	1.00	0.42	1.00	0.61	1.00	0.70	1.00	6.95	orange
2-Hydroxymethyl-4(or 5)-methylimidazole	0.43	0.79	0.02	0.09	0.15	0.30	0.46	0.76	0.30	0.45	—	red
4(or 5)-(2-Hydroxyethyl)-imidazole	0.30	0.54	0.06	0.27	0.11	0.21	0.49	0.82	0.41	0.61	—	red
4(or 5)-Hydroxymethylimidazole	0.34	0.61	0.04	0.18	0.10	0.20	0.46	0.75	0.30	0.45	6.38	orange-red
2-Mercaptoimidazole	0.74	1.33	0.14	0.64	0.52	1.24	0.57	0.95	0.51	0.91	—	orange
Histamine dihydrochloride	0.04	0.07	0.02	0.09	0.00	0.00	0.02	0.07	0.02	0.09	5.98	red
Histidine hydrochloride	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	red
4(or 5)-D-Arabotetrahydroxybutylimidazole	0.03	0.07	0.01	0.04	0.01	0.02	0.00	0.00	0.00	0.00	—	red
Imidazole-4(or 5)-carboxylic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.08	yellow
Imidazole-4(or 5)-acrylic acid	0.11	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	yellow-orange
Imidazole-4(or 5)-pyruvic acid	0.01	0.14	0.00	0.00	0.01	0.02	0.01	0.01	0.00	0.00	—	yellow-orange

* A = chloroform–methanol–ammonia (80:25:0.1); B = toluene–ethyl acetate–ammonia (1:3:0.1); C = chloroform–pyridine (2:1); D = toluene–95% ethanol (1:1); E = toluene–100% ethanol (1:1).

After 15 min at room temperature the silica plates were activated in an oven at 110° for 1 h while the aluminium oxide plates were allowed to stand in the air overnight.

The base line was fixed at a distance of 3 cm from the edge of the plate and the compounds were applied in ethanol or diethyl ether solution from a micropipette. Each chromatoplate was developed to a height of 12.5 cm at 19°. After air-drying, the plates were sprayed with alkaline diazotized sulphanilic acid, which gives red, orange or yellow dyes with imidazoles[5]. R_F and R_{Im}* values in five solvent systems are reported in Table I. Documentation is best accomplished by colour photography.

Discussion

The use of TLC with basic layers or solvents has proved most useful for the rapid separation and identification of imidazole bases. Compact spots are obtained with all except the carboxyl-substituted imidazoles. There is little significant difference in R_F values between activated and unactivated aluminium oxide G chromatoplates.

Methyl-substituted imidazoles can be differentiated from hydroxyalkylimidazoles on activated silica gel G chromatoplates in solvent A and on aluminium oxide G chromatoplates in all of the solvent systems studied. In complex mixtures of imidazoles TLC has given good differentiation between methyl-substituted imidazoles which are difficult to separate by conventional paper chromatography. Further improvement has been achieved using two-dimensional TLC on aluminium oxide G chromatoplates and identification is aided by the different colours of the azo-dyes produced on spraying (see Fig. 1).

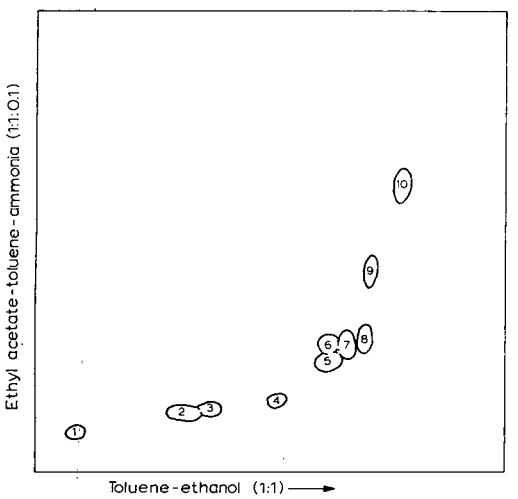

Fig. 1. Two-dimensional TLC on aluminium oxide G of a mixture of methyl- and hydroxy-alkylimidazoles. Alkaline diazotized sulphanilic acid spray. 1 = 4 (or 5)-D-arabotetrahydroxybutylimidazole (red); 2 = 4(or 5)-hydroxymethylimidazole (orange-red); 3 = 4(or 5)-(2-hydroxyethyl)-imidazole (red); 4 = 2-hydroxymethyl-4(or 5)-methylimidazole (red); 5 = imidazole (orange); 6 = 4(or 5)-methylimidazole (red); 7 = 4,5-dimethylimidazole (bright yellow); 8 = 2-methylimidazole (lemon); 9 = 2,4(or 2,5)-dimethylimidazole (lemon); 10 = 2-acetyl-4(or 5)-methylimidazole (orange-yellow).

* $R_{Im} = \dfrac{\text{distance travelled by compound}}{\text{distance travelled by imidazole (parent free base)}}$.

It has been noted with paper chromatography that R_F values increase with an increase in basic strength of the imidazolic compound[1]. Electron-releasing groups, particularly in the 2-position, increase the pKa values while electron-attracting groups have the opposite effect[6,7]. We have found that this relationship is followed in general for the imidazoles of published pKa values with TLC on alumina chromatoplates (see Table I).

The use of R_{Im} values has been found by the authors to be preferable to the use of R_F values as the former are more reproducible and usually compensate for solvent or layer irregularities.

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Separation of hexahydro-1,3,5-trinitro-s-tetrazine (RDX) and octahydro-1,3,5,7-tetranitro-s-tetrazine (HMX) by thin layer chromatography

The qualitative separation of nitramines by thin-layer chromatography (T.L.C.) as reported by HARTHON[1] has been extended to traces of RDX present in explosive-grade HMX. The physical and chemical properties of the two are similar and their solubilities quite low[2]. Since RDX occurs as a contaminant in HMX, separation processes require methods for estimating residual RDX in the range of 0.5 % to 1.5 %

The HMX and RDX (Holston Defense Corporation) were dried by infrared heating and dissolved in acetone. Concentrations above 25 g/l were obtained by dissolving in γ-butyrolactone and diluting. A Research Specialties Co. Kit[3] provided most of the tools for T.L.C., although sprayers had to be improvised for the work. The techniques used have been described by RUSSEL[4]. The developer found most suitable was 10 % acetonitrile in methylene chloride.

T.L.C. plates were scored with a sharp pencil to provide identically prepared strips of adsorbent. On such strips, the sample to be tested was spotted for comparison with a strip containing no RDX and with a monitor strip. The monitor strip alone was dyed with Griess' reagent[5] to indicate the locations of RDX and HMX. Separated RDX was recovered from the scrapings of blank strips and sample strips by extraction with spectrograde acetonitrile. Spectrometry at 228 μ showed that the blanks

did not exceed 0.05 %, the instrument limit of error. Separation has been found possible at RDX concentrations as low as 0.05 % provided that a minimum of $5 \cdot 10^{-4}$ mg of RDX is present. This concentration closely approaches the limit of visual detection and depends on the solubility of HMX in γ-butyrolactone to obtain a high concentration of HMX sample in solution. Densitometric measurements were made using a Photovolt Photometer (Model 520-A) connected to a suitable recorder (Speedomax G). With these instruments, the spots caused by the RDX separated with HMX on alumina-coated plates and sprayed with Griess' reagent, were measured. The area under the absorption peaks was estimated and compared with that corresponding to a reference spot.

For low levels of RDX (not exceeding 0.8 %) the relation between amount of RDX and relative peak area is linear. Since this is the region of interest, the method is suitable for quality control of RDX in HMX.

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Chromatographic determination of riboflavin and flavin nucleotides in yeast

Chromatographic methods, in particular paper partition chromatography, are one of the preferred methods for quantitatively determining flavins. Chromatography was first applied for flavin separation by CRAMMER[1] and then by HAIS[2]. Further modifications have been described by CARTER[3], WHITBY[4], FOSTER AND KARRER[5] and DIAMANT *et al.*[6]. However, determination of flavins in samples of biological material still presents technical difficulties.

We have introduced a new solvent system for paper chromatography of flavins, composed of butanol–pyridine–water–glacial acetic acid (3:3:3:1) and have modified the elution of flavins from paper strips, riboflavin (Rb), flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) being eluted at pH 5.5, 3.5 and 2.8, respectively.

Experiments were performed using pure riboflavin (Rb), (Merck) flavin nucleotides, FMN and FAD (National Biochemical Corporation, Cleveland, Ohio) and flavin extracts from yeast prepared according to the method of YAGI[7].

TABLE I

CHROMATOGRAPHIC SEPARATION OF STANDARD FLAVIN SOLUTIONS AND YEAST EXTRACTS

Solvent systems (amounts in parts by volume)		R_F *values*			
		Rb	*FMN*	*FAD*	*Flavin Fraction IV* *
1 *n*-Butanol	4				
Acetic acid	1	0.3	0.11	0.04	
Water	5				
2 *n*-Butanol	4				
Acetic acid	3	0.49	0.33	0.19	0.13
Water	3				
3 Isobutanol	4				
Acetic acid	1	0.4	0.12	0.04	
Water	5				
4 *n*-Butanol	50				
Ethanol	25	0.36	0.20	0.13	0.22
5 *n*-Butanol	77				
Formic acid	10	0.13	0.02	0.01	
Water	13				
6 5% Na_2HPO_4 (aqueous solution)		0.30	0.49	0.34	
7 *n*-Butanol	3				
Pyridine	3	0.67	0.37	0.30	0.47
Water	3				
Glacial acetic acid	1				

* Values calculated from experiments with yeast hydrolysates.

The results of chromatographic separations of standards and yeast extracts in various solvent systems are presented in Table I.

Paper strips were cut as described by MATTHIAS[8] and the chromatograms were developed by the ascending technique for about 14 h. This method of cutting the paper strips considerably improved the chromatographic separation. After development the chromatograms were dried at room temperature, washed twice with xylene and once with ether to remove traces of phenol, which suppresses the fluorescence of flavins. The chromatograms were then photographed in U.V. light through the yellow filter marked 14-212 (PG-2) according to Coleman; the film used was "Agfa Röntgen Fluorapid für Schirmbildphotographie".

Solvent No. 2 gave a better separation of the flavins than No. 1, which was used more frequently. The least satisfactory results were obtained with the monophase solvent No. 6. Spots obtained in this particular solvent were diffuse and some of the chromatographed flavins were decomposed to lumichromes.

Solvent No. 7 containing pyridine proved to be the best. It was superior to all solvents mentioned above due to its higher resolving power, giving more compact spots and better distribution coefficients. This solvent was checked with standards and then successfully applied to the chromatographic separation of yeast hydrolysate Rb, FMN, FAD, and the so-called flavin fraction IV (Fig. 1).

The quantitative determination of the flavins was modified as follows: The chromatograms were photographed, the spots of the individual flavins were marked with a pencil in U.V. light, washed with chloroform, then cut out and eluted with buffers of various pH's. We elute Rb, FMN and FAD at pH 5.5, 3.5 and 2.8, respec-

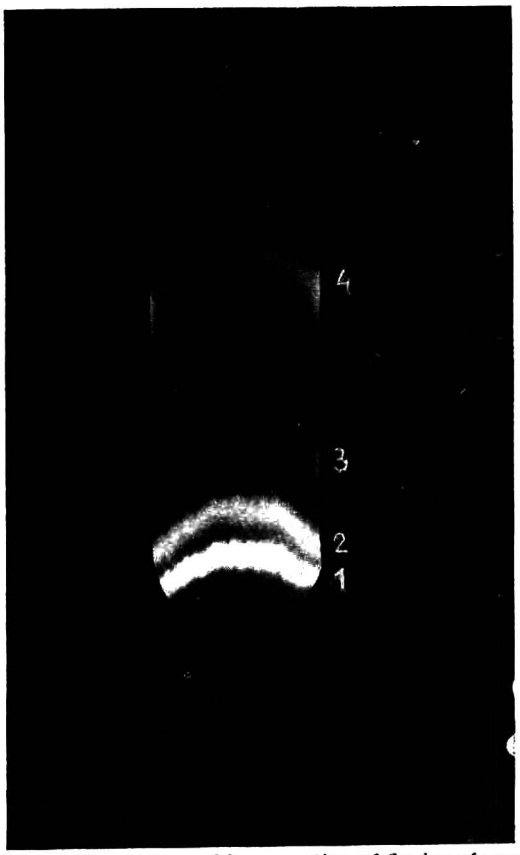

Fig. 1. Chromatographic separation of flavins of yeast hydrolysates. 1 = FAD; 2 = FMN; 3 = Flavin fraction IV; 4 = Rb.

tively. The flavins eluted were fluorimetrically determined in an electrophotofluorimeter using a standard of riboflavin (concentration 0.08 μg/ml). Recovery of the flavins was about 80%.

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Thin-layer chromatography of phosphorylated glycolysis intermediates*

Investigations concerned with the chromatographic separation of the intermediates of the EMBDEN-MEYERHOF glycolytic pathway have been confined mainly to paper and column methods[1-12]. These methods, while yielding good results, are handicapped by the length of time required for development. In some instances, partial purification must precede actual chromatography[2]. A procedure using gas chromatography has been described[13].

This note describes a method for the rapid thin-layer partition chromatography of several glycolytic intermediates.

Apparatus and materials

The Camag** spreading apparatus was employed for the preparation of 20 × 20 cm plates. The developing chamber consisted of two glass plates, one coated, one clean, separated by a cardboard gasket which covered three borders of the plates[14]. Equally good results were obtained by using 3-mm thick polyethylene instead of cardboard. Development was achieved by placing the ungasketed edge of the "sandwich" in a glass trough 2.5 × 22.5 cm which contained about 15 ml of solvent. Saturation of the chamber between the plates was extremely rapid since its overall volume was quite small (about 6 ml). Development took place quickly, often in as little as 20 min.

The cellulose employed was Camag Type D (without binder). Cellulose from Research Specialties Co., Bio-Rad Laboratories, and Macherey-Nagel*** was tested. Only Macherey-Nagel Type MN 300 was found comparable to the Camag D grade in our system.

The solvents were reagent grade and were used without further purification. In some cases practical grade acetonitrile (b.p. 80–82°) was used and found to give identical results. The compounds tested were obtained from the Sigma Chemical Co. and used without further purification. Visualization was attained by the spray reagent of HANES AND ISHERWOOD[4] followed by 5–10 min exposure to a Westinghouse Sterilamp G15T8 at a distance of 10 cm. Micropipets were used to spot the samples.

Procedure

The slurry consisted of 15 g cellulose and 90 ml distilled water. Plates coated with slurries which had been allowed to stand 12–18 h were consistently more uniform and gave greater reproducibility than those coated immediately after slurry preparation. The plates were coated to a thickness of 0.3 mm and dried in metal racks at 105° for 30 min. The dry cellulose layer was scraped from two opposite edges of the plate to a depth of 7–10 mm, a piece of filter paper (20 cm wide) was placed on the layer at the top, and the "sandwich" prepared. The filter paper touched the layer and extended through the gasket to the outside. The plate was then washed with the solvent to be used until the impurities in the cellulose were swept above the area to be utilized. The cellulose contained a brownish impurity which almost completely obliterated the separation until it was removed.

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** Available from Microchemical Specialties Co., Berkeley, Calif.

*** Available from Brinkmann Instruments, San Francisco, Calif.

2–4 μl of a solution containing 4 mg/ml of each compound were spotted 1.5 cm from the bottom of the plate. The applications were made in 1-μl portions, in order to limit the spot diameter to 5 mm or less. The spots were allowed to dry, the "sandwich" prepared with the top edge now scraped and without filter paper, and the development allowed to proceed 17–18 cm at room temperature (20–22°). The plate was then removed, dried at room temperature and sprayed. The movement of the compounds relative to the solvent front (R_F) and relative to orthophosphate (R_P) was then measured. R_F values were found to be less reproducible than R_P values.

TABLE I

POSITION CONSTANTS OF GLYCOLYTIC PHOSPHATE ESTERS

Phosphate ester	R_P	R_F
Orthophosphate	1.00	0.76
Phosphoenolpyruvate (PEP)	1.14	0.87
3-Phosphoglycerate (3PGA)	0.96	0.73
2-Phosphoglycerate (2PGA)	0.96	0.73
Fructose-6-phosphate (F6P)	0.38	0.29
Fructose-1,6-diphosphate (FDP)	0.38	0.29
Glucose-1-phosphate (G1P)	0.19	0.14
Glucose-6-phosphate (G6P)	0.19	0.14
Adenosine-5′-triphosphate (ATP)	0.00	0.00

Some 160 solvent systems were tested on cellulose plates, using a synthetic mixture of the glycolytic phosphate esters and orthophosphate listed in Table I. Based on the rapidity of solvent development, resolution in one dimension, and reproducibility of resolution, the following solvent system was chosen: acetone–acetonitrile–1 *N* hydrochloric acid (64:26:10, v/v). It gave rapid resolution into six distinct groups, of which three were pairs and three were singlets.

Results and discussion

The results obtained from the unidimensional development of the phosphate esters are shown in Table I and in Fig. 1. Excellent separation is achieved between compounds possessing different moieties attached to the phosphate group. However, isomer separation does not appear possible in this system. Alkaline solvents gave some separation of the fructose mono- and diphosphates, but excessive streaking led to abandonment of these systems. In order to separate the phosphoglyceric acids from orthophosphate it was necessary to allow the solvent to rise 17–18 cm from the bottom. If, however, orthophosphate is known to be absent, then the height may be limited to 10 cm, with excellent separation. In addition to the compounds listed in Table I, an equilibrium mixture of 3-phosphoglyceraldehyde and dihydroxyacetone phosphate was also tested with this system, and found to give a streaky spot of R_P 0.1–0.3.

In investigating solvent mixtures containing an acidic component, it was found that large concentrations of weak acids were not beneficial to the separation of our synthetic mixture. On the other hand, large amounts of either hydrochloric or trichloroacetic acid had a very desirable effect, apparently by insuring that all the

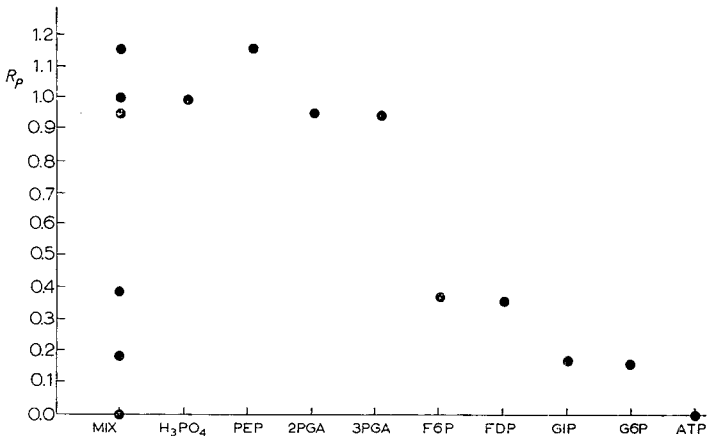

Fig. 1. Graphic plot of phosphate esters after thin-layer chromatography.

phosphates tested were in a single ionic form, namely the phosphate acid. Neither of these two acids hydrolyzes glucose-1-phosphate, the most labile of the phosphate esters tested, under the conditions used. Hydrochloric acid was the final choice since it is more easily handled than trichloroacetic acid.

Note added in proof

A paper by WARING AND ZIPORIN[15], using thin-layer chromatography for the separation of hexosephosphates and triosephosphates has appeared after completion of this work.

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The capillary absorption of liquids in paper and other porous materials

In studying the one-dimensional capillary absorption of liquids into porous materials, particularly in connection with paper chromatography, several observers[1–6] have noticed that a concentration* gradient exists between the liquid source and the advancing liquid front, that the shape of the concentration profile is independent of the distance of penetration (*i.e.* the concentration at any specified fraction of the distance between source and front is independent of time) and that the distance moved by any point of given concentration is proportional to the square root of the time elapsed since the start of the absorption. By adopting models for the capillary system, some authors[6–8] have derived theoretical equations in agreement with these practical observations.

RUOFF *et al.*[9] have pointed out that the flow of a liquid into paper under capillary suction forces is similar to the flow observed in diffusion though they "make no pretence that the molecular basis (of capillary flow) is a diffusion controlled process in the random molecular definition, admitting that it is diffusion by analogy".

GILLESPIE[6] assumed empirical relationships between capillary pressure (suction) and concentration, and between permeability and concentration, and by substituting these in a combination, due to MUSKAT AND MERES[10], of Darcy's law and the equation of continuity, derived relations for the rate of advance and distribution of liquid in paper.

It is the purpose of this communication to point out that, in *any* homogeneous porous material in which Darcy's law and the equation of continuity are obeyed, liquids will be absorbed at a rate determined by the square root relationship, and the shape of the reduced concentration profile will be independent of time. No assumptions need be made as to the structure of the porous material and no particular model for the capillary system need be adopted. It is only necessary to assume that the capillary pressure and permeability at any point in the porous material are dependent only on the structure of the material and the local liquid concentration[11].

The capillary pressure in the material will be determined by the size of the largest filled pores. As the liquid is drawn into the material, the small pores fill first, their capillary pressure being the greater. For any given concentration, liquid will distribute itself so that, depending on the pore size distribution, only pores up to a given size will be filled. The capillary pressure at any point will then be uniquely determined by the structure of the porous material and the amount of liquid present.

It is known that the permeability of a porous material to a particular fluid depends to some extent on whether the fluid concentration in the material is increasing or decreasing, that is, whether the liquid is penetrating or leaving the porous solid. However, as an advancing liquid is being considered here, hysteresis effects of this nature need not concern us. It has been pointed out above, that for any given liquid concentration, only pores up to a given size will be filled. Flow can pass only through these pores and the permeability will therefore depend on the size and distribution of filled pores, and hence upon the pore structure and liquid concentration.

If Darcy's law is assumed, the flow through any point in the porous material is given by (K/μ) grad P, where K is the local permeability, μ the liquid viscosity,

* "Concentration" here refers to the amount of liquid per unit volume of porous solid.

and grad P the pressure gradient—in this case the capillary pressure gradient. When this expression is combined with the equation of continuity we obtain:

$$\text{div } [(K/\mu) \text{ grad } P] = \frac{\partial C}{\partial t} \tag{1}$$

C being the local liquid concentration.

A similar equation has been given by RICHARDS[11].

But as K and P depend only on C for any given porous material, and $\partial P/\partial C$ will also depend only on C, this equation may be written:

$$\text{div } (D \text{ grad } C) = \frac{\partial C}{\partial t} \tag{2}$$

where $D = (K/\mu)\ (\partial P/\partial C)$ and is also a function of C only. In its one dimensional form this equation reduces to:

$$\frac{\partial}{\partial x}\left(D \frac{\partial C}{\partial x}\right) = \frac{\partial C}{\partial t} \tag{3}$$

which is similar to the one dimensional forms of Fick's law of diffusion.

As RUOFF[9] points out, it was shown by BOLTZMAN (*cf.* ref. 12) that for certain boundary conditions, where D is a function of C only, C may be expressed in terms of a single variable $\lambda_C = x/t^{1/2}$, eqn. (3) then being reducible to an ordinary differential equation in C and λ_C only. Any particular constant value of C will therefore move according to the equation:

$$x = \lambda_C t^{1/2}$$

and so the movement of the advancing front will also follow this equation and the shape of the reduced concentration profile will be independent of time.

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Book Reviews

Gas Chromatography Abstracts, 1963, edited by C. E. H. KNAPMAN, published by the Institute of Petroleum, London, 1964, x + 286 pp., price 42 s.

This is the 6th volume of the series and the first which has been published by the Institute of Petroleum. It is indistinguishable as far as type and production is concerned from the previous volumes (which were published by Butterworths) and contains 1051 abstracts, an author and a subject index. Like the previous volumes this book will be indispensable for all chemists working with gas chromatography.

G. P. CARTONI (Rome)

Gas Analysis by Gas Chromatography, by P. G. JEFFREY AND P. J. KIPPING, Pergamon Press, Oxford, xi + 216 pp., price £ 3.10 s.

This is volume 17 of an International series of monographs on Analytical Chemistry and aims to give the industrial analyst methods which will replace some of the traditional techniques. It is limited to "materials that are normally transferred in the gaseous state and are present in such amounts that warrant determination". This limitation restricts the book quite severely, but gives the authors the opportunity to include more experimental detail than normally occurs in books on gas chromatography. The whole of the book, in fact, is written with emphasis on the experimental aspect and therefore is of great value to the practising analyst.

The first section of the book deals with the general principles and equipment of gas chromatography, and the second with the experimental details of applying the technique to separations of individual gases: hydrogen, and other common gases like oxygen, nitrogen, methane, carbon monoxide, and carbon dioxide, hydrocarbons, oxides of nitrogen, ammonia, sulphur dioxide, hydrogen sulphide, and carbonyl sulphide, halogen compounds. A third section deals with some complete analyses of coal gas, and related mixtures, gases in solid materials, gases in liquid sample material, and finally gas sampling.

This is a useful compilation for the gas analyst and can be recommended for this purpose, though it will be of interest to others as well.

F. H. POLLARD (Bristol)

Electrochemistry of Molten and Solid Electrolytes, Vol. 2, edited by M. V. SMIRNOV (authorised translation from the Russian), Transactions (Trudy) No. 4 of the Institute of Electrochemistry, Urals Academy of Sciences, published by Consultants Bureau, New York, 1964, price $ 17.50.

In this volume several interesting communications dealing with various techniques in molten salts are reported.

Some of the papers, concerning the electrolytic production and refining of metallic uranium and uranium dioxide in fused alkali chlorides, contribute further to the knowledge of the nature of the reactions occurring at the electrodes during these processes.

Investigations on the electro-plating of metals in molten salts, and the formation of an alloy of the metal deposited with the metal of the backing are described, and the relationship between cathode potential, phase composition and structure of the platings are carefully examined.

Further results on the structure and the nature of the electrical conductivity of solid oxide systems are reported.

The collection of papers in this volume will be of interest not only to investigators in the above-mentioned fields, but also to those interested in general problems of molten salt chemistry.

A. CONTE (Rome)

Stability Constants of Metal-ion Complexes, by L. G. SILLÉN AND A. E. MARTELL, Chemical Society Special Publication No. 17, xviii + 754 pp., price £ 8.0.0.

This is the second, revised and enlarged edition of the well-known compilation which appeared for the first time in 1957–58. In the new edition, E. A. MARTELL has replaced G. SCHWARZENBACH as the compiler of the part on organic ligands.

This very valuable collection of equilibrium data has been extended to cover inorganic redox equilibria and also includes some data on solvent extraction of metal complexes. Nomenclature and symbols are now somewhat more uniform in the inorganic and the organic parts. A detailed key gives the interpretation of the symbols and criteria of classification used throughout the book.

The usefulness of such a compilation is beyond any doubt, and the present volume meets very satisfactorily the requirements of a complete critical selection of data available from the literature until 1960 (and sometimes later). It is thought, however, that the selection criteria used by the compilers, or to be used by the reader (in those cases where several data pertaining to the same equilibrium under different experimental conditions or due to different techniques are reported) are not always clear to readers not professionally interested in techniques for stability constant determinations, and therefore an introductory description of such selection methods, like that in the analogous compilation by YATSIMIRKII, would have been useful.

C. FURLANI (Trieste)

Manuel Pratique de Chromatographie en Phase Gazeuse, edited by J. TRANCHANT, Masson et Cie., Paris, 1964, 232 pp. and 62 figs., price 36 F.

The value of this book is that it provides much useful information on the principles and practice of gas chromatography. The general theory is treated simply and an explanation of the fundamental concepts is given, thus supplying the necessary background. The instrumentation for gas chromatography is described in detail and the more recent improvements receive particular attention. Ionization detectors and capillary columns are well described and much information concerning their basic principles and optimum working conditions are reported. The general aspects of qualitative analysis and quantitative determination are treated and criticized. The more advanced methods such as the retention index method and the programmed temperature procedure are clearly explained.

A few, but well selected, examples of the application of gas chromatography are reported with a large number of references. This book provides a guide of great practical utility for all workers in this field.

G. P. CARTONI (Naples)

Biomedical Applications of Gas Chromatography, edited by H. A. SZYMANSKI, Plenum Press, N.Y., 1964, 324 pp., price $ 12.50.

This book is based on a series of lectures given during the Fifth Annual Gas Chromatography Course, held at Canisius College, Buffalo, N.Y., 1963.

The large number of papers which have appeared recently shows the increasing interest in the application of gas chromatography to biomedical analysis. With the development of more advanced instruments, furnished with high sensitivity ionization detectors, and high resolution columns, the separation of many similar substances and their determination in very small amounts, can be easily carried out.

This volume covers almost all fields where gas chromatography can be successfully applied to biomedical and biochemical problems.

In a short introduction the theory of gas chromatography is briefly summarized and many practical aspects explained; this part is necessarily very concise and some important concepts are only outlined. Gas chromatography of amines, amino acids and alkaloids is dealt with in detail and practical problems usually encountered in this type of analysis such as columns, supports, stationary phases, are discussed with reference to the wide experience of the authors. The analysis of steroids, perhaps one of the more important applications of gas chromatography to medicine, is very well presented with many data and explanatory chromatograms.

Other topics such as the analysis of carbohydrates, bile and fatty acids are carefully explained and reviewed. In all the sections complete procedures are given which would allow anyone to undertake any type of analysis in this field.

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CHROMATOGRAPHIC DATA

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TABLE 1

R_F VALUES OF CYSTINE PEPTIDES AND THEIR DERIVATIVES

(H. N. RYDON AND F. O. DOS S. P. SERRAO, *J. Chem. Soc.*, (1964) 3638)

Solvents: S_1 = Acetic acid–butan-1-ol–water (16.7:100:37.6, v/v, equilibrated for 30 days at room temperature).
S_2 = Pyridine–butan-1-ol–water (21:39:39, v/v).
Paper: Whatman No. 1.

Compound	R_F	
	S_1	S_2
N-Monobenzyloxycarbonyl-L-cystine	0.33	0.41
N-Monobenzyloxycarbonyl-L-cystine diethyl ester hydrochloride	0.84	0.90
N-Benzyloxycarbonyl-N′-(N-formylglycyl)-L-cystine diethyl ester	0.89	0.90
N,N′-Bis-benzyloxycarbonyl-L-cystine diethyl ester	0.89	—
N,N′-Bis-(N-formylglycyl)-L-cystine diethyl ester	0.70	—
L-Cystine diethyl ester bis-toluene-*p*-sulphonate	0.53	—
N,N′-Bis-(N-formylglycyl)-L-cystine diethyl ester	0.70	0.80
N,N′-Bis-(N-tritylglycyl)-L-cystine diethyl ester	0.95	0.96
N,N′-Bis-(N-benzyloxycarbonylglycyl)-L-cystine diethyl ester	0.93	0.92
N,N′-Bis-glycyl-L-cystine diethyl ester dihydrochloride	0.24	0.55
N,N′-Bis-(N,S-bis-benzyloxycarbonyl-L-cysteinylglycyl)-L-cystine diethyl ester	0.97	—
N-(N-Tritylglycyl)-L-cystine diethyl ester toluene-*p*-sulphonate monohydrate	0.80	0.75
N-(N-Benzyloxycarbonylglycyl)-N′-(N-tritylglycyl)-L-cystine diethyl ester	0.89	—
N-(N-Tritylglycyl)-N′-(N-tritylglycyl)-L-cystine diethyl ester	0.96	—
N-(S-Benzyl-N-benzyloxycarbonyl-L-cysteinyl)-N′-(N-tritylglycyl)-L-cystine diethyl ester	0.89	—
N-(S-Benzyl-N-benzyloxycarbonyl-L-cysteinylglycyl)-N′-(N-benzyloxycarbonylglycyl)-L-cystine diethyl ester	0.81	—
N,S-Bis-benzyloxycarbonyl-cysteine ethyl ester	0.85	0.90
N,S-Bis-benzyloxycarbonyl-L-cysteinyl-L-leucine methyl ester	—	0.80
N,S-Bis-benzyloxycarbonyl-L-cysteinyl-L-tyrosine methyl ester	0.91	0.93
N,S-Bis-benzyloxycarbonyl-L-cysteinylglycylglycine ethyl ester	0.90	—

TABLE 2

R_F VALUES OF PEPTIDES RELATED TO OXYTOCIN

(S. DRABAREK, *J. Am. Chem. Soc.*, 86 (1964) 4477)

Solvent: Butan-1-ol–acetic acid–water (4:1:5).
Paper: Whatman No. 1.

Compound	R_F
L-Isoleucyl-glycyl-L-asparaginyl-S-benzyl-L-cysteinyl-propyl-L-leucyl-glycinamide	0.68
4-Glycine-oxytocin	0.58
Glycyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide	0.25
3-Glycine-oxytocin	0.36
2-Glycine-oxytocin	0.38

TABLE 3

R_F VALUES OF PEPTIDES RELATED TO ADRENOCORTICOTROPIN

(C. H. LI, J. RAMACHANDRAN, D. CHUNG AND B. GORUP, *J. Am. Chem. Soc.*, 86 (1964) 2703)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:1).
S_2 = Butan-2-ol–10% ammonia (85:15).
Paper: Whatman No. 1.

Compound	R_F	
	S_1	S_2
Carbobenzoxy-valyl-glycine methyl ester	0.87	0.85
Carbobenzoxy-prolyl-valyl-glycine methyl ester	0.87	0.87
Prolyl-valyl-glycine methyl ester	0.56	0.66
N^{α}-Butyloxycarbonyl-N^{ε}-tosyl-lysine	0.76	0.50
N^{α}-*tert.*-Butyloxycarbonyl-N^{ε}-tosyl-lysine *p*-nitrophenyl ester	0.91	0.88
N^{α}-*tert.*-Butyloxycarbonyl-N^{ε}-tosyl-lysyl-prolyl-valyl-glycine	0.91	0.37
N^{ε}-Tosyl-lysyl-propyl-valyl-glycine	0.59	0.09
N^{ε}-Tosyl-lysine-benzyl ester hydrochloride	0.76	0.82
N^{α}-Carbobenzoxy-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-lysine benzyl ester	0.89	0.88
N^{α}-Carbobenzoxy-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester	0.89	0.88
N^{ε}-Tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester	0.80	0.82
N^{α}-*tert.*-Butyloxycarbonyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester	0.87	0.88
N^{ε}-Tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginine	0.67	0.22
N^{α}-*tert.*-Butyloxycarbonyl-N^{ε}-tosyl-lysyl-prolyl-valyl-glycyl-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester	0.89	0.86
N^{ε}-Tosyl-lysyl-prolyl-valyl-glycyl-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester trifluoroacetate	0.76	0.84
N^{ε}-Tosyl-lysyl-prolyl-valyl-glycyl-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-lysyl-arginine benzyl ester	0.78	0.75

TABLE 4

R_F VALUES OF PEPTIDES RELATED TO ADRENOCORTICOTROPIN

(C. H. LI, J. RAMACHANDRAN AND D. CHUNG, *J. Am. Chem. Soc.*, 86 (1964) 2711)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:1).
S_2 = Butan-2-ol–10% ammonia (85:15).
Paper: Whatman No. 1.

Compound	R_F	
	S_1	S_2
N^{α}-Carbobenzoxy-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.79	0.89
N^{α}-Carbobenzoxy-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.88	0.86
N^{α}-Carbobenzoxy-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.87	0.89
N^{α}-Carbobenzoxy-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.93	0.93
N^{ε}-*tert.*-Butyloxycarbonyl-lysyl-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.81	—
N^{α}-Carbobenzoxy-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.92	0.92
N^{α}-Carbobenzoxy-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.90	0.92
N^{ε}-Tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.77	0.79

TABLE 5

R_F VALUES OF PEPTIDES RELATED TO ADRENOCORTICOTROPIN

(C. H. LI, D. CHUNG AND J. RAMACHANDRAN, *J. Am. Chem. Soc.*, 86 (1964) 2715)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:1).
S_2 = Butan-2-ol–10% ammonia (85:15).

Compound	R_F	
	S_1	S_2
N^{α}-Carbobenzoxy-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-prolyl-valyl-glycine methyl ester	0.93	0.94
N^{α}-Carbobenzoxy-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-prolyl-valyl-glycine	0.83	0.51
N^{α}-Carbobenzoxy-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.81	0.81
N^{ε}-Butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{ε}-*tert.*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.76	0.81
N^{α}-Carbobenzoxy-N^{ε}-tosyl-lysyl-prolyl-valyl-glycyl-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.91	0.82
N^{ε}-Tosyl-lysyl-propyl-valyl-glycyl-N^{ε}-tosyl-lysyl-N^{ε}-tosyl-lysyl-N^{G}-tosyl-arginyl-proline *tert.*-butyl ester	0.86	0.88

TABLE 6

R_F VALUES OF POLYMYXINS

(S. WILKINSON AND L. A. LOWE, *J. Chem. Soc.*, (1964) 4107)

Solvents: S_1 = Butan-1-ol–acetic acid–water (120:30:50).
S_2 = Butan-1-ol–acetic acid–water (4:1:5) (upper phase).
S_3 = Butan-1-ol–acetic acid–water (4:1:5) (lower phase).
S_4 = Butan-1-ol–pyridine–acetic acid–water (30:20:6:24).
S_5 = Butan-1-ol–acetic acid–1% aqueous NaCl (120:30:50).
S_6 = Butan-1-ol–acetic acid–5% aqueous NaCl (120:30:50).
S_7 = Butan-1-ol–pyridine–acetic acid–1% aqueous NaCl (30:20:6:24).
Paper: a = Whatman No. 1.
b = Whatman No. 2.
c = Whatman No. 4.
d = Whatman No. 20.

Compound	R_F															
	S_1				S_2			S_3	S_4			S_5	S_6		S_7	
	a	*b*	*c*	*d*	*a*	*c*	*d*	*a*	*a*	*c*	*d*	*a*	*a*	*c*	*a*	
Polymyxin A	0.39	0.29	—	0.15	—	—	—	0.92	0.58	—	—	—	—	—	—	
Polymyxin B	0.54	0.42	0.52	0.31	0.50	0.46	—	0.86	0.73	0.73	—	0.42	0.37	0.46	0.24	
Polymyxin E (Colistin)	0.51	0.40	0.51	0.30	0.46	0.45	0.20	0.90	0.77	0.72	0.64	0.42	0.37	0.46	0.24	

TABLE 7

R_F VALUES OF CYCLOSERINE AND SOME ANALOGUES

(F. C. NEUHAUS AND J. L. LYNCH, *Biochemistry*, 3 (1964) 472)

Solvents: S_1 = *n*-Butanol–acetic acid–water (4:1:5).
S_2 = 77% ethanol.
S_3 = Methyl ethyl ketone–pyridine–water (20:5:8).
S_4 = Isopropanol–ammonia–water (80:2:18).
Paper: Whatman No. 3 MM.

Compound	R_F			
	S_1	S_2	S_3	S_4
D-Cycloserine	0.31	0.43	0.37[a]	0.17
cis-DL-Cyclothreonine	0.39	0.57	0.54[a]	0.21
trans-DL-Cyclothreonine	0.41	0.61	0.60[a]	0.23
β-Aminoxy-D-alanine methyl ester	[b]	0.66	0.88[c]	[d]
β-Aminoxy-D-alanine	[b]	0.22	0.55[c]	0.08[a]
D-Serine	0.18	0.27	0.18	0.12
D-Alanine	0.28	0.40	0.24	0.20

[a] Major spots but some streaking present.
[b] Poor solvent system as indicated by extensive decomposition.
[c] Chromatographed as the oxime.
[d] Possible cycloserine formation.

TABLE 8

R_F VALUES OF SUBSTITUTED PYRIMIDINES

(J. D. FISSEKIS, A. MYLES AND G. B. BROWN, *J. Org. Chem.*, 29 (1964) 2670)

Solvents: S_1 = Butan-1-ol–acetic acid–water (12:3:5).
S_2 = Butan-1-ol–water (85:15).
S_3 = Propan-2-ol–water–ammonia (7:2:1).
Paper: Whatman No. 1 (ascending).

Compound						
Position of substituents of pyrimidine				R_F		
2	*4*	*5*	*6*	S_1	S_2	S_3
SH	OH	$CH_2CHOHCH_3$	NH_2	0.70	0.54	0.50
SH	OH	CH_2CH_2OH	NH_2	0.53	—	0.46
OH	OH	$CH_2CHOHCH_3$	NH_2	0.53	0.31	0.43
OH	OH	CH_2CH_2OH	NH_2	0.41	0.17	0.34
OH	OH	CH_2CH_2OAc	NHAc	0.70	0.53	0.53
NH_2	OH	CH_2CH_2OH	H	0.49	0.24	0.48
SH	OH	CH_2CH_2OH	H	0.63	0.43	0.58
OH	OH	CH_2CH_2OH	H	0.46	0.28	0.48
SCH_3	OH	CH_2CH_2OH	H	0.82	0.73	0.66
OH	OH	CH_2CH_2OAc	H	0.71	0.55	0.65
OH	SH	CH_2CH_2OAc	H	—	—	0.82
OH	NH_2	CH_2CH_2OH	H	0.39	0.12	0.53

TABLE 9

R_X VALUES OF SOME PYRIMIDINES AND THEIR DERIVATIVES

(Y. KURIKI, *Biochim. Biophys. Acta*, 80 (1964) 361)

Solvents: S_1 = Isobutyric acid–1 *N* ammonia (10:6).
S_2 = *n*-Butanol–water (86:14).
S_3 = *n*-Butanol–ethanol–water (50:15:30).
S_4 = 80% ethanol in 0.02 *N* acetic acid.
Paper: Whatman No. 1.
Detection: U.V. light (Mineralight Model SL 2537, Ultraviolet Products, Inc.).

Compound	$R_{Uridine}$			
	S_1	S_2	S_3	S_4
Uracil	1.23	2.37	1.44	1.19
Uridine	1.00	1.00	1.00	1.00
deU	1.18	2.66	1.27	1.03
UMP	0.42	0	0.18	0.25
deUMP	0.58	0	0.33	0.39
Cytosine	1.68	3.90	1.86	1.12
C	1.48	1.95	1.36	1.12
CMP	0.82	0	0.29	0.36
deCMP	0.10	0	0.36	0.50
Thymine	1.60	5.63	2.36	1.32
T	1.46	5.60	2.26	1.21
TMP	0.78	0	0.42	0.62
ψ-Uridine	0.68	0.74	0.71	0.88
ψ-UMP	0.39	—	—	—
5-Hydroxymethyluracil	1.50	2.50	1.34	1.06
5-Hydroxymethylcytosine	1.65	2.79	1.73	1.23
5-Hydroxymethylcytidine	1.05	1.10	1.05	0.91

TABLE 10

R_F VALUES OF 5′-THYMIDYLIC ACID DERIVATIVES

(F. N. HAYES AND E. HANSBURY, *J. Am. Chem. Soc.*, 86 (1964) 4172)

Solvent: Propan-1-ol–conc. ammonia–water (55:10:35).
Paper: Whatman No. 41 H (No. 541).

Compound	R_F
Thymidine	0.73
5′-Thymidylic acid	0.41
Thymidine 3′,5′-diphosphate	0.25
5′-Thymidylic acid pentamer	0.23
5′-Thymidylic acid decamer	0.11

TABLE 11

R_F VALUES OF CYTIDINE AND GUANOSINE DERIVATIVES

(R. LOHRMANN AND H. G. KHORANA, *J. Am. Chem. Soc.*, 86 (1964) 4188)

Solvents: S_1 = Propan-2-ol–conc. ammonia–water (7:1:2, v/v).
S_2 = Butan-1-ol–30% acetic acid (2:1, v/v).
S_3 = Ethanol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v).
S_4 = Isobutyric acid–conc. ammonia–water (57:4:39, v/v).
Paper: Whatman No. 1.

Compound	R_F			
	S_1	S_2	S_3	S_4
Cytidine	0.49	0.22	0.63	—
Guanosine	0.31	0.20	0.57	—
$O^{5'}$-Monomethoxytritylguanosine	0.82	—	0.82	—
N^2,$O^{2'}$,$O^{3'}$-Triacetylguanosine	—	0.72	0.81	—
$O^{2'}$,$O^{3'}$-Diacetylguanosine	—	0.60	0.72	—
Cytidine 3′-phosphate	0.10	0.051	0.26	0.61
Guanosine 3′-phosphate	0.057	0.033	0.17	0.43
N,$O^{2'}$,$O^{5'}$-Triacetylcytidine 3′-phosphate	—	0.20	0.56	—
N^2,$O^{2'}$,O^{5}-Triacetylguanosine	—	0.21	0.53	—
$O^{2'}$,$O^{5'}$-Diacetylguanosine 3′-phosphate	—	—	0.40	—
Cytidylyl-(3′ → 5′)-adenosine	0.21	0.023	0.40	0.73
Cytidylyl-(3′ → 5′)-cytidine	0.18	0.019	0.41	0.65
Cytidylyl-(3′ → 5′)-guanosine	0.087	0.019	0.34	0.51
Cytidylyl-(3′ → 5′)-uridine	0.17	0.05	0.42	0.52
Guanylyl-(3′ → 5′)-adenosine	0.10	0.021	0.33	0.56
Guanylyl-(3′ → 5′)-cytidine	0.10	0.03	0.29	0.51
Guanylyl-(3′ → 5′)-guanosine	0.040	0.020	0.24	0.34
Guanylyl-(3′ → 5′)-uridine	0.10	0.03	0.36	0.39

TABLE 12

PAPER ELECTROPHORESIS OF SOME NUCLEOTIDES

(R. LOHRMANN AND H. G. KHORANA, *J. Am. Chem. Soc.*, 86 (1964) 4188)

Buffer: 0.03 *M* potassium phosphate, pH 7.1.
Voltage: 4000 V.
Paper: Whatman No. 3 MM.

Compound	*Mobility**
Cytidine 3′-phosphate	1.0
Guanosine 3′-phosphate	0.95
N,$O^{2'}$,$O^{5'}$-Triacetylguanosine 3′-phosphate	0.82
N^2,$O^{2'}$,$O^{5'}$-Triacetylguanosine 3′-phosphate	0.86
Cytidine 2′,3′-cyclic phosphate	0.63
Guanosine 2′,3′-cyclic phosphate	0.60
Cytidylyl-(3′ → 5′)-adenosine	0.37
Cytidylyl-(3′ → 5′)-cytidine	0.35
Cytidylyl-(3′ → 5′)-guanosine	0.38
Cytidylyl-(3′ → 5′)-uridine	0.36
Guanylyl-(3′ → 5′)-adenosine	0.34
Guanylyl-(3′ → 5′)-cytidine	0.33
Guanylyl-(3′ → 5′)-guanosine	0.29
Guanylyl-(3′ → 5′)-uridine	0.32

* Relative to cytidine 3′-phosphate.

TABLE 13

PAPER ELECTROPHORESIS OF URIDINE POLYNUCLEOTIDES

(C. COUTSOGEORGOPOULOS AND H. G. KHORANA, *J. Am. Chem. Soc.*, 86 (1964) 2926)

Buffers: B_1 = 0.03 *M* potassium phosphate (pH 7.1).
B_2 = 0.05 *M* ammonium formate–formic acid (pH 3.6).
Electric potential: 5000 V.

Compound	*Mobility*	
	B_1	B_2
Uridine 3′-phosphate	1.00*	1.00*
2′-O-Acetyluridine 3′-phosphate	0.84	—
2′-O-Acetyl-3′-uridylyl acetate	0.48	—
C_5′-Pyridinium Up	0.42	0.00
Uridine 3′,5′-cyclic phosphate	0.61	1.08
Cyclic dinucleotide	0.82	1.41
UpUp	1.00	1.24
Cyclic trinucleotide	0.92	1.60
UpUpUp	1.05	1.49
UpUpUpUp	1.04	1.51
UpUpUpUpUp	1.03	1.65
UpUpUpUpUpUp	1.03	1.52

* 15–17 cm run for uridine 3′-phosphate.

TABLE 14

R_F VALUES OF URIDINE POLYNUCLEOTIDES

(C. COUTSOGEORGOPOULOS AND H. G. KHORANA, *J. Am. Chem. Soc.*, 86 (1964) 2926)

Solvent: Propan-1-ol–conc. ammonia–water (55:10:35).
Paper: Whatman No. 1 or 3 MM.

Compound	R_F
Uridine 3′-phosphate	0.36
Uridine	0.52
C_5′-Pyridinium uridine 3′-phosphate	0.34
Uridine 3′,5′-cyclic phosphate	0.54
Cyclic dinucleotide	0.32
UpUp	0.25
UpU	0.40
Cyclic trinucleotide	0.22
UpUpUp	0.18
UpUpU	0.28
UpUpUpUp	0.12
UpUpUpU	0.20
UpUpUpUpUp	0.11
UpUpUpUpU	0.14
UpUpUpUpUpUp	0.07
UpUpUpUpUpU	0.10
$U(pU)_6p$	0.31*
$U(pU)_7p$	0.23*
$U(pU)_8p$	0.17*
$U(pU)_9p$	0.10*

* Relative to Up.

TABLE 15

R_F VALUES (RELATIVE) OF SUBSTITUTED PURINES RELATED TO ADENINE

(L. B. TOWNSEND, R. K. ROBINS, R. N. LOEPPKY AND N. J. LEONARD, *J. Am. Chem. Soc.*, 86 (1964) 5320)

Solvents: S_1 = Dimethylformamide–28% aqueous ammonia–propan-2-ol (25:10:65, v/v).
S_2 = Propan-2-ol–5% aqueous ammonium sulphate (1:19, v/v).

Compound	R_F*	
	S_1	S_2
Adenine		
1-Methyl-	0.94	0.69
3-Methyl-	1.20	1.61
7-Methyl-	1.38	1.28
9-Methyl-	1.40	1.04
Aminopurine		
6-Methyl-	1.83	1.19
1-Methyl-6-methyl-	1.47	1.78
3-Methyl-6-methyl-	1.69	1.65
7-Methyl-6-methyl-	2.24	1.40
9-Methyl-6-methyl-	2.24	1.24
6-Dimethyl-	2.38	1.29
Methylpurine		
6-Dimethylamino-1-	1.60	1.83
6-Dimethylamino-3-	2.29	1.68
6-Dimethylamino-7-	2.50	1.68
6-Dimethylamino-9-	2.95	1.31

* Relative to adenine

TABLE 16

R_F VALUES (THIN LAYER) OF CERTAIN METHYLATED PURINES AND NUCLEOSIDES

(A. D. BROOM, L. B. TOWNSEND, J. W. JONES AND R. K. ROBINS, *Biochemistry*, 3 (1964) 495)

Solvents: S_1 = 5% aqueous ammonium bicarbonate.
S_2 = 5% aqueous sodium dihydrogen phosphate saturated with isoamyl alcohol.
S_3 = Conc. aqueous ammonia–N,N-dimethylformamide–isopropanol (10:25:65).
Thin layer: MN-Cellulose powder 300G, 250 μ.

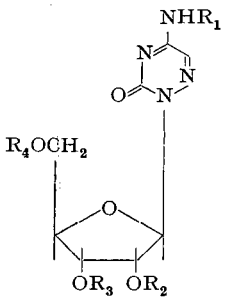
Compound	R_F		
	S_1	S_2	S_3
1-Methyldeoxyinosine	0.87	0.80	0.66
2′-Deoxyinosine	0.80	0.75	0.40
1-Methylhypoxanthine	0.73	0.68	0.48
Hypoxanthine	0.63	0.61	0.34
1-Methylguanosine	0.67	0.73	0.57
Guanosine	0.61	0.65	0.20
1-Methylguanosine	0.49	0.33	0.37
Guanine	0.42	0.25	0.17
2′-Deoxy-1-methylguanosine	0.69	0.69	0.62
2′-Deoxyguanosine	0.64	0.65	0.53
1,7-Dimethylguanosine iodide	0.77	0.86	
7-Methylguanine	0.48	0.58	0.39
1,7-Dimethylguanine	0.59	0.43	0.49

TABLE 17

R_F VALUES OF NUCLEOSIDES

(J. BERÁNEK AND J. PIŤHA, *Collection Czech. Chem. Commun.*, 29 (1964) 628)

Paper: Whatman No. 1.
Impregnation: I_1 = Formamide.
I_2 = 1,2-Propanediol.
Mobile phase: S_1 = *n*-Butanol saturated with water.
S_2 = *n*-Butanol–acetic acid–water (5:2:3).
S_3 = Chloroform.
S_4 = Chloroform–benzene (1:1).
S_5 = Benzene.
Detection: D_1 = U.V. light (Chromatolite).
D_2 = Potassium periodate–benzidine reagent (M. VISCONTINI, D. HOCH AND P. KARRER, *Helv. Chim. Acta*, 38 (1955) 642).



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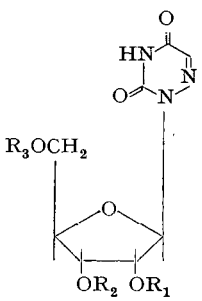

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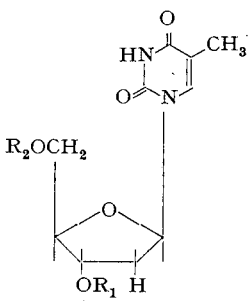

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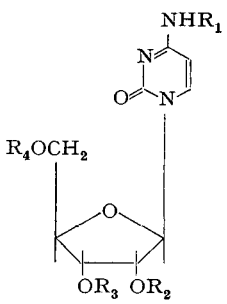

(IV)

Compound					R_F					
Type	R_1	R_2	R_3	R_4	S_1	S_2	S_3I_1	S_4I_1	S_5I_1	S_5I_2
I	H	H	H	H	0.10	0.32	—	—	—	—
I	H	H	H	Trityl	—	—	0.04	0	0	0
I	H	CH_3CO	CH_3CO	Trityl	0.82	—	0.89	0.84	0.40	0.40
I	CH_3CO	CH_3CO	CH_3CO	Trityl	0.86	0.90	0.92	0.93	0.84	0.91
I	CH_3CO	CH_3CO	CH_3CO	CH_3CO	0.80	0.85	0.87	0.52	0.15	0.46
I	H	CH_3CO	CH_3CO	CH_3CO	0.71	0.80	0.17	0.27	—	—
I	H	CH_3CO	CH_3CO	H	0.57	0.71	0.03	0.03	—	—
II	H	H	H		0.15	—	—	—	—	—
II	H	H	Trityl		—	—	0.42	0.15	0.01	0.01
II	CH_3CO	CH_3CO	Trityl		0.94	—	0.88	0.85	0.79	0.84
II	CH_3CO	CH_3CO	H		0.66	0.75	0.10	0.08	—	—
II	CH_3CO	CH_3CO	CH_3CO		0.80	—	0.69	0.23	0.02	0.22
	5′-O-Acetyl-6-azauridine				0.43	—	—	—	—	—
	Thymidine				0.49	—	—	—	—	—
III	H	Trityl			—	—	0.92	0.85	0.39	0.35
	Cytidine				0.13	—	—	—	—	—
IV	CH_3CO	CH_3CO	CH_3CO	CH_3CO	0.82	0.87	0.87	0.37	0.07	0.32
IV	H	CH_3CO	CH_3CO	CH_3CO	0.71	0.80	0.31	0.02	0	0.01

TABLE 18

R_F VALUES OF NUCLEOTIDES

(J. BERÁNEK AND J. PIŤHA, *Collection Czech. Chem. Commun.*, 29 (1964) 629)

Solvents: S_1 = Saturated aqueous solution of ammonium sulphate–1 *M* ammonium acetate–2-propanol (79:19:2).
S_2 = Ethanol–1 *M* ammonium acetate (5:2).
Paper: Whatman No. 1.
Detection: D_1 = U.V. light (Chromatolite).
D_2 = Potassium periodate–benzidine reagent (M. VISCONTINI, D. HOCH AND P KARRER, *Helv. Chim. Acta*, 38 (1955) 642).

Compound	R_F	
	S_1	S_2
6-Azauridine 5′-phosphate	0.85	0.27
2′,3′-Di-O-acetyl-6-azauridine 5′-phosphate	0.64	0.61
2′,3′-Di-O-propionyl-6-azauridine 5′-phosphate	0.65	0.73
2′,3′-Isopropylidene-6-azauridine 5′-phosphate	0.60	0.60

TABLE 19

R_F VALUES OF PTERIDINE DERIVATIVES

(A. ALBERT AND E. P. SERJEANT, *J. Chem. Soc.*, (1964) 3357)

Solvents: S_1 = Butanol–5 *N* acetic acid (7:3) (ascending).
S_2 = 3% ammonium chloride (ascending).
Paper: Whatman No. 1.

Compound	R_F	
	S_1	S_2
4,5-Diaminopyrimidine	0.30	0.65
7-Hydroxy-6-methylpteridine	0.65	0.70
6-Hydroxy-7-methylpteridine	0.60	0.70
7-Hydroxypteridine	0.50	0.65
6-Hydroxypteridine	0.4–0.8	0.05
6,7-Dihydroxypteridine	0.25	0.55
Dipteridinylmethane	0.20	0.50 and 0.40
7-Methyl-dipteridinylmethane	0.35	0.65 and 0.55
Quinazoline	0.90	0.0
3,4-Dihydro-4-(7-hydroxypteridin-6-yl-methyl)-quinazoline	0.50	0.60
7,8-Dihydro-6-hydroxy-7-(quinazolin-4-yl-methyl)-pteridine	0.3–0.6	0.45
7,8-Dihydro-7-methyl-6-hydroxy-7-(quinazolin-4-yl-methyl)-pteridine	0.75	0.60
3,7-Dihydro-6-hydroxy-7-(7-hydroxypteridin-6-yl-methylene)-pteridine	0.0–0.2	0.0
7-Diacetylmethyl-7,8-dihydro-6-hydroxypteridine	0.45	0.75
6-Ethoxycarbonyl-7-hydroxypteridine	0.80	0.75
7-Hydroxy-6-methylcarbonamidopteridine	0.45	0.65

TABLE 20

R_F VALUES (THIN LAYER) OF FLAVAN-3,4-DIOLS

(B. H. BROWN AND J. A. H. MACBRIDE, *J. Chem. Soc.*, (1964) 3822)

Solvent: 5% ethanol in chloroform (v/v).
Thin-layer: Kieselgel H.

Flavan-3,4-diol	R_F	
	cis	*trans*
Unsubstituted	0.33	0.30
6-Methyl-	0.46	0.40
4′-Methoxy-	0.37	0.31
4′-Methoxy-6-methyl-	0.40	0.34
7-Methoxy-	0.37	0.32
4′,7-Dimethoxy-	0.38	0.33
3′,4′,7-Trimethoxy-	0.37	0.34

TABLE 21

R_F VALUES OF SOME STEROIDS IN A MODIFIED BUSH SYSTEM

(R. DECKX, K. DELAERE, W. HEYNS AND P. DE MOOR, *Clin. Chim. Acta*, 9 (1964) 70–71)

Solvent: Water–methanol–toluene (35:65:300).
Detection: U.V. at 254 mμ, Blue Tetrazolium, antimony trichloride, A.A.C.S. test of Katz, and phosphoric acid.

Steroid	R_F *values at*	
	36°	*22°*
9α-Fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione (Triamcinolone)	0.02	
11β,17α,21-Trihydroxypregna-1,4-diene-3,20-dione (Prednisolone)	0.12	
9α-Fluoro-16β-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione (Betamethasone)	0.15	
9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione (Dexamethasone)	0.18	
11β,17α,21-Trihydroxypregn-4-ene-3,20-dione (Cortisol)	0.19	0.13
17α,21-Dihydroxypregna-1,4-diene-3,11,20-trione (Prednisone)	0.25	
17α,21-Dihydroxypregn-4-ene-3,20-dione (Cortisone)	0.33	0.29
11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione (Allodihydrocortisol)	0.34	0.27
17α,21-Dihydroxy-5β-pregnane-3,11,20-trione (Dihydrocortisone)	0.42	0.45
17α,21-Dihydroxy-5α-pregnane-3,11,20-trione (Allodihydrocortisone)	0.41	0.42
3α,11β,21-Trihydroxy-5β-pregnan-20-one (Tetrahydrocorticosterone)	0.43	
5β-Pregnane-3α,17α,20α-triol (Pregnanetriol)	0.47	
11β,21-Dihydroxypregn-4-ene-3,20-dione (Corticosterone)	0.53	
17α,21-Dihydroxypregn-4-ene-3,20-dione (Substance S)	0.57	
3α,11β-Dihydroxy-5β-androstan-17-one (11β-Hydroxyetiocholanolone)	0.70	0.72
3β,11β-Dihydroxy-5α-androstan-17-one (11β-Hydroxyepiandrosterone)	0.74	0.78
Oestra-1,3,5(10)-triene-3,17β-diol (Oestradiol)	0.76	
3α-Hydroxy-5β-androstane-11,17-dione (11-O-Etiocholanolone)	0.81	0.82
3β-Hydroxyandrost-5-en-17-one (Dehydroepiandrosterone)	0.82	
5β-Pregnane-3α,20α-diol (Pregnanediol)	0.84	
11β-Hydroxyandrost-4-ene-3,17-dione	0.85	0.88
3β-Hydroxypregn-5-en-20-one (Pregnenolone)	0.88	
17β-Hydroxyandrost-4-en-3-one (Testosterone)	0.91	
Androst-4-ene-3,11,17-trione	0.93	0.93

TABLE 22

R_X VALUES OF SOME CORTISOL METABOLITES

(R. DECKX, K. DELAERE, W. HEYNS AND P. DE MOOR, *Clin. Chim. Acta*, 9 (1964) 70)

Solvent: Water–methanol–toluene (35:65:300).

Steroid	R_X *values* at*	
	36°	*22°*
6β,11β,17α,21-Tetrahydroxypregn-4-ene-3,20-dione (6β-Hydroxycortisol)	0.10	0.02
5β-Pregnane-3α,11β,17α,20α,21-pentol (α-Cortol)	0.24	0.13
5β-Pregnane-3α,11β,17α,20β,21-pentol (β-Cortol)	0.25	0.19
5α-Pregnane-3β,11β,17α,20β,21-pentol (Allo-β-cortol)	0.26	0.18
3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one (α-Cortolone)	0.36	0.42
3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one (β-Cortolone)	0.43	0.36
11β,17α,20β,21-Tetrahydroxypregn-4-en-3-one (20β-Hydroxycortisol)	0.59	0.57
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one (Tetrahydrocortisol)	1.00	1.00
3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one (Allotetrahydrocortisol)	1.23	1.16
17α,20β,21-Trihydroxypregn-4-ene-3,11-dione (20β-Hydroxycortisone)	1.29	1.60
3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione (Tetrahydrocortisone)	1.43	1.75
11β,17α,21-Trihydroxypregn-4-ene-3,20-dione (Cortisol)	1.77	2.40

* Relative to tetrahydrocortisol.

TABLE 23

R_F VALUES OF *Ormosia jamaicensis* ALKALOIDS

(C. H. HASSALL AND E. M. WILSON, *J. Chem. Soc.*, (1964) 2657)

Solvents: S_1 = Butan-1-ol–hydrochloric acid–water (25:5:9, v/v).
S_2 = Butan-2-ol–hydrochloric acid–water (25:5:9, v/v).
S_3 = Butan-1-ol–acetic acid–water (4:1:5, v/v).
S_4 = Saturated aqueous ammonium sulphate–ethanol (75:1, v/v).

Paper: Whatman No. 1.

Compound	R_F			
	S_1	S_2	S_3	S_4
Piptamine	0.56	0.68	0.38	0.69
Angustifoline	0.77	0.87	0.63	0.45
Ormosajine	0.41	0.54	0.42	0.54
Ormojanine	0.32	0.47	0.36	0.56
Ormosajine–formaldehyde adduct	0.40	0.55	—	0.42
Ormojine	0.21	0.33	0.23	0.36
Alkaloid A	0.65	0.75	0.55	0.48
Alkaloid B	0.94	0.98	0.74	0.42

TABLE 24

R_X VALUES (THIN-LAYER) OF 2,4-DINITROPHENYLHYDRAZONES OF ALIPHATIC ALDEHYDES AND KETONES

(A. ZAMOJSKI AND F. ZAMOJSKA, *Chem. Anal. (Warsaw)*, 9 (1964) 589)

Solvents: S_1 = Benzene.
S_2 = Light petroleum (60–80°)–ether (7:3).
S_3 = Carbon tetrachloride–acetone (9:1).
S_4 = Hexane–ethyl acetate (7:3).
S_5 = Hexane–ethyl propionate (7:3).
Thin-layer: Silica gel G.
Detection: U.V. light.

2,4-Dinitrophenylhydrazone of	$R_{Formaldehyde}$					
	S_1	S_2	S_3	S_4	S_5	S_6
Formaldehyde	1	1	1	1	1	1
Acetaldehyde	1.04	1.19	1.17	1.18	1.03	1.03
Propionaldehyde	1.19	1.70	1.40	1.54	1.22	1.27
Butyraldehyde	1.25	1.99	1.46	1.65	1.34	1.45
Isobutyraldehyde	1.26	2.07	1.47	1.69	1.35	1.52
Valeraldehyde	1.31	2.14	1.51	1.82	1.39	1.58
Isovaleraldehyde	1.32	2.20	1.52	1.85	1.36	1.60
Oenanthaldehyde	1.46	2.24	1.55	1.99	1.46	1.73
Acrolein	1.27	1.60	1.34	1.61	1.18	1.36
Crotonaldehyde	1.28	1.77	1.47	1.71	1.30	1.58
α-Methyl-β-ethylacrolein	1.57	2.43	1.61	2.07	1.60	1.86
Cyclopentanone	1.07	1.98	1.42	1.63	1.38	1.41
Cyclohexanone	1.26	2.22	1.47	1.74	1.46	1.57
Acetone	1.00	1.55	1.19	1.30	1.15	1.14
Methyl ethyl ketone	1.31	2.12	1.34	1.67	1.34	1.47
Methyl propyl ketone	1.40	2.46	1.46	1.82	1.50	1.72
Methyl *tert.*-butyl ketone	1.67	2.90	1.54	2.02	1.65	2.01
3-Methylcyclohexen-2-one	1.30	2.25	1.43	1.79	1.47	1.62

TABLE 25

R_F VALUES (THIN LAYER) OF CYCLOHEXANONES

(J. S. LITTLER AND I. G. SAYCE, *J. Chem. Soc.*, (1964) 2545)

Solvent: Light petroleum (b.p. 60–80°)–ethyl acetate (50:50, v/v).
Thin layer: Kieselgel G (Merck).

Compound	R_F
Cyclohexanone	0.9–0.92
Cyclohexane-1,2-dione	0.5–0.58
2-Hydroxycyclohexanone	0.35–0.4
Cyclohexane-1,4-dione	0.17
4-Hydroxycyclohexanone	0.10

TABLE 26

R_F VALUES OF HYDROQUINONE DERIVATIVES

(J. POSPÍŠIL AND L. TAIMR, *Collection Czech. Chem. Commun.*, 29 (1964) 377)

Paper: Whatman No. 3.
Impregnation: I_1 = 20% formamide in methyl alcohol.
I_2 = 25% dimethylformamide in benzene.
Mobile phase: S_1 = Diisopropyl ether.
S_2 = Diisopropyl ether–heptane (1:2).
S_3 = Chloroform.
S_4 = Benzene.
S_5 = Diisopropyl ether–heptane (1:8).
S_6 = Benzene–heptane (1:1).
S_7 = Chloroform–heptane (1:2).
S_8 = Heptane.
Detection: 15% ferric chloride + 1% potassium ferricyanide (1:1).

Compound	R_F								
	S_1I_1	S_2I_1	S_3I_1	S_4I_1	S_5I_1	S_6I_1	S_7I_1	S_8I_1	S_2I_2
Hydroquinone	0.12								
2-Methylhydroquinone	0.23								
2,6-Dimethylhydroquinone	0.30								
2,5-Dimethylhydroquinone	0.38								
2-*tert.*-Butylhydroquinone	0.76	0.28	0.23	0.12					
2-Methyl-5-*tert.*-butylhydroquinone	0.83	0.50	0.41	0.30	0.14	0.09			
2-Methyl-6-*tert.*-butylhydroquinone		0.40	0.49	0.36	0.11	0.11			
2-*tert.*-Octylhydroquinone		0.75	0.61	0.56	0.39	0.22	0.10		
2-Methyl-5-*tert.*-octylhydroquinone		0.85	0.75	0.77	0.63	0.53	0.33		0.16
2,5-Di-*tert.*-butylhydroquinone		0.84	0.74	0.79	0.70	0.58	0.33		0.16
2,6-Di-*tert.*-butylhydroquinone		0.81		0.86	0.71	0.76	0.70	0.30	0.20
2-*tert.*-Butyl-5-*tert.*-octylhydroquinone	0.14*				0.90	0.85	0.80	0.45	0.41
2,5-Di-*tert.*-octylhydroquinone	0.35*	0.27**						0.78	0.67

* In system 50% dimethylformamide/S_2.
** In system S_8I_2.

TABLE 27

R_F VALUES OF HYDROQUINONE MONOETHER DERIVATIVES

(J. POSPÍŠIL AND L. TAIMR, *Collection Czech. Chem. Commun.*, 29 (1964) 378)

Paper: Whatman No. 3.
Impregnation: I_1 = 20% formamide in methyl alcohol.
I_2 = 25% dimethylformamide in benzene.
I_3 = 50% dimethylformamide in benzene.
Mobile phase: S_1 = Chloroform.
S_2 = Benzene.
S_3 = Diisopropyl ether–heptane (1:2).
S_4 = Benzene–heptane (1:1).
S_5 = Diisopropyl ether–heptane (1:8).
S_6 = Chloroform–heptane (1:2).
S_7 = Heptane.
Detection: 15% ferric chloride + 1% potassium ferricyanide (1:1).

Compound	R_F										
	S_1I_1	S_2I_1	S_3I_1	S_4I_1	S_5I_1	S_6I_1	S_7I_1	S_3I_2	S_3I_3	S_7I_2	S_7I_3
4-*tert.*-Butoxyphenol	0.80	0.65	0.55	0.37	0.36	0.33					
3,5-Dimethyl-4-*tert.*-butoxyphenol				0.62	0.58	0.55	0.21	0.40	0.17	0.16	
2,5-Dimethyl-4-*tert.*-butoxyphenol				0.76	0.71	0.71	0.43	0.46	0.20	0.20	
2-*tert.*-Butyl-4-methoxyphenol				0.81	0.79	0.73	0.43	0.38	0.15	0.16	
2,6-Dimethyl-4-*tert.*-butoxyphenol				0.84	0.74	0.82	0.63	0.51	0.24	0.29	0.10
2-*tert.*-Butyl-4-*tert.*-butoxyphenol				0.86	0.87	0.85	0.62	0.64	0.35	0.42	0.16
2-*tert.*-Octyl-4-*tert.*-butoxyphenol							0.82		0.66	0.65	0.36
2,6-Di-*tert.*-butyl-4-*tert.*-butoxyphenol									0.81	0.85	0.69

TABLE 28

R_F VALUES OF HYDROQUINONE MONOACETATE DERIVATIVES

(J. POSPÍŠIL AND L. TAIMR, *Collection Czech. Chem. Commun.*, 29 (1964) 379)

Paper: Whatman No. 3.
Impregnation: I_1 = 20% formamide in methyl alcohol.
I_2 = 25% dimethylformamide in benzene.
I_3 = 50% dimethylformamide in benzene.
Mobile phase: S_1 = Benzene.
S_2 = Diisopropyl ether–heptane (1:2).
S_3 = Chloroform–heptane (1:2).
S_4 = Benzene–heptane (1:1).
S_5 = Diisopropyl ether–heptane (1:8).
S_6 = Heptane.
Detection: 15% ferric chloride + 1% potassium ferricyanide (1:1) and short heating at 120°.

Compound	R_F									
	S_1I_1	S_2I_1	S_3I_1	S_4I_1	S_5I_1	S_6I_1	S_2I_2	S_2I_3	S_6I_2	S_6I_3
3,5-Dimethyl-4-acetoxyphenol	0.33	0.22	0.18	0.10						
2,5-Dimethyl-4-acetoxyphenol	0.46	0.27	0.23	0.17	0.10					
2,6-Dimethyl-4-acetoxyphenol	0.63	0.28	0.40	0.33	0.12					
2-*tert.*-Octyl-4-acetoxyphenol			0.85	0.88	0.84	0.64	0.53	0.20	0.19	
2,5-Di-*tert.*-butyl-4-acetoxyphenol			0.87	0.85	0.84	0.61	0.52	0.25	0.22	
2-*tert.*-Octyl-5-methyl-4-acetoxyphenol			0.89		0.88	0.73	0.56	0.26	0.23	0.09
2,6-Di-*tert.*-butyl-4-acetoxyphenol						0.90	0.68	0.38	0.52	0.20
2-*tert.*-Octyl-5-*tert.*-butyl-4-acetoxyphenol							0.73	0.45	0.52	0.20
2,5-Di-*tert.*-octyl-4-acetoxyphenol							0.85	0.69	0.72	0.45

TABLE 29

R_F VALUES (THIN LAYER) OF ORGANIC IODINE COMPOUNDS

(E. STAHL AND J. PFEIFLE, *Z. Anal. Chem.*, 200 (1964) 382)

Solvents: S_1 = Ethyl acetate–isopropyl alcohol–25 % NH_3 (55:35:20).
S_2 = Acetone–isopropyl alcohol–25 % NH_3 (40:40:20).
S_3 = Isopropyl alcohol–25 % NH_3 (80:20).
S_4 = Acetic acid–chloroform (5:95).
S_5 = Chloroform–methanol–pyridine (85:5:10).
S_6 = Ethyl acetate–methanol–diethylamine (50:40:20).

Thin layer: Kieselgel HF_{254} (Merck) + 2 % starch.

Detection: Spraying with 50 % acetic acid and irradiation with U.V. light 2537 Å (Sylvania Germicidal-Leuchtstoff-Lampe, Typ G8T5, No. 20817, Desaga).

Compound	*Trade name*	R_F					
		S_1	S_2	S_3	S_4	S_5	S_6
N,N'-Adipic-di-(3-amino-2,4,6-triiodo-benzoic acid)	Billigrafin	0.09	0.27	0.33	0	0	0.33
N,N'-Diacetyl-3,5-diamino-2,4,6-triiodo-benzoic acid	Gastrografin	0.12	0.30	0.37	0.40	0	0.37
α-Ethyl-β-(2,4,6-triiodo-3-hydroxy-phenyl)-propionic acid	Teridax	0.19	0.30	0.36	0	0.33	0.37
3-Acetylamino-2,4,6-triiodobenzoic acid	Triopac	0.26	0.40	0.47	0	0	0.44
Monoiodomethanesulphonic acid	Abrodil	0.28	0.43	0.48	0	0	0.44
3,5-Diiodo-4-pyridone-N-acetic acid	Ioduron	0.29	0.43	0.50	0.33	0	0.43
N-(3-Amino-2,4,6-triiodobenzoyl)-N-phenyl-β-aminopropionic acid	Osbil	0.31	0.42	0.49	0.10	0.15	0.44
β-(3-Dimethylamino-methyleneamino-2,4,6-triiodophenyl)-propionic acid	Biloptin	0.35	0.43	0.50	0.40	0.28	0.50
α-(2,4,6-Triiodophenoxy)-butyric acid	Baygnostil	0.37	0.43	0.50	0.27	0.07	0.57
3,5-Diiodo-4-pyridone-N-acetic acid *n*-propyl ester	Propyliodan	0.87	0.63	0.71	0	0.60	0.67
3-Iodo-L-tyrosine		0.07	0.20	0.23	0	0	0.08
Iodogorgoic acid (3,5-diiodo-L-tyrosine)		0.07	0.20	0.23	0	0	0.08
3,5-Diiodo-L-thyronine		0.31	0.42	0.38	0	0	0.13
3,3',5-Triiodo-L-thyronine		0.30	0.39	0.33	0	0	0.17
DL-Thyroxine		0.22	0.35	0.30	0	0	0.12
Potassium iodide		0.33	0.48	0.47	0	0	0.40
Potassium iodate		0	0	0	0	0	0

TABLE 30

R_F VALUES (THIN LAYER) OF VARIOUS ANALGESICS

(S. J. MULÉ, *Anal. Chem.*, 36 (1964) 1912)

Solvents: S_1 = Ethanol–pyridine–dioxane–water (50:20:25:5).
S_2 = Ethanol–glacial acetic acid–water (60:30:10).
S_3 = Ethanol–dioxane–benzene–ammonium hydroxide (5:40:50:5).
S_4 = Methanol–*n*-butanol–benzene–water (60:15:10:15).
S_5 = *tert.*-Amyl alcohol–*n*-butyl ether–water (80:7:13).
S_6 = *n*-Butanol–glacial acetic acid–water (4:1:2).
S_7 = *n*-Butanol–conc. HCl, saturated with water (90:10).

Thin layer: Silica gel G (Merck) with solvents S_1–S_7. MN-Cellulose powder 300G (Macherey, Nagel & Co.) with solvents S_4[a] and S_5[a].

Detection: Potassium iodoplatinate.

Compound	R_F*								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_4[a]	S_5[a]
Morphine	0.29	0.27	0.11	0.21	0.07	0.54	0.34	0.86	0.85
Normorphine	0.08	0.48	0.04	0.07	str.	0.66	0.62	0.48	0.25
Codeine	0.30	0.29	0.39	0.25	0.08	0.53	0.30	0.86	0.91
Norcodeine	0.12	0.50	0.13	0.09	0.06	0.63	0.49	0.59	0.56
Heroine	0.37	0.35	0.76	0.35	0.15	0.61	0.32	0.90	0.95
Nalorphine	0.71	0.55	0.35	0.67	0.25	0.59	0.41	0.88	0.96
Methyldihydromorphinone	0.16	0.24	0.25	0.15	str.	0.45	0.26	0.76	0.92
Dihydromorphinone	0.11	0.21	0.17	0.13	str.	0.41	0.25	0.65	0.85
Ethylmorphine	0.33	0.25	0.46	0.27	0.08	0.53	0.33	0.84	0.96
Dihydrohydroxymorphinone	0.46	0.29	0.34	0.24	0.10	0.45	0.28	0.63	0.81
Dihydromorphine	0.15	0.21	0.10	0.10	str.	0.43	0.29	0.67	0.73
Dihydrocodeinone	0.17	0.25	0.41	0.19	str.	0.42	0.23	0.76	0.94
Dihydrohydroxycodeinone	0.46	0.24	0.87	0.29	0.16	0.32	0.34		
6-Monoacetylmorphine	0.38	0.40	0.64	0.29	0.19	0.37	0.37		
l-3-Hydroxy-N-methylmorphinan	0.11	0.47	0.80	0.10	0.07	0.51	0.60		
l-3-Hydroxymorphinan	0.05	0.68	0.19	0.10	0.08	0.72	0.80		
l-3-Methoxy-N-methylmorphinan	0.13	0.43	0.91	0.08	0.07	0.55	0.59		
l-3-Methoxymorphinan	0.07	0.65	0.38	str.	str.	0.66	0.81		
l-3-Hydroxy-N-allylmorphinan	0.65	0.70	0.98	0.41	0.44	0.64	0.73		
dl-Methadone	0.34	0.59	0.99	0.17	0.17	0.55	0.62		
l-Acetylmethadol	0.64	0.60	0.99	0.40	0.38	0.52	0.62		
d-Propoxyphene	0.73	0.68	0.97	0.54	0.56	0.53	0.61		
Pethidine	0.42	0.41	0.97	0.36	0.20	0.46	0.44		
Norpethidine	0.12	0.65	0.51	0.10	0.11	0.58	0.63		
Ketobemidone	0.31	0.39	0.47	0.24	0.12	0.42	0.40		
dl-Alphaprodine	0.39	0.40	0.93	0.34	0.20	0.42	0.40		
Piminodine	0.88	0.73	0.99	0.85	0.76	0.69	0.58		
dl-2′-Hydroxy-5,9-dimethyl-2-phenetyl-6:7-benzomorphan	0.88	0.87	0.97	0.82	0.70	0.76	0.77		
l-2′-Hydroxy-2,5,9-trimethyl-6:7-benzomorphan	0.12	0.36	0.56	0.08	0.05	0.43	0.51		
2′-Hydroxy-5,9-dimethyl-2-(3,3-dimethylallyl)-6:7-benzomorphan	0.73	0.81	0.96	0.25	0.34	0.65	0.77		
2′-Hydroxy-5,9-dimethyl-2-cyclopropylmethyl-6:7-benzomorphan	0.45	0.71	0.92	0.15	0.16	0.55	0.67		

* str. = streaking.

TABLE 31

R_F VALUES (THIN LAYER) OF SOME ANALGESICS AND ANTIPYRETICS

(J. ZARNACK AND S. PFEIFER, *Pharmazie*, 19 (1964) 216)

Solvents: S_1 = Butyl acetate–chloroform–85% formic acid (6:4:2).
S_2 = Butyl acetate–acetone–*n*-butanol–10% aqueous ammonia (5:4:3:1).
Thin-layer adsorbent: K_1 = Silicagel VEB Laborchemie Apolda.
K_2 = Silicagel G Merck.
Detection: Kieffer reagent.

Compound	R_F			
	S_1K_1	S_1K_2	S_2K_1	S_2K_2
Amidopyrine	0.04	0.04	0.69	0.75
Sodium noramidopyrine-methanesulphonate	0.10	0.10	0.05	0.03
Phenazone	0.22	0.21	0.58	0.60
Paraacetamole	0.39	0.46	0.66	0.67
Phenacetin	0.52	0.57	0.74	0.81
Salicylamide	0.58	0.61	0.57	0.59
Acetosalicylic acid	0.70	0.83	0.16	0.19
Phenylbutazone	0.76	0.90	0.34	0.40
Quinine	0.02	0.02	0.53	0.55
Codeine	0.04	0.03	0.33	0.38
Papaverine	0.12	0.11	0.72	0.80
Caffeine	0.25	0.25	0.52	0.53

TABLE 32

R_F VALUES (THIN LAYER) OF SOME PURINES

(J. ZARNACK AND S. PFEIFER, *Pharmazie*, 19 (1964) 216)

Solvent: Acetone–chloroform–*n*-butanol–25% ammonia (3:3:4:1).
Thin-layer adsorbent: K_1 = Silicagel VEB Laborchemie Apolda.
K_2 = Silicagel G Merck.
Detection: 2% iodine + 5% $FeCl_3 \cdot 6H_2O$ in 20% aqueous tartaric acid and acetone (1:1).

Compound	R_F		*Detection*
	K_1	K_2	
Theophylline	0.22	0.26	violet-blue
Theobromine	0.41	0.47	greyish blue
Hydroxyethyltheophylline	0.60	0.62	greyish blue
Hydroxyethyltheobromine	0.67	0.69	greyish blue
Caffeine	0.74	0.78	red-brown

TABLE 33

R_F VALUES (THIN LAYER) OF SOME ALKALOIDS

(J. ZARNACK AND S. PFEIFER, *Pharmazie*, 19 (1964) 216)

Solvents: S_1 = Benzene–acetone–ether–10 % aqueous ammonia (4:6:1:0.3).
S_2 = Benzene–acetone–ether–25 % ammonia (4:6:1:0.3).
Thin-layer adsorbents: K_1 = Silicagel VEB Laborchemie Apolda.
K_2 = Silicagel G Merck.

Compound	R_F			
	S_1K_1	S_1K_2	S_2K_1	S_2K_2
Atropine	0.09	0.08	0.18	0.20
Morphine	0.13	0.09	0.14	0.09
Hydromorphone	0.13	0.09	0.15	0.13
Sparteine	0.14	0.11	0.49	0.32
Brucine	0.15	0.10	0.22	0.25
Colchicine	0.16	0.15	0.22	0.20
Ergometrine	0.18	0.14	0.27	0.17
Apoatropine	0.24	0.19	0.36	0.43
Hydrocodon	0.25	0.18	0.32	0.25
Codeine	0.28	0.18	0.36	0.24
Strychnine	0.28	0.20	0.37	0.40
Pilocarpine	0.28	0.25	0.37	0.37
Quinine	0.31	0.23	0.35	0.35
Dihydroergotamine	0.34	0.28	0.39	0.29
Ergotamine	0.36	0.29	0.42	0.32
Quinidine	0.37	0.28	0.42	0.43
Scopolamine	0.38	0.37	0.46	0.52
Dihydroergocornine	0.47	0.47	0.56	0.48
Dihydroergocristine	0.47	0.47	0.56	0.48
Dihydroergocryptine	0.47	0.47	0.56	0.48
Thebaine	0.49	0.46	0.57	0.51
Physostigmine	0.49	0.47	0.59	0.60
Pethidine	0.52	0.48	0.61	0.60
Oxycodon	0.61	0.64	0.63	0.69
Papaverine	0.62	0.64	0.65	0.69
Reserpine	0.63	0.72	0.67	0.75
Cocaine	0.69	0.78	0.72	0.80
Narcotine	0.72	0.78	0.74	0.81

TABLE 34

R_F VALUES (THIN LAYER) OF SOME SULFONAMIDES

(J. ZARNACK AND S. PFEIFER, *Pharmazie*, 19 (1964) 216)

Solvents: S_1 = Butyl acetate–*n*-butanol–acetone–10% aqueous ammonia (3:3:4:1).
S_2 = Chloroform–*n*-butanol–acetone–85% formic acid (8:2:2:2).
Thin-layer adsorbents: K_1 = Silicagel VEB Laborchemie Apolda.
K_2 = Silicagel G Merck.
Detection: 0.1% *p*-dimethylaminobenzaldehyde solution in conc. HCl–96% ethanol (9:1).

Compound	R_F			
	S_1K_1	S_1K_2	S_2K_1	S_2K_2
Phthalylsulfathiazole (Talisulfazol)	0.03	0.04	0.46	0.55
Sulfacarbamide (Euvernil)	0.06	0.04	0.38	0.44
Sulfacetamide (Albucid)	0.12	0.11	0.54	0.59
Sulfathiourea	0.14	0.13	0.69	0.73
Sulfafurazole (Sulfasol)	0.18	0.17	0.70	0.70
Carbutamide (Oranil)	0.18	0.21	0.72	0.78
Sulfamethoxypyridazine (Depovernil)	0.21	0.23	0.58	0.60
Sulfisomidine (Sulfamethin)	0.22	0.24	0.25	0.25
Sulfaethidole (Globucid)	0.21	0.25	0.65	0.69
Sulfathiazole	0.23	0.26	0.42	0.44
Sulfaphenazole (Depocid)	0.33	0.33	0.75	0.80
Tolbutamide (Orabet)	0.29	0.32	0.84	0.88
Sulfaguanidine	0.48	0.47	0.24	0.24
Sulfanilamide	0.68	0.75	0.36	0.37

TABLE 35

R_F VALUES (THIN LAYER) OF SOME SULFONAMIDES

(E. A. MOREIRA, *Tribuna Farm. (Brazil)*, 31 (1964) 53)

Solvent: Chloroform–methanol–*n*-butanol–2% aqueous ammonia (80:10:9:1).
Detection: *p*-Dimethylaminobenzaldehyde.
Thin layer: Kieselgel G Merck.

Sulfonamide	R_F
Sulfaguanidine	0.16
Sulfacetamide	0.31
Sulfathiazole	0.42
Sulfadiazine	0.47
Sulfanilamide	0.55
Sulfamerazine	0.69
Sulfamethazine	0.77

TABLE 36

R_F VALUES OF SOME GUANIDINE DERIVATIVES IN DIFFERENT SOLVENTS

(R. PANT AND H. C. AGRAWAL, *Z. Physiol. Chem.*, 335 (1964) 203)

Solvents: S_1 = Pyridine–isoamyl alcohol–water (8:4:7).
S_2 = 3% *sec.*-Butanol–ammonia.
S_3 = Propanol–water (3:1).
S_4 = Ethyl acetate–acetic acid–water (3:1:1).
S_5 = *sec.*-Butanol–formic acid–water (15:3:2).

Paper: Whatman No. 1 (ascending).

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Arginine	0.32	0.1	0.17	0.45	0.34
Agmatine	0.3	0.12	0.07	0.23	0.25
Arcaine	0.57	0.22	0.41	0.33	0.64
Asterubine	0.56	0.14	0.36	0.1	0.26
Canavanine	—	0.07	0.43	0.17	0.02
*G-Cystine	0.37	0.06	0.17	0.72	0.22
Diethylguanidine	0.37	0.28	0.44	0.92	0.67
Dimethylguanidine	0.47	0.32	0.54	0.98	0.8
Ethylguanidine	0.68	0.76	0.73	0.97	0.69
Galegine	0.84	0.94	0.82	0.98	0.94
Glycocyamine	0.43	0.16	0.21	0.66	0.58
Guanidoethanol	0.48	0.6	0.53	0.78	0.71
Guanidoethanol phosphate	0.49	0.41	0.17	0.5	0.7
Hirudonine	0.08	0.03	0.05	0.1	0.04
Homoarginine	0.64	0.13	0.33	0.51	0.38
*G-Isoleucine	0.74	0.78	0.73	0.99	0.92
Lombricine	0.32	0.02	0.1	0.27	0.07
Methylguanidine	0.56	0.61	0.58	0.92	0.76
*G-Methionine	0.71	0.68	0.64	0.98	0.81
Octopine	0.4	0.03	0.18	0.54	0.41
Streptomycin	0.16	—	—	—	—
*G-Serine	0.46	0.22	0.33	0.78	0.51
Taurocyamine	0.57	0.27	0.36	0.5	0.25
*G-Tryptophan	0.78	0.68	0.65	0.97	0.89
*G-Valine	0.65	0.66	0.66	0.97	0.9
γ-Guanido-butyric acid	0.59	0.49	0.6	0.96	0.86
Creatine	0.34	0.09	0.49	0.9	0.63

*G = guanylated.

TABLE 37

R_F VALUES OF PEPTIDES OF TYROSINE, DIIODOTYROSINE AND THYROXINE

(T. SHIBA AND H. J. CAHNMANN, *J. Org. Chem.*, 29 (1964) 3063)

Solvents: S_1 = Butan-1-ol–dioxane–2 *N* ammonia (4:1:5).
S_2 = Butan-1-ol–acetic acid–water (78:10:12).

Compound	R_F	
	S_1	S_2
Glycyl-L-tyrosine	0.12	0.11
Glycyl-L-3,5-diiodotyrosine	0.13	0.28
Glycyl-L-thyroxine	0.27	0.57
L-Tyrosyl-glycine	0.14	0.12
3,5-Diiodo-L-tyrosyl-glycine	0.09	0.32
L-Thyroxyl-glycine	0.37	0.58
Glycyl-L-tyrosyl-glycine	0.09	0.12
Glycyl-3,5-diiodo-L-tyrosyl-glycine	0.05	0.27
Glycyl-L-thyroxyl-glycine	0.33	0.51

TABLE 38

ELECTROPHORETIC MOBILITIES OF 2,4-DINITROPHENYLHYDRAZONES OF KETO ACIDS

(E. PAPKE AND R. POHLOUDEK-FABINI, *Pharmazie*, 19 (1964) 27)

Electrolyte: Britton–Robinson buffer pH 9.91.
Potential: 52–55 V/cm.
Temperature: 15–18°.
Paper: Schleicher & Schüll 2043b Mgl.

Keto acid 2,4-dinitrophenyl-hydrazone	*Mobility*
Dehydroascorbic acid	+0.0–2.0
Acetoacetic acid*	+0.0–4.0
α-Ketobutyric acid II**	+11.4
Pyruvic acid II	+12.1
α-Keto-isovaleric acid	+13.9
α-Keto-*n*-valeric acid	+14.8
Phenylpyruvic acid	+14.9
Levulinic acid	+15.0
α-Ketobutyric acid I**	+15.3
Glyoxylic acid	+15.4
Pyruvic acid I	+15.5
Oxalosuccinic acid	+20.6
α-Ketoglutaric acid	+20.8
γ-Ketopimelic acid	+24.1
Oxaloacetic acid	+25.1
Mesoxalic acid	+27.2
2,4-Dinitrophenylhydrazine	+0.0

* Decarboxylation to acetone.
** Two spots.

TABLE 39

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONES OF KETO ACIDS

(E. PAPKE AND R. POHLOUDEK-FABINI, *Pharmazie*, 19 (1964) 27)

Solvents: S_1 = *n*-Butanol–3 % aqueous ammonia (1:1).
S_2 = Veronal buffer, pH 8.6 (sodium diethylbarbiturate, 10.3 g/l and diethylbarbituric acid, 1.84 g/l).
Paper: Schleicher & Schüll 2043b Mgl.
Detection: D_1 = U.V. light.
D_2 = 2 % NaOH.

Keto acid 2,4-dinitrophenylhydrazone	R_F		D_1	D_2
	S_1	S_2		
Dehydroascorbic acid	tailing	0.0	dark spot	violet, later brown
Oxalosuccinic acid	0.10	0.50	dark spot	yellow
α-Ketoglutaric acid	0.10	0.52	dark spot	yellow
γ-Ketopimelic acid	0.10	0.77	dark spot	brown
Mesoxalic acid	0.11	0.68	dark spot	brownish yellow
Oxaloacetic acid	0.11	0.65	dark spot	yellow
Glyoxylic acid	0.36	0.47	dark spot	orange
Pyruvic acid	0.39	0.50	dark spot	brown
	(0.62)	(0.31)	dark spot	
Levulinic acid	0.46	0.60	dark spot	brown
α-Keto-*n*-valerianic acid	0.48	0.58	dark spot	brown
Acetoacetic acid	0.58	0.61	dark spot	brown
	(0.91)	(0.26)	dark spot	
Phenylpyruvic acid	0.67	0.57	dark spot	brown
α-Ketobutyric acid	0.67	0.32	dark spot	brown
α-Keto-isovaleric acid	0.75	0.49	dark spot	yellow
2,4-Dinitrophenylhydrazine	0.92	0	dark spot	brown

TABLE 40

R_F VALUES (THIN LAYER) OF IONONES AFTER SIX MULTIPLE RUNS

(J. H. DHONT AND G. J. C. DIJKMAN, *Analyst*, 89 (1964) 682)

Solvent: Benzene.
Thin layer: Silica gel G.
Detection: D_1 = 0.2 % solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl.
D_2 = A solution of 200 mg of vanillin in 50 ml of methanol containing 4 % of *p*-toluenesulphonic acid, and heating to 110–115° for 5–15 min.

Compound	R_F (*mean value*)
Pseudoionone	0.384
α-Ionone	0.580
β-Ionone	0.505
α-Methylionone	0.756
β-Methylionone	0.699

TABLE 41

R_F VALUES OF PHENOLIC DERIVATIVES OF PHENYLETHYLAMINE AND SOME OTHER DIAZO-REACTIVE BASES IN URINE

(R. F. COWARD, P. SMITH AND O. S. WILSON, *Clin. Chim. Acta*, 9 (1964) 385)

Solvents: S_1 = *tert.*-Amyl alcohol–0.88 ammonia (4:1).
S_2 = *tert.*-Amyl alcohol–17% methylamine (4:1) at 32°.
S_3 = *sec.*-Butanol–buffer pH 3.9 (4:1); composition of buffer: water–pyridine–acetic acid (100:10:41).
S_4 = Nitroethane–70% aqueous acetic acid (9:4).

Detection: D_1 = Diazotized *p*-nitroaniline.
D_2 = Diazotized sulphanilic acid.

Abbreviations of colours: B = blue; Br = brown; G = green; Gy = grey; O = orange; Pk = pink; R = red; V = violet; Y = yellow; OX = oxidizes.

Compound	R_F				*Colour with*	
	S_1	S_2	S_3	S_4	D_1	D_2
β-Phenylethylamine						
2-Hydroxy-	0.81	0.84	0.58	0.56	R	O-Y
3-Hydroxy-	0.72	0.60	0.49	0.49	R	O-Y
4-Hydroxy-	0.73	0.68	0.46	0.39	Dull V	R
4-Hydroxy-N,N-dimethyl-	0.92	0.93	0.49	0.69	Dull V-R	R
3,4-Dihydroxy-	OX	OX	0.23	0.21	OX with Na_2CO_3	
4-Hydroxy-3-methoxy-	0.59	0.53	0.35	0.49	Gy	Weak R
3,4-Dihydroxy-5-methoxy-	OX	OX	0.18	0.26	OX with Na_2CO_3	
3-Hydroxy-4,5-dimethoxy-	0.46	0.35	0.39	0.46	Br-R	O-Y
4-Hydroxy-3,5-dimethoxy-	0.43	0.42	0.25	0.47	Pale B	Pk
β-Phenyl-β-hydroxyethylamine						
2-Hydroxy-	0.52	0.41	0.47	0.35	R	O-Y
3-Hydroxy-	0.39	0.25	0.38	0.24	R	O-Y
3-Hydroxy-N-methyl-	0.66	0.51	0.44	0.44	R	O-Y
4-Hydroxy-	0.33	0.23	0.31	0.20	R	Y
4-Hydroxy-N-methyl-	0.60	0.42	0.40	0.33	R	Y
3,4-Dihydroxy-	OX	OX	0.16	0.12	OX with Na_2CO_3	
3,4-Dihydroxy-N-methyl-	OX	OX	0.20	0.24	OX with Na_2CO_3	
3,4-Dihydroxy-N-isopropyl-	OX	OX	0.38	0.42	OX with Na_2CO_3	
3-Hydroxy-4-methoxy-	0.22	0.14	0.22	0.25	B-V	O
3-Hydroxy-4-methoxy-N,N-dimethyl-	0.63	0.51	0.29	0.63	V	O
4-Hydroxy-3-methoxy-	0.24	0.15	0.23	0.26	V	O
4-Hydroxy-3-methoxy-N-methyl-	0.47	0.33	0.30	0.48	V	O
4-Hydroxy-3-methoxy-N-isopropyl-	0.74	0.65	0.52	0.63	V	O
4-Hydroxy-3-methoxy-N,N-dimethyl-	0.66	0.51	0.29	0.63	V	O
4-Hydroxy-3,5-dimethoxy-	0.16	0.12	0.19	0.30	B	R
4-Hydroxy-3,5-dimethoxy-N-methyl-	0.37	0.30	0.21	0.57	B	R
Miscellaneous compounds						
Serotonin	0.56	0.68	0.24	streaks	R	R
Imidazole	0.78	0.84	0.28	0.44	O	Y
4(5)-Hydroxymethylimidazole	0.55	0.59	0.27	0.24	Br-O or Br-R	R
Histamine	0.43	0.53	0.03	0.13	Br-O or Br-R	R
ω-Methylhistamine	0.70	0.75	0.08	0.23	Br-O or Br-R	R
ω-Ethylhistamine	0.80	0.85	0.10	0.27	Br-O or Br-R	R
4(5)-Aminoimidazole-5(4)-carboxamide	0.27	0.25	0.37	0.33	G Gy → B	B

TABLE 42

R_F VALUES OF ARYLAMIDES OF THE NAPHTHOL AS SERIES

(J. GASPARIČ, *Collection Czech. Chem. Commun.*, 29 (1964) 1724)

Paper: Whatman No. 3.
Impregnation: 10% 1-bromonaphthalene in chloroform.
Mobile phase: 80% aqueous acetic acid saturated with 1-bromonaphthalene.
Detection: D_1 = U.V. light.
D_2 = 0.1% stabilized 1-diazo-2-chloro-4-nitrobenzene.

Compound			R_F	D_1 *fluorescence*	D_2
Arylide	*Parent acid**	*Trade name*			
Anilide	BON	Naphthol AS Brenthol AS	0.64	violet-blue	red
2-Methylanilide	BON	Naphthol AS-D Brenthol OT Azotol OT	0.63	violet	red
4-Chloroanilide	BON	Naphthol AS-E	0.51	violet-blue	red
4-Chloro-2-methylanilide	BON	Naphthol AS-TR Brenthol CT	0.48	grey-violet	red
2-Methoxyanilide	BON	Naphthol AS-OL Azotol OA	0.36	grey-violet	red
4-Methoxyanilide	BON	Naphthol AS-RL Azotol PA	0.64	grey-green	red
4-Methoxy-2-methylanilide	BON	Naphthanilid LT	0.66	grey-green	red
5-Chloro-2-methylanilide	BON	Naphthanilid KB	0.50	grey-green	red
2,4-Dimethylanilide	BON	Naphthol AS-AM	0.53	yellow-green	red
5-Chloro-2-methoxyanilide	BON	Naphthol AS-CA Naphthanilid EL	0.28	grey-violet	red
2,5-Dimethoxyanilide	BON	Naphthol AS-BG Brenthol FO	0.39	yellow-green	red
4-Chloro-2-methoxy-5-methylanilide	BON	Naphthol AS-RS	0.20	violet	red
2-Ethoxyanilide	BON	Naphthol AS-PH	0.31	grey-violet	red
4-Ethoxyanilide	BON	Naphthol AS-VL	0.53	grey-violet	red
3-Nitroanilide	BON	Naphthol AS-BS Brenthol MN Azotol MNA	0.62	grey-violet	red
1-Naphthylamide	BON	Naphthol AS-BO Brenthol AN Azotol ANF	0.50	yellow-green	red
2-Naphthylamide	BON	Naphthol AS-SW Naphthanilid SW	0.39	grey-violet	red
3-Methoxydiphenylene oxide 2-amide	BON	Naphthol AS-S	0.12	light blue	red
o-Tolidide	BON	Naphthanilid M	0.68	green-grey	red
2,4-Dimethoxy-5-chloroanilide	TO	Naphthanilid LG	0.30	yellow-green	red
2-Ethoxyanilide	HDOK	Naphthol AS-KG Naphthanilid KG	0.15	blue-grey	grey
1-Naphthylamide	HDOK	Naphthol AS-KN	0.38	grey-blue	grey
4-Chloroanilide	HKK	Brenthol BT	0.48	rose	brown
4-Methoxyanilide	HBKK	Naphthol AS-SG Naphthanilid SR Brenthol GB	0.32 S	blue	green grey
2-Methylanilide	HAK	Naphthol AS-GR	0.38	green-yellow	violet
1-Naphthylamide	HAK	Naphthol AS-RP	0.34	yellow-green	violet
o-Tolidide	AO	Naphthol AS-G	0.00 0.90	— —	yellow yellow
2,5-Dimethoxy-4-chloroanilide	AO	Naphthol AS-13GH Naphthol AS-IRG	0.85	—	yellow

* BON = 2-hydroxy-3-naphthoic acid; TO = terephthaloylacetic acid; HDOK = 3-hydroxydiphenylene oxide-2-carboxylic acid; HKK = 3-hydroxycarbazole-2-carboxylic acid; HBKK = 3′-hydroxy-7:8-benzocarbazole-2′-carboxylic acid; HAK = 2-hydroxyanthracene-3-carboxylic acid; AO = acetoacetic acid.

TABLE 43

R_F VALUES OF ACETOACETYLARYLAMIDES

(J. GASPARIČ, *Collection Czech. Chem. Commun.*, 29 (1964) 1725)

Paper: Whatman No. 3.
Impregnation: 20% formamide in ethyl alcohol.
Mobile phase: Benzene–hexane (1:1).
Detection: Stabilized 1-diazo-2-chloro-4-nitrobenzene and 5% NaOH in ethyl alcohol–water (1:1).

Derivative	R_F
Anilide	0.25
2-Methylanilide	0.36
4-Methylanilide	0.36
2,5-Dimethylanilide	0.51
2-Nitroanilide	0.64
3-Nitroanilide	0.14
4-Nitroanilide	0.14
2-Chloroanilide	0.71
4-Chloroanilide	0.45
2,5-Dichloroanilide	0.87
2-Methoxyanilide	0.55
4-Methoxyanilide	0.15
2-Ethoxyanilide	0.76
4-Ethoxyanilide	0.34
2,5-Dimethoxyanilide	0.58
2,5-Dimethoxy-4-chloroanilide**	0.81
2-Methoxy-5-chloroanilide	0.75
5-Chloro-2-methylanilide	0.65
2-Methoxy-5-methylanilide	0.71
2-Methoxy-4-chloro-5-methylanilide	0.87
4-Nitro-2-methoxyanilide	0.55
6-Ethoxybenzothiazole-2-amide	0.56
1-Naphthylamide	0.44
o-Tolidide***	0.00

* Naphthol AS-AG.
** Naphthol AS-13GH and Naphthol AS-IRG.
*** Naphthol AS-G.

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