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VAPOR-PHASE BUTANE NITRATION PRODUCT ANALYSIS BY PARALLEL COLUMN GAS CHROMATOGRAPHY

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In previous work on this problem[1,2] an analytical procedure was developed for the analysis of the pure nitroparaffins resulting from the vapor-phase nitration of commercial butane (Phillips Petroleum Co., technical grade) by gas–liquid partition chromatography. This method made use of both constant temperature, linear programmed temperature operation, and ramp function temperature programmed operation of a 6-ft. length of 1/4-in. copper refrigeration tubing packed with a 2:1 mixture by weight of two separate packings, Armeen SD and Apiezon N, each containing 10 g of substrate per 100 g of the $-48+65$ Tyler standard screen fraction of Johns-Manville Type C-22 firebrick. The presence of oxygenated compounds in the product stream from the butane nitrator, chiefly water, formaldehyde, acetaldehyde, and methanol made further work on this problem necessary.

EXPERIMENTAL

Apparatus

The unit used in this work was the F & M Scientific Corp. Model 500A programmed temperature gas chromatograph, modified as previously described[1]. The injection port was maintained at 200°. Liquid samples were injected through a self-sealing silicone rubber septum with a 10-μl Hamilton microsyringe. The carrier gas used in this investigation was helium (minimum purity, 99.98 mole %, The Matheson Co.) which was dried before use by passing through a 12-in. length of 3/8-in. pipe filled with No. 5A Linde molecular sieves installed in the inlet line to the thermal conductivity cell. The flow rate through the reference side of the cell was maintained constant at 30 ml of helium/min, measured at 28°. The flow rate through the columns was also measured at 28° with a calibrated Brooks "Shorate" 150 rotameter, tube No. 1-15-6, stainless steel float. Gas samples for scouting work were collected in 250 ml sample bulbs, fitted with two stopcocks. After the sample was obtained and the stopcocks closed, a rubber serum cap was fitted to the stub end through which the samples were withdrawn (by opening the cock, inserting the needle, sampling,

withdrawing the needle and closing the cock) with a 1-ml gas-tight syringe (Teflon-tipped nylon plunger, Wilkens Instrument and Research). Liquid samples used were 4 μl. The gas samples used were 200 μl and 20 μl. The output signal from the thermal conductivity cell was recorded at a chart speed of 30 in./h on a Bristol Dynamaster Potentiometer, Model 1PH-570.

DISCUSSION

The nitrator product is obtained as a liquid phase and an off-gas. The liquid product is recovered and immediately separated into two layers: an oil layer, containing approximately 80–90 % nitroparaffins, 1–10 % water, 2 % oxygenated organics, and about 2 % dissolved gases; and an aqueous layer containing, in addition to the majority of the water and other oxygenated compounds produced in the reactor, less than 1 % nitroparaffins, 1–2 % dissolved gases (primarily CO_2 and NO_2), and nitric acid.

Oil layer

It was found that linear temperature programming at 2.9°/min, starting at 40°, of the mixed Armeen SD/Apiezon N column, A, was effective in the separation of the nitroparaffins in the oil layer in the presence of small amounts of light hydrocarbon gases, water, and the lower molecular weight alcohols, aldehydes, and ketones. The flow rate used was 60 ml of helium/min. The ratio of substrate to support was 1:10 by weight unless otherwise stated. The very slight tailing of nitromethane, methanol, and water which is typical of this column under all operating conditions, did not affect the precision and accuracy of the analyses.

The columns tested which provided reasonably good separation of the nitroparaffins caused considerable tailing of water, methanol, formaldehyde and acetaldehyde. Those columns which provided good separation for the oxygenated compounds caused leading of the nitroparaffins.

Aqueous layer

Upon mixing the different classes of compounds to form new test mixtures having compositions similar to the expected compositions[3] of the oil and aqueous layers, it was found that a column, C, 24 ft. 11 in. long × 1/4 in. O.D. copper tubing, filled with a packing consisting of 10 g of squalane per 100 g of the −30 + 80 U.S standard screen fraction of Fluoropak, when operated at 50° at a helium flow of 77 ml/min gave excellent separation of the oxygenated constituents of the aqueous layer. Under these conditions, the nitroparaffins were retarded for 20 min, allowing the aqueous layer analyses to be made on a nitroparaffin-free basis. The first four nitroparaffins (nitromethane through 1-nitropropane) can be determined quantitatively in the squalane column if sufficient time is allowed. 1-Nitropropane has a retention time of 69.5 min under these conditions.

It was found that in general, substrates which are commonly used[1,2,4–8] for the separation of water (Armeen SD); alcohols (dibutyl sebacate, 1-decanol, dibutyl phthalate, Carbowax 600 and 1500); aldehydes (Carbowax 20M); and dissolved gases such as CO, CO_2, N_2, O_2, CH_4, C_2H_6, C_3H_8, C_4H_{10}, etc. (squalane, hexamethyldisilazane, activated carbon, activated alumina, silica gel, and a 1:1 mixture by weight of glutaronitrile and propylene carbonate) lose their effectiveness when used for the separation of samples containing compounds from the other classes. This is especially

evident if the liquid partitioning agents have been made up on Type C-22 firebrick or the Johns-Manville acid-washed Chromosorb P. This was also found to be the case when the substrates were prepared using either of the silicone-treated, non-acid-washed Chromosorbs P or W as the support material. When using the silicone-treated Chromosorbs (separately) as the support for the 2:1 Armeen SD/Apiezon N column, water, methanol, ethanol, and acetaldehyde seem to be almost permanently adsorbed, as they did not appear, even after a six-hour wait. This may be of considerable advantage to other researchers who require analyses on a water- and oxygenated-free basis. We have found that the substitution of Fluoropak (Wilkens Instrument and Research) for the Type C-22 firebrick and the Chromosorb P greatly reduces or even eliminates the tailing so often encountered in the analysis of samples containing water by GLPC. However, when this change of support material was tried for the mixed Armeen SD/Apiezon N substrate for the analysis of the nitroparaffins in the oil layer, a decrease in resolving power was observed.

Gas phase

This stream contains primarily *n*-butane, 9–11 mole % NO and NO_2, 2–3 mole % propane, 1.5–2.5 mole % carbon dioxide, and traces of carbon monoxide, methanol, water, and formaldehyde. The gas stream is water saturated. Of the packings tested for the separation of the gas-phase components, the aqueous layer squalane column, C, gave satisfactory quantitative separation of ethane, propane, *n*- and *iso*-butane, carbon dioxide, methanol, acetaldehyde, formaldehyde, and nitromethane through 1-nitropropane when operated at 50° and a helium flow rate of 77 ml/min. Under these conditions, N_2, NO, NO_2 and CO are eluted as a single peak just before CO_2. Operation of this column at 30° and 27 ml helium/min gave quantitative separation of CO and NO, but N_2 and NO_2 still appeared as a single peak. Under these conditions, however, the separations of water, methanol, formaldehyde, and acetaldehyde were unsuitable for quantitative work. The NO_2 and NO were also separated completely by running duplicate samples on a 1/4-in. O.D. column, B, 20 ft. long filled with the $-65 + 80$ Tyler standard screen fraction of activated charcoal impregnated with 2 parts by weight of squalane per 100 parts of the activated charcoal. Operating conditions were 22° and 66 ml of helium/min.

Hexamethyldisilazane treated Chromosorb P[6], a 1:1 mixture by weight of glutaronitrile and propylene carbonate, Carbowaxes 600 and 20M, a 1:1 mixture by weight of Armeen SD and Apiezon N, all in the ratio of 10 g substrate per 100 g support; 2 wt. % squalane on activated carbon; 2 wt. % dibutyl sebacate on silica gel; activated carbon; were also tested for the analysis of the off-gas leaving the reactor and were found to be unsatisfactory.

Analytical procedure

The analysis of the nitrator products was broken down as follows. The oil layer samples were analyzed on the Armeen SD/Apiezon N column, A, for the quantitative separation of the nitroparaffins and the oxygenated compounds (Fig. 1, lower chromatogram). The dissolved gases in the oil layer, which appeared as three small incompletely resolved peaks, were separated by running duplicate oil layer samples through the squalane/Fluoropak column, C, (Fig. 1, upper chromatogram), and through the squalane-activated charcoal column, B, (Fig. 2, lower chromatogram).

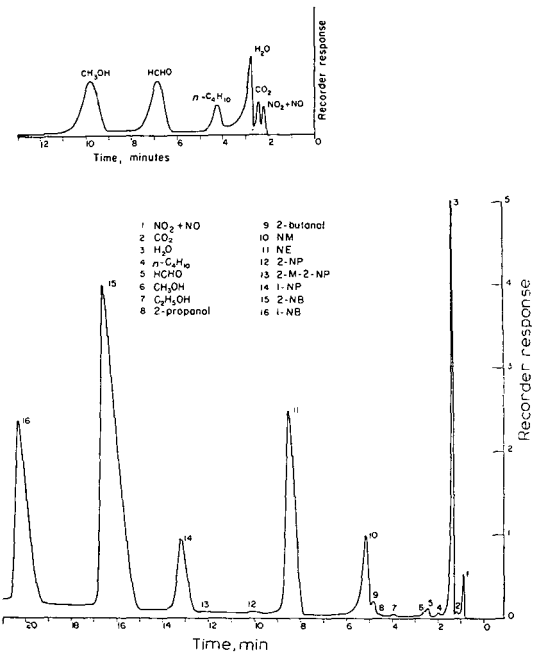

Fig. 1. Chromatograms of the oil layer. Top: Column C: 50°; 77 ml helium/min. Bottom: Column A: linear temperature programmed at 2.9°/min starting from 40°; 60 ml helium/min.

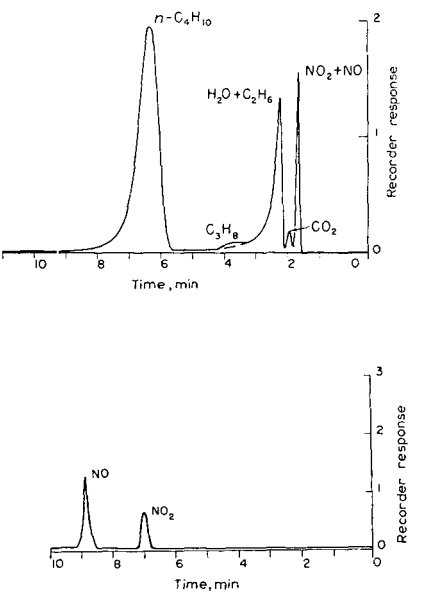

Fig. 2. Chromatograms of the off-gas. Top: Column C: 50°; 77 ml helium/min. Bottom: Column B: 22°; 66 ml helium/min.

The aqueous layer samples were analyzed on the squalane/Fluoropak column, C, at the higher temperature and flow rate to pick out the water, oxygenated compounds, and the nitroparaffins (Fig. 3, upper chromatogram). Duplicate water layer samples were then run through column C at the lower temperature and flow rate (Fig. 3, lower chromatogram), and initially through column B for the analysis of the dissolved gases. This analysis showed that the acidity of the aqueous layer was due almost entirely to the presence of nitric acid. In later runs, an immediate titration with standard base provided the same information as column B.

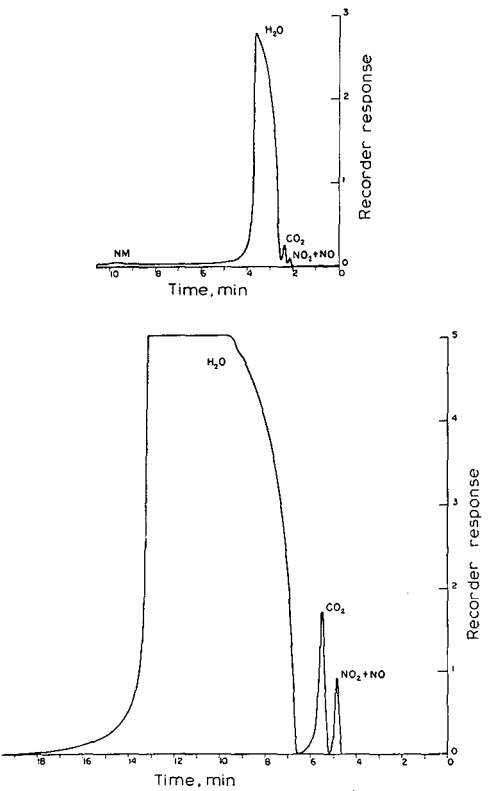

Fig. 3. Chromatograms of the aqueous layer. Top: Column C: 50°; 77 ml helium/min. Bottom: Column C: 30°; 27 ml helium/min.

The off-gas samples were analyzed in column C at the higher temperature and flow rate (Fig. 2, upper chromatogram), for water, nitroparaffins, and oxygenated aliphatics and at the lower temperature and flow rate for CO_2, oxides of nitrogen, ethane, propane, and *n*-butane (Fig. 4). Duplicate samples were analyzed in column B (Fig. 2), for the NO–NO_2 split.

In all cases, the supplementary analyses (oil layer samples in columns B and C, off-gas and aqueous layer samples in column B and under both sets of conditions in column C) for each product stream were made to obtain quantitative analyses of overlapping or crowded peaks. The peak areas from these supplementary analyses

were used as a basis of partitioning the areas of the primary analysis. For example, the upper chromatogram of Fig. 1 shows the separation of the first 6 components of a typical oil layer sample (lower chromatogram is from column A). The areas from the

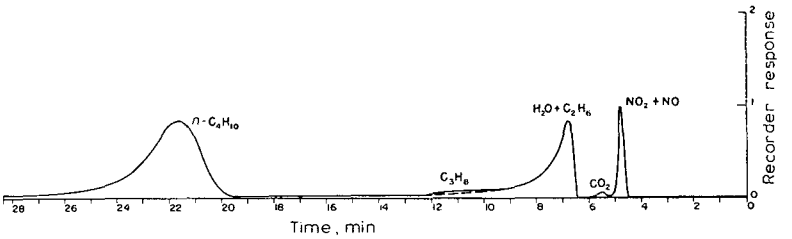

Fig. 4. Chromatogram of the off-gas. Column C: 30°; 27 ml helium/min.

methanol and formaldehyde peaks in the upper chromatogram were used to partition the total area under peaks 5 and 6 in the lower chromatogram. A similar technique was used for any other crowded or overlapping peaks. This procedure appears to be quite reproducible and precise when tested against known, gravimetrically-prepared test mixtures. These results can best be summarized by Table I which shows the procedure followed for routine analysis.

The presence of certain compounds (hydrogen, nitrogen, oxygen, methane, alkenes, alkynes) which might be expected in the product streams was ruled out by

TABLE I

PROCEDURE FOR PRODUCT ANALYSIS BY GLPC

Use	*Column*	*Flow rate*[a]	*Temperature (°C)*	*Compounds separated quantitatively*
Oil	A[b]	60	L.T.P.[c]	NP's, H_2O, oxygenated aliphatics[d], C_2H_6, C_3H_8, n-C_4H_{10}, *iso*-C_4H_{10}, CO_2, CO + NO + NO_2 + N_2
Oil	B[e]	66	22	CO, HCHO, NO_2 + N_2, N_2O_4, NO
Oil	C[f]	77	50	C_2H_6, C_3H_6, n-C_4H_{10}, *iso*-C_4H_{10}, CO_2, NO_2, NO + CO
Off-gas	B[e]	66	22	CO, HCHO, NO_2 + N_2, N_2O_4, NO
Off-gas	C[f]	27	30	CO_2, NO_2 + N_2, H_2O + C_2H_6, C_3H_8, C_4H_{10}
Off-gas	C[f]	77	50	H_2O + C_2H_6, oxygenated aliphatics[d], NM, NE, 2-NP, 2-M-2-NP, 1-NP, NO_2, CO_2
Aqueous	B[e]	66	22	CO, HCHO, NO_2 + N_2, N_2O_4, NO
Aqueous	C[f]	27	30	CO_2, NO_2 + N_2, H_2O + C_2H_6, C_3H_6, C_4H_{10}
Aqueous	C[f]	77	50	H_2O + C_2H_6, oxygenated aliphatics[d], NM, NE, 2-NP, 2-M-2-NP, 1-NP, NO_2, CO_2

Abbreviations: NM = nitromethane; NE = nitroethane; 1-NP = 1-nitropropane; 2-NP = 2-nitropropane; 2-M-1-NP = 2-methyl-1-nitropropane; 2-M-2-NP = 2-methyl-2-nitropropane.

[a] ml helium/min measured at 28°, atmospheric pressure.

[b] Column A: a 2:1 weight mixture of Armeen SD and Apiezon N, each 10/100 on C-22, —48 + 65 Tyler standard mesh, 1/4 in. O.D. × 6 ft.

[c] L.T.P.: Linear temperature programming at 2.9°/min starting from 40°.

[d] Includes all C_1 to C_4 RCHO, ROH, ROR', RCOOR', and RR'C=O.

[e] Column B: 2/100, squalane on activated charcoal, —65 + 80 Tyler standard mesh, 1/4 in. O.D. × 20 ft.

[f] Column C: 10/100, squalane on Fluoropak, —30 + 80 U.S. standard mesh, 1/4 in. O.D. × 24 ft. 11 in.

the use of certain columns which are most satisfactory for qualitative work. Hydrogen and methane can be qualitatively separated from the other components expected to be present in the oil and off-gas samples in a 1/4-in. O.D. column 4 ft. long filled with a packing consisting of 5 g of squalane per 100 g of the —65 + 80 Tyler standard screen fraction of reagent grade silica gel. When the oil layer and off-gas samples were analyzed in this column at 0° and a helium flow rate of 5 ml/min, no hydrogen or methane was found. The off-gas samples were also analyzed at 30° in a 1/4-in. O.D. column 10 ft. 5 in. long filled with the —65 + 80 Tyler standard screen fraction of activated carbon using hydrogen at a pressure of 11 p.s.i.g. at a flow rate of 84 ml/min. Neither oxygen nor nitrogen was found. The absence of alkenes and alkynes in the oil and gaseous products was demonstrated when those samples were analyzed at 0° and a helium flow rate of 20 ml/min (measured at 28°) in a 1/4-in. O.D. column filled with a mixture of equal parts by weight of two separate packings, glutaronitrile and propylene carbonate, each containing 10 g of substrate per 100 g of the —80 + 100 U.S. standard screen fraction of non-acid-washed, silicone-treated Type C-22 Johns-Manville firebrick. Since these compounds were not present in the samples taken from several of the nitrator runs at different conditions, no further attempts were made to develop quantitative separations for them.

The limits of accuracy and reproducibility for any given component in columns A and C were ±2 % of the true value of that component when calibrated against known gravimetric samples containing that component, *i.e.* nitroethane in Table II would be between 16.5 and 17.1 wt. %. The GLPC analyses were obtained by measuring the

TABLE II

ANALYSIS OF REACTION PRODUCTS FROM MOLTEN SALT BUTANE NITRATOR*

	Oil layer	*Aqueous layer*	*Off-gas***
NO	—	—	8.6
NO_2	1.0	0.4	0.9
CO	—	—	—
CO_2	0.2	1.5	2.0
H_2O	9.8	98.1	—
C_3H_8	—		2.1
n-C_4H_{10}	0.2	100.0	86.4
HCHO	0.3		
			100.0
CH_3OH	0.2		
C_2H_5OH	0.2		
$CH_3CH(OH)CH_3$	0.1		
$CH_3CH(OH)CH_2CH_3$	0.7		
NM	4.7		
NE	16.8		
2-NP	trace		
2-M-2-NP	0.1		
1-NP	4.2		
2-NB	45.7		
2-M-1-NP	—		
1-NB	15.8		
	100.0		

* All analyses are in weight %.
** Off-gas analysis is on a water-free basis.

TABLE III

MATERIAL BALANCES AROUND MOLTEN SALT BUTANE NITRATOR

(gram-atoms/h)

Substance	*In*	*Out*				
		Oil layer	*Aqueous layer*	*Off-gas*	*Total*	*% Accounted for*
C	31.240	1.532	0.060	26.905	28.497	91.22
N	2.160	0.472	0.202	1.333	2.007	92.88

peak areas with a Model K2-1 integrator (Disc Instruments, Inc.). Working with known volumes of pure gases, column B was found to have similar limits. The purity of the compounds used for calibration was the highest normally available. The nitroparaffin samples (minimum purity, 99.8 mole %) were kindly supplied by Commercial Solvents Corp.

The analysis of the products from a typical nitration run made at a butane flow rate of 6.181 SCFH using 178.5 ml/h of 12.10 *N* nitric acid and a molten salt temperature of 785°F is shown in Table II. The input and output material balances are shown in Table III. Closing a material balance within $\pm 20\%$ is considered quite satisfactory for developmental work of this type. Deviations from perfect (*i.e.* 100 %) material balances may be attributed to product loss during sampling, product deterioration between sampling and analysis, admitted errors in analysis, and certain unavoidable operating losses. However, the fact that the carbon and nitrogen material balances consistently check within ± 7 to 9 % and are both of the same order of magnitude is a strong inducement for their acceptance and use for scale-up from an engineering point of view.

From this work, it was concluded that a gas chromatographic analysis technique has been developed which will routinely give adequate quantitative analysis for the reaction products from the vapor-phase nitration of commercial butane. A full report concerning the construction and operation of the nitrator will be made in the near future.

SUMMARY

A routine gas chromatographic analytical procedure has been developed for the reaction products of a vapor-phase butane nitrator. Replicate samples are used in 3 columns to give satisfactory quantitative separation of the C_1 to C_4 mononitroparaffins, alcohols, aldehydes, C_1 to C_4 paraffins, and NO, NO_2, CO, CO_2, all in the presence of water. Accuracy and precision are at least ± 2 % of the true value of any component. Carbon and nitrogen material balances of approximately 92 % are obtained.

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DIE TRENNUNG STEREOISOMERER VERBINDUNGEN DURCH VERTEILUNG

IV. MITTEILUNG. DIE GASCHROMATOGRAPHISCHE TRENNUNG STEREOISOMERER CYCLOHEXYLAMINE

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Die gaschromatographische Trennung von Aminen mit geringen Siedepunktsunterschieden ist auf den üblichen Säulen häufig nicht möglich, weil starkes "tailing" die Trennung verhindert. So blieben auch frühere Versuche, stereoisomere Methylcyclohexylamine an Carbowax- oder Siliconsäulen zu trennen, erfolglos[1]; lediglich die Trennung der unmethylierten, der monomethylierten und der dimethylierten Methylcyclohexylamine voneinander war auf der Siliconsäule möglich, indem etwa beim Siedepunkt des Amins gearbeitet wurde.

Über eine Möglichkeit das störende "tailing" auszuschalten, berichtete zuerst JAMES[2], dem es durch Vorbehandeln der Trägersubstanz mit Alkali gelang, die Trennungen wesentlich zu verbessern. Durch Anwendung derartiger alkalihaltiger Säulen gelang es dann auch SMITH UND RADFORD[3] neben anderen nahe beieinander siedenden Aminen die stereoisomeren 1,2-Diaminocyclohexane zu trennen.

Die weitere Suche nach einfachen Analysenverfahren für Stereoisomerengemische verschieden substituierter Cyclohexylamine, die im Rahmen anderer Arbeiten benötigt wurden, gab den Anlass zu prüfen, ob Alkali enthaltende Säulen auch bei stereoisomeren mono-Aminocyclohexanen brauchbare Ergebnisse liefern. Hierbei zeigte sich, dass die mit einer nach den Angaben von SMITH UND RADFORD gebauten Siliconsäule erzielten Trennungen für analytische Zwecke zumeist ausreichten (vgl. Fig. 1–3). Die an dieser Säule bei verschiedenen Stereoisomerenpaaren festgestellten Retentionszeiten sind in Tabelle I zusammengefasst.

Die beiden Stereoisomeren eines Stereoisomerenpaares weisen jeweils ähnliche Unterschiede in den Retentionszeiten und in den Siedepunkten auf[4]. Beim Vergleich verschiedener Stereoisomerenpaare miteinander zeigt sich aber keine strenge Abhängigkeit der Retentionszeit vom Siedeverhalten. So besitzen z.B. bei 100° und 45 p.s.i. das N-Methyl-*cis*-2-methylcyclohexylamin $Sdp._{727}$ = 162.7° und das N,N-Dimethyl-*trans*-2-methylcyclohexylamin $Sdp._{729}$ = 170.5° die gleiche Retentionszeit von 37 Min. Bei 130° und 45 p.s.i. zeigen die gleichen Amine allerdings einen geringen Unterschied in der Retentionszeit (s. Tabelle I).

Diese Beobachtungen über den Einfluss der stationären Phase auf die Retentionszeiten waren der Anlass, die Trennung an polareren Säulen zu untersuchen. Eine in der gleichen Weise gebaute mit 10 % Fluorsilicon QF 1 beschichtete Säule, die

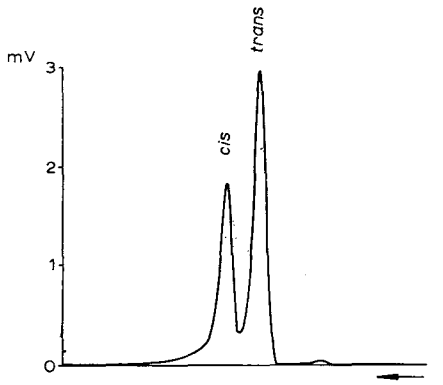

Fig. 1. Trennung der 2-Methylcyclohexylamine. 8 m Silicon DC 710, 130°, 45 p.s.i.

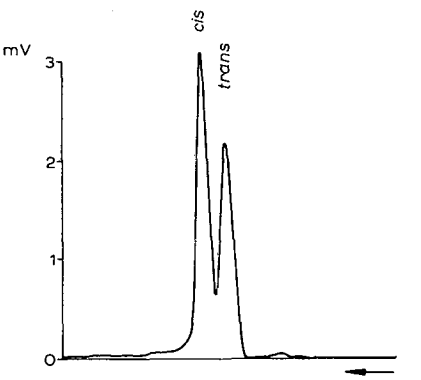

Fig. 2. Trennung der N-Methyl-3-methylcyclohexylamine. 8 m Silicon DC 710, 100°, 45 p.s.i.

im Prinzip gleichartige, aber weniger gute Trennungen ergab, erwies sich als unbeständig. Anscheinend zersetzt sich das Fluorsilicon unter dem Einfluss von Kaliumhydroxyd. Da Amine mit freien Hydroxylgruppen zu assoziieren* vermögen und das Ausmass derartiger Assoziationen zumeist sehr wesentlich von der Zugänglichkeit der hierzu befähigten Gruppen abhängt, war für stereoisomere Cyclohexylamine ein unterschiedliches Verhalten zu erwarten. Amine mit einer in Äquatorialstellung verhältnismässig frei liegenden Aminogruppe sollten besser eine Wasserstoffbindung

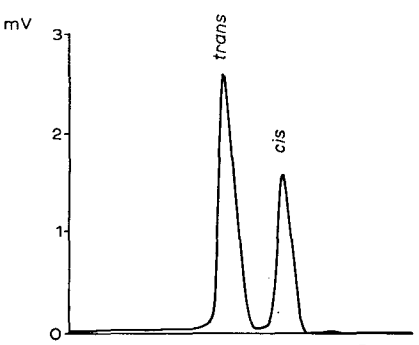

Fig. 3. Trennung der N,N-Dimethyl-4-methylcyclohexylamine. 8 m Silicon DC 710, 100°, 45 p.s.i.

eingehen können als axiale Amine, deren Stickstoff mit seinem freien Elektronenpaar durch die beiden benachbarten axialen Wasserstoffatome gehindert wird. Dass diese Überlegung zutrifft, hat bereits die flüssig/flüssig-Verteilung ergeben[5]. Es lag daher nahe, auch in der Gaschromatographie den Einfluss einer flüssigen Phase mit freien Hydroxylgruppen zu studieren, weshalb in der beschriebenen Weise eine Säule mit 10 % Hyprose als flüssiger Phase gebaut wurde. Die an dieser Säule erhaltenen Chro-

* Unter Assoziation ist hier nur die Ausbildung einer Wasserstoffbrückenbindung ("hydrogen bond") zwischen Amino- und Hydroxylgruppe gemeint.

TABELLE I

	Retentionszeit			
	8 m Silicon DC 710/KOH		*8 m Hyprose/KOH*	*4 m Carbowax/KOH*
	130°, 30 p.s.i.	*130°, 45 p.s.i.*	*130°, 45 p.s.i.*	*70°, 45 p.s.i.*
2-Methylcyclohexylamin				
nicht methyliert				
trans	19.5	14.5	9.5	21
cis	21.5	16.5	9.5	26
monomethyliert				
trans	23.5	17.5	7	20
cis	25	18.5	6.5	22
dimethyliert				
trans	25	19.5	5.5	18
cis	31.5	24.5	7.5	25
3-Methylcyclohexylamin				
nicht methyliert				
trans	21	15	11	25
cis	19.5	14	11	25
monomethyliert				
trans	23.5	17	7	20.5
cis	24.5	18.5	9	24.5
dimethyliert				
trans	25.5	20	6.5	17.5
cis	29.5	22.5	8.5	22.5
4-Methylcyclohexylamin				
nicht methyliert				
trans	19.5	14.5	11	23
cis	20.5	15.5	10.5	25
monomethyliert				
trans	24	19	9.5	24.5
cis	24	18.5	7.5	22.5
dimethyliert				
trans	29	23	7	23.5
cis	27	21	6	19
4-tert.-Butylcyclohexylamin		*130°, 50 p.s.i.*	*160°, 60 p.s.i.*	
nicht methyliert				
trans		51.5	14.5	
cis		50.5	11.5	

matogramme unterscheiden sich grundsätzlich von denen der Siliconsäule. Die unmethylierten Amine kommen jetzt weit nach ihren über 20° höher siedenden N,N-Dimethylierungsprodukten. Dazwischen liegen mit ihren Retentionszeiten die N-monomethylierten Amine. Offenbar nimmt die Fähigkeit zur Ausbildung einer Wasserstoffbindung mit fortschreitender Methylierung des Stickstoffs ab. Auch innerhalb der einzelnen Stereoisomerenpaare der unmethylierten Amine macht sich der Einfluss des unterschiedlichen Assoziationsvermögens bemerkbar, indem erwartungsgemäss die Amine mit hauptsächlich äquatorialer Aminogruppe im Verhältnis stärker

zurückgehalten werden als die mit vorwiegend axialer. Das hat zur Folge, dass bei den 2- und 3-Methylcyclohexylaminen die auf Grund der unterschiedlichen Siedepunkte auf der Siliconsäule erzielten Trennungen aufgehoben werden, während beim 4-Methylcyclohexylamin sogar das niedriger siedende, aber stärker assoziierende *trans*-Amin zuletzt kommt. Beim 4-*tert.*-Butylcyclohexylamin, bei dem auf Grund der Siedepunkte an der Siliconsäule nur eine schlechte Trennung eintrat, konnte eine wesentliche Verbesserung der Trennung erreicht werden, da hier das stärker assoziierende Isomere auch den höheren Siedepunkt besitzt.

Aus dem gleichen Grunde ist auch die Trennung der N-monomethylierten Methylcyclohexylamine besser als auf der Siliconsäule. Ein gewisser Unterschied in der Assoziationsfähigkeit innerhalb der Stereoisomerenpaare ist also auch hier vorhanden, wenngleich die Assoziation im ganzen gesehen sicherlich weit geringer ist als bei den unmethylierten Aminen. Hingegen war bei den dimethylierten Aminen kein Einfluss einer H-Bindung festzustellen. In grundsätzlich gleicher Weise können die Trennungen auf einer Carbowaxsäule erklärt werden, wenn auch hier die Einflüsse der Wasserstoffbrücken geringer sind, da nur noch wenige freie Hydroxylgruppen vorhanden sind. Immerhin besitzt, genau wie auf der Hyprosesäule, das am niedrigsten siedende unmethylierte Amin die höchste Retentionszeit gegenüber seinen Methylierungsprodukten.

Ob die mit zunehmender Methylierung abnehmende Assoziation auf einer sterischen Hinderung durch die eingetretenen Methylgruppen beruht, kann hier nicht entschieden werden.

Die an Hyprose- und Carbowaxsäulen beobachteten Retentionszeiten sind ebenfalls in Tabelle I zusammengefasst.

Trennungen desselben Gemisches dreier Amine — eines unmethylierten, eines momomethylierten und eines dimethylierten — an beiden Säulen zeigen die Fig. 4 und 5. Die unterschiedliche Assoziation gibt sich nicht nur in der deutlich sichtbaren Umkehr der Reihenfolge, sondern auch im Breiterwerden der Banden auf dem Chromatogramm der Hyprosesäule zu erkennen.

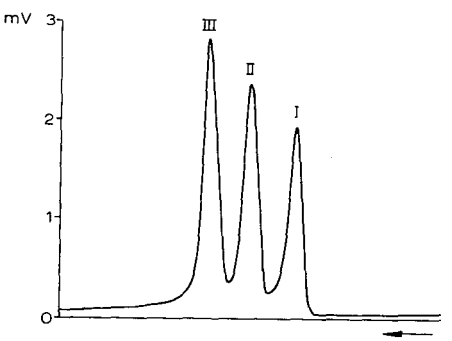

Fig. 4. Trennung der *trans*-3-Methylcyclohexylamine. (I) unmethyliert; (II) monomethyliert; (III) dimethyliert. Silicon DC 710, 130°, 45 p.s.i.

Die in dieser Arbeit beschriebenen Säulen haben sich zur Analyse von Gemischen stereoisomerer Cyclohexylamine und zur Reinheitsprüfung der einzelnen Isomeren bisher gut bewährt.

(Die Reinheitsprüfung bereitete allerdings zunächst Schwierigkeiten, weil bei häufigem Spritzen immer geringe Mengen zuvor chromatographierter Amine in den Chromatogrammen festzustellen waren. Es wurde daher vermutet, dass ein Teil des Amins — wahrscheinlich als Carbaminat — im Verdampfungsraum zurückbleibt

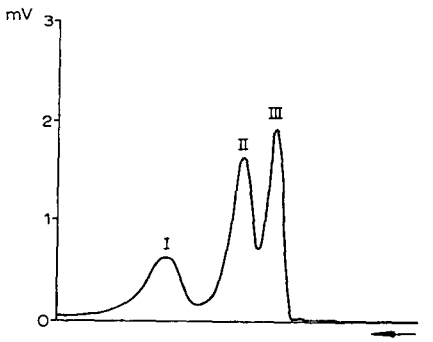

Fig. 5. Trennung der *trans*-3-Methylcyclohexylamine. (I) unmethyliert; (II) monomethyliert; (III) dimethyliert. Hyprose, 130°, 45 p.s.i.

und beim Einspritzen der nächsten Aminprobe teilweise freigesetzt bzw. mitgerissen wird. Durch Einspritzen eines anderen, mit seiner Retentionszeit nicht störenden Amins sollte sich der Verdampfungsraum reinigen lassen, was auch in diesem Falle mit Äthylendiamin gelang).

BESCHREIBUNG DER VERSUCHE

Bau der Chromatographiesäulen

100 g Chromosorb W (W. H. Curtin Co., Houston, Tex. 30/60 mesh) wurden mit Methanol gut angefeuchtet und mit einer Lösung von 5 g Kaliumhydroxyd in 100 ml Methanol eine Stunde lang im Rotationsverdampfer ohne Anwendung von Wärme und Vakuum vermischt. Darauf wurde das Lösungsmittel im Vakuum abgezogen. Die völlig trockene Masse wurde gesiebt (Din Gewebe No. 14) und die groben Anteile verworfen.

Silicon- und Carbowaxsäule

50 g der so vorbereiteten Trägersubstanz wurden mit Methylenchlorid angefeuchtet, mit einer Lösung von 5 g Siliconöl DC 710 (Dow Corning Corp., Midland, Mich.), bzw. 5 g Carbowax 20000 in 50 ml Methylenchlorid versetzt und in der beschriebenen Weise gemischt, getrocknet und gesiebt.

Hyprosesäule

50 g der präparierten Trägersubstanz wurden mit 60 ml Chloroform angefeuchtet und mit einer Lösung von 5 g Hyprose in 60 ml Chloroform versetzt. Sonst wurde wie oben verfahren.

Füllen der Säulen

Das Füllmaterial wurde unter ständigem leichtem Klopfen in senkrecht hängende Kupferrohre (ø 4 mm) entsprechender Länge eingefüllt und das Klopfen unter ge-

legentlichem Nachfüllen eine Stunde lang fortgesetzt. Es wurden *ca.* 5 g Füllmaterial auf 1 m Kolonnenlänge verbraucht.

Versuchsbedingungen

Die Versuche wurden sämtlich im Beckman Gaschromatographen GC 2 durchgeführt. Temperatur 100–160°, Druck 30–45 p.s.i., H_2 als Trägergas. Hitzdrahtdetektor, Empfindlichkeit 2, Probenmenge 1–2 μl.

Reinigung

Zur Entfernung von Amin- bzw. Carbaminresten aus der Apparatur genügte zumeist dreimaliges Einspritzen von je 5 μl Äthylendiamin.

Die Darstellung der untersuchten Amine ist an anderer Stelle[4] beschrieben.

ZUSAMMENFASSUNG

Durch Überziehen der Trägersubstanz mit Kaliumhydroxyd liess sich das "tailing" beim Gaschromatographieren von Aminen nicht nur an Silicon- sondern auch an Hyprosesäulen weitgehend verhindern. 10 Stereoisomerenpaare von Alkyl-cyclohexylaminen bzw. ihrer N-Mono- und ihrer N,N-Dimethylierungsprodukte konnten getrennt werden. Der Einfluss des Siliconöls als stationäre Phase war gering, so dass hier die Retentionszeiten den Siedepunkten ungefähr vergleichbar waren. Dagegen wurde auf der Hyprosesäule die Retentionszeit eines Amins von dessen Fähigkeit zur Ausbildung einer H-Bindung entscheidend beeinflusst.

SUMMARY

In the gas chromatography of amines on silicone and hyprose columns, tailing could be prevented to a great extent by treating the support with potassium hydroxide. Ten pairs of stereoisomers consisting of alkylcyclohexylamines and their N-monomethyl and N,N-dimethyl derivatives could be separated. Silicone oil as stationary phase had only a slight effect on the retention times, so that with this phase there is some relation between the retention times and the boiling points of the amines. On the other hand with hyprose columns the capacity of an amine to form a hydrogen bond has a considerable influence on the retention time.

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GAS CHROMATOGRAPHIC ANALYSIS OF CYCLOPENTENYL FATTY ACIDS

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Some seed oils of the Flacourtiaceae species contain cyclopentenyl-substituted fatty acids in their glycerides[1]. The fatty acid composition of these oils was first studied by POWER AND GORNALL[2], who also determined the presence of a cyclopentenyl structure in the fatty acid molecules. The major components are chaulmoogric (13-cyclopent-2-enyltridecanoic), hydnocarpic (11-cyclopent-2-enylhendecanoic) and gorlic (13-cyclopent-2-enyltridec-6-enoic) acids.

The composition of Flacourtiaceae seed oils was later investigated by COLE AND CARDOSO[3]. In addition to the above fatty acids, they found traces of lower homologues, *i.e.* alepric (9-cyclopent-2-enylnonanoic), aleprylic (7-cyclopent-2-enylheptanoic), aleprestic (5-cyclopent-2-enylpentanoic) and aleprolic (cyclopent-2-enylcarboxylic) acids and small amounts of normal straight-chain fatty acids, mainly palmitic and oleic acids.

Chaulmoogra oil is the best known product prepared from the seeds of various Flacourtiaceae, formerly obtained mainly from *Taraktogenos Kurzii*, now mostly from *Hydnocarpus Wightiana*. Chaulmoogra oil is used in medicine for the treatment of leprosy. The technical product, however, is rarely sufficiently pure and most frequently contains a mixture of extracts from several related plants. In spite of their importance in medicine, very little attention has been payed to the further investigation of this group of oils.

It is assumed that the fatty acid composition of Flacourtiaceae seed oils may conveniently be determined by means of gas–liquid chromatography, but it was necessary to investigate the chromatographic behaviour of cyclopentenyl fatty acids and to compare them with the normal straight-chain fatty acids.

EXPERIMENTAL

Material

All the chemicals used were analytical reagents, produced by Lachema, nat. corp., Brno.

Chaulmoogra oil was extracted from the seeds of *Hydnocarpus Wightiana* with hexane, and had the following properties: acid value 27.2, ester value 159.9, saponification value 187.1, iodine value (Hanuš) 94.8, unsaponifiable matter 2.4 %, melting point 22.8°, water and volatile products 2.2 %, total fatty acids 91.4 %, iodine value of isolated fatty acids (Hanuš) 97.7, acid value of fatty acids 202.0, peroxide value (iodometric "cold" method) 2.4 mequiv./kg.

Preparation of methyl esters

Approximately 5 g of chaulmoogra oil were refluxed for 2 h with 100 ml of 5% ethanolic potassium hydroxide solution, 100 ml of 10% aqueous hydrochloric acid were added and the liberated fatty acids extracted with pentane. The residue after the evaporation of pentane was esterified by refluxing for 2 h with 100 ml of anhydrous methanol containing 2% of sulphuric acid. About 100 ml of water were then added and the methyl esters were extracted with pentane. The unreacted fatty acids were removed by washing with 5% aqueous potassium carbonate solution. After the removal of the solvent the methyl esters were used for analysis by gas chromatography.

Analysis by gas chromatography

The analysis was carried out with the Chrom I apparatus manufactured by Laboratorní přístroje, nat. corp., Prague, with a flame ionization detector, described by NOVÁK, RUSEK AND JANÁK[4]. The conditions of the determination using a non-polar stationary phase were: 8.50 g of packing (Celite, particle size of 0.2–0.3 mm, impregnated with 20% of Apiezon M), temperature of 240°, column length of 80 cm and flow rate of 35 ml N_2/min. The conditions of the determination using a polar stationary phase were: 15.23 g of packing (Celite of the same particle size, impregnated with 20% of polyethylene glycol succinate), temperature of 190°, column length of 160 cm and flow rate of 35 ml N_2/min. The fatty acid composition was calculated on the basis of the areas of the peaks.

RESULTS

Typical chromatograms on Apiezon and polyester stationary phases are shown in Figs. 1 and 2. Numerical values of relative retention volumes of chaulmoogra fatty acids are summarized in Table I. Normal straight-chain fatty acids were identified by means of pure standards: even-numbered saturated fatty acids of 10–22 carbon atoms, oleic and linoleic acids. Individual fatty acids were identified by comparing graphically logarithms of relative retention volumes on both phases tested, according

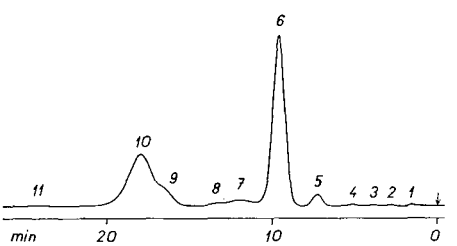

Fig. 1. Separation of methyl esters of fatty acids from *Hydnocarpus Wightiana* seed oil on Apiezon M. For operating conditions see text. Fatty acids: 1 = alepristic; 2 = aleprylic; 3 = myristic; 4 = alepric; 5 = palmitic; 6 = hydnocarpic; 7 = oleic; 8 = stearic; 9 = gorlic; 10 = chaulmoogric; 11 = eicosanoic.

to JAMES[5]; small amounts of myristic, palmitic, stearic, eicosanoic and oleic acids were detected. No linoleic acid was found by gas chromatography, although 0.2–0.3% of dienoic acids with isolated double bonds and 0.1% of dienoic acids with conjugated double bonds were determined by spectral methods. Six other components (three

TABLE I

RELATIVE RETENTION VOLUMES OF NORMAL AND CYCLOPENTENYL FATTY ACID METHYL ESTERS

Methyl ester of fatty acid	*Shorthand designation*	*Relative retention volumes in*	
		Apiezon M at 240°	*Polyethylene glycol succinate at 190°*
Decanoic	10:0	0.15	0.20
Lauric	12:0	0.28	0.34
Myristic	14:0	0.52	0.58
Palmitic	16:0	1.00	1.00
Stearic	18:0	1.88	1.69
Eicosanoic	20:0	3.51	2.92
Behenic	22:0	6.60	5.00
Oleic	18:1	1.72	2.03
Linoleic	18:2	1.70	2.45
Aleprestic		0.19	0.45
Aleprylic		0.37	0.73
Alepric		0.70	1.21
Hydnocarpic		1.31	2.04
Chaulmoogric		2.51	3.38
Gorlic		2.33	3.92

major components and three trace components) were detected, possessing unusual retention volumes on the JAMES' diagram and differing substantially from normal fatty acids (Fig. 3).

As is evident from Fig. 3, the specific component fatty acids of chaulmoogra oil (A–E) lie on one straight line at distances corresponding to an increase of chain

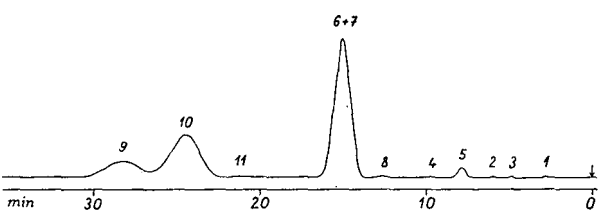

Fig. 2. Separation of methyl esters of fatty acids from *Hydnocarpus Wightiana* seed oil on polyethylene glycol succinate. For operating conditions see text. Fatty acids: the same as in Fig. 1.

length by 2 carbon atoms, similarly to the case of normal aliphatic fatty acids, which are situated on a nearby line. The component F alone lies outside and its position with regard to the nearest component (E) is analogous to that of oleic acid to stearic acid. The series A–E evidently corresponds to the homologous cyclopentenyl fatty acids with saturated straight chains (aleprestic, aleprylic, alepric, hydnocarpic, and chaulmoogric acid, the lowest homologue, *i.e.* aleprolic acid, was not detected), whilst the component F corresponds to an unsaturated derivative of chaulmoogric acid (gorlic acid).

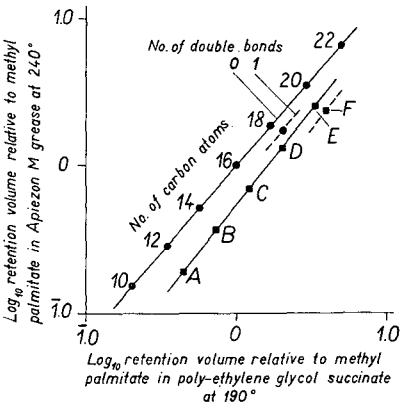

Fig. 3. Log_{10} relative retention volumes of chaulmoogra oil fatty acid methyl esters on Apiezon M plotted against log_{10} relative retention volumes on polyester. Completed by acids not occurring in chaulmoogra oil: decanoic (10:0), lauric (12:0) and behenic (22:0). Straight-chain fatty acids ●; Cyclopentenyl fatty acids ■. A = aleprestic; B = aleprylic; C = alepric; D = hydnocarpic; E = chaulmoogric; F = gorlic.

DISCUSSION

All the cyclopentenyl acids detected in chaulmoogra oil were identified on assumptions in agreement with the known behaviour of homologous series in analysis by gas chromatography[6]. The evidence of this behaviour is a linear relationship of the logarithm of relative retention volumes and the number of carbon atoms in the series, and in particular, the linear relationship obtained by comparison of the logarithm of relative retention volumes obtained on two stationary phases of different polarities. The latter relation is very important as it enables reliable identification of individual members of a homologous series. The same method was successfully applied *e.g.* for the identification of branched-chain fatty acids and less common unsaturated fatty acids[5] because the components of different structures are then situated on different lines on the graph.

The relation between the components assumed to be gorlic and chaulmoogric acids are in good agreement with that of stearic and oleic acids, *i.e.* that oleic acid precedes stearic acid on non-polar stationary phases, while the unsaturated acid follows the saturated acid on polar phases. The percentages of the thus indirectly identified cyclopentenyl acids agree very well with those reported by COLE AND CARDOSO for the oil from *Hydnocarpus Wightiana*[3]. This comparison is given in Table II.

The behaviour of cyclopentenyl and straight-chain fatty acids may be easily compared on the basis of the separation factors calculated from the retention volumes according to JAMES[5,7]; these factors are summarized in Table III. It may be concluded from the position of the line of the homologous series on the JAMES' diagram (Fig. 3) and from the separation factors that the presence of a cyclopentenyl group in the molecule causes a retardation in analysis by gas chromatography on both types of stationary phases. The retardation is greater in the case of the more polar (polyester) phase as a result of an increase of polarity by comparison with normal fatty acids. This increase of polarity is probably caused not only by the presence of a ring in the molecule, but also by the presence of a double bond in the ring.

TABLE II

FATTY ACID COMPOSITION OF CHAULMOOGRA OIL FROM *Hydnocarpus Wightiana*

Fatty acid	*Percentage in fatty acids (wt. %)*	
	Cole and Cardoso[3]	*Authors*
Myristic	—	0.2
Palmitic	1.8	3.0
Stearic	—	0.7
Eicosanoic	—	0.7
Oleic	6.5	2.6
Aleprolic	3.4	not found
Aleprestic		0.1
Aleprylic		0.2
Alepric		0.3
Hydnocarpic	48.9	52.4
Chaulmoogric	27.1	28.4
Gorlic	12.3	11.4

The line of the homologous series of cyclopentenyl fatty acids (Fig. 3) converges to that of the normal fatty acids. The separation factor for the $-CH_2-$ group of cyclopentenyl fatty acids also is smaller on polyester phase than the same factor of straight-chain fatty acids, whilst the $-CH_2-$ separation factors of both straight-chain and cyclopentenyl fatty acids on Apiezon M have the same value. Therefore, the relative polarity of cyclopentenyl fatty acids decreases gradually with increasing molecular weight since the cyclopentenyl group is situated at the end opposite to the carboxyl or ester group and the non-polar part of the molecule (*i.e.* the normal hydrocarbon chain) becomes gradually more important.

Thus, it is evident, that the chromatographic behaviour of cyclopentenyl fatty acids is affected by their structure and depends on the proportion of polar and non-polar parts of the molecule.

TABLE III

SEPARATION FACTORS CALCULATED ON THE BASIS OF THE RETENTION VOLUMES (TABLE I)

Separation factor	*Stationary phase*	
	Apiezon M 240°	*Polyethylene glycol succinate 190°*
For the $-CH_2-$ group of normal fatty acids	1.37	1.31
For the $-CH_2-$ group of cyclopentenyl fatty acids	1.37	1.29
For the introduction of a double bond in the 9-position of normal fatty acids	0.92	1.20
For the introduction of a double bond in the 6-position of cyclopentenyl fatty acids	0.93	1.16
For the formation of a cyclopentenyl group in the molecule*	1.3	2.0
For the introduction of a cyclopentenyl group at the ω-position**	6.6	7.7

* Determined by the comparison of relative retention volumes of hydnocarpic and chaulmoogric acids with those of palmitic and stearic acids.

** Determined by the comparison of relative retention volumes of hydnocarpic and chaulmoogric acids with those calculated for the acids with 5 carbon atoms less, *i.e.* hendecanoic and tridecanoic acids.

SUMMARY

The fatty acid composition of chaulmoogra oil was investigated by means of gas–liquid chromatography. Normal straight chain fatty acids were identified directly, cyclopentenyl fatty acids, characteristic for chaulmoogra oil, were identified indirectly on the basis of present knowledge of their structure and occurrence in the oil and on the relations of the retention volumes on two stationary phases of different polarities. The chromatographic behaviour of cyclopentenyl fatty acids differs from that of normal fatty acids and depends on their structure.

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THE PAPER CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SIMPLE PHENOLS

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INTRODUCTION

During the past few years a considerable amount of work has been reported in the literature on the paper chromatographic analysis of a wide range of phenols, but very few of the methods described lead to clean separations of the isomers of low molecular weight alkylphenols. The latter materials are of interest to the petroleum industry in connection with the analysis of the low boiling phenolic constituents that can occur in various products *e.g.* gasolines and kerosines.

Attempts to overcome the difficulties caused by the volatility of simple phenols have been made in a variety of ways, including separations on papers impregnated with amides[1,2] or polyamides[3] and on papers held between glass plates[4,5]. However, most workers have used derivatives of phenols *e.g.* the sodium salts of phenylazobenzenesulphonic acid[6,7], the *p*-nitrophenylazo dyes[8,9] and the 3,5-dinitrobenzoate esters[10]. The separations of low molecular weight phenols reported in the literature were frequently poor, *e.g.* *m*- and *p*-cresol were rarely resolved[1,11].

A case therefore existed for an improved method of separating mixtures of phenols, by paper chromatography, which would be applicable to a wide variety of samples, particularly petroleum products.

This report describes a method in which phenols are coupled with diazotised aromatic amines to form stable azo dyes. This reaction forms the basis of many well-known quantitative methods for the determination of phenols. In the present method mixtures of these azo dyes are separated into single components on a paper impregnated with formamide. A cyclohexane–benzene mixture containing dipropylene glycol is used as the developing solvent.

The azo dyes are further characterized by the colour reactions they give in alkaline solutions. Examples are given illustrating the successful application of the method in the separation and identification of phenols in a variety of products.

EXPERIMENTAL AND RESULTS

1. *Coupling reagents and the formation of azo dyes*

The choice of amine was governed by several considerations.

(a) The use of the nitro–aci-nitro reaction in alkaline media would be useful in

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characterizing individual phenols, therefore un-nitrated amines such as aniline, toluidines, anisidines, benzidines were not used.

(b) It was intended that the chromatographic papers should not be pretreated with acids or alkalis. It seemed likely that dyes containing sulphonic acid functions, *i.e.* those based on sulphanilic acid, would have a strong affinity for the paper, and hence with developing solvents of low polarity they would have R_F values close to zero; consequently sulphonic acid dyes were not examined.

(c) Since *meta*-nitro dyes would not have quinonoid aci-nitro forms, this investigation was confined to the *ortho*- and *para*-nitro-anilines.

Solutions of the diazotised amines were prepared as follows:

The nitro amine (1.5 g) was dissolved in 1000 ml of *N* aqueous hydrochloric acid. Just before the coupling with the phenol samples, 25 ml of amine hydrochloride solution were diazotised at 0° with 0.75 ml of sodium nitrite solution (10% aqueous) giving a solution of *p*-nitrophenyldiazonium chloride (soln. A); the yellow colour of the amine was completely discharged. A convenient portion of the phenol solution was treated with 1–2 ml of the soln. A, and after 1 min the mixture was made alkaline with sodium carbonate solution (2 ml 20% w aq.). When phenols were present in the

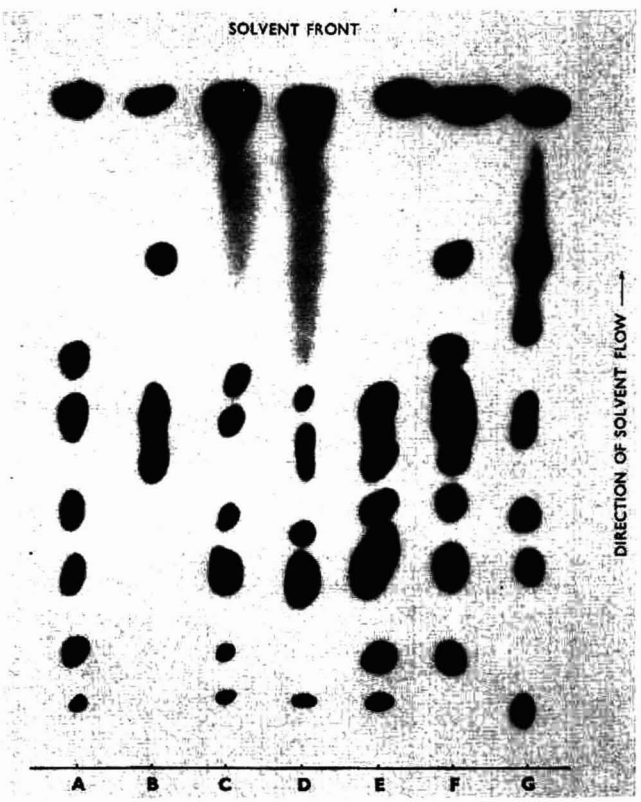


Fig. 1. A = Mixture of phenol, cresols and ethylphenols. B = Mixture of xylenols. C = Phenols from commercial tritolyl phosphate (I). D = Phenols from commercial trixylenyl phosphate. E = Phenols from commercial tritolyl phosphate (II). F = Reference blend of simple alkylphenols. G = Phenols isolated from a Curaçao kerosine.

sample under test a brilliant red/purple dye was formed. The azo dyes, still phenolic, were in solution as their sodium salts. After acidification with excess dilute hydrochloric acid or by adding excess solid carbon dioxide the azo dyes were extracted with ether (2 or 3 extractions with 10 ml ether usually sufficed to remove all the azo dye from the aqueous phase).

The ethereal phase was dried over anhydrous magnesium sulphate. The solvent was distilled off and the azo dyes immediately redissolved in chloroform (*ca.* 0.2 ml).

This reaction was carried out for all thirteen simple alkylphenols, with both *o*- and *p*-nitrophenyldiazonium chlorides. A reagent blank was prepared in the same way, but omitting the phenols. The azo dyes and the blank were examined by paper chromatography (Fig. 1).

2. *Development of a suitable solvent system*

Most of the systems reported in the literature for the analysis of phenols have required the use of a variety of aqueous solvents. In the case of azo dyes derived from phenols little work has been done, and an example is the use of sodium carbonate treated papers with phenylazobenzenesulphonic acids[6,7].

Since the nitrophenylazobenzene dyes were almost insoluble in water it was decided to use only organic phases. The azo dyes could be partitioned between formamide and hydrocarbon solvents. Therefore in order to achieve separations of the azo dyes, papers impregnated with formamide were used, with hydrocarbon solvents as developing phases. It was found that the best separations were obtained if the hydrocarbon solvent contained a small proportion of dipropylene glycol.

Several conventional systems were also examined, and the nature and behaviour of these systems are listed in Table I. System 9 was finally adopted.

TABLE I

PAPER CHROMATOGRAPHIC SYSTEMS USED FOR THE SEPARATION OF *p*-NITROPHENYLAZOBENZENE DYES (DERIVED FROM PHENOLS)

System No.	*Mobile phase*	*Paper* pretreatment*	*Observation*
1	Acetic acid–butanol–water (50:50:10 vol.)	None	Dyes move with solvent front
2	Methyl isobutyl ketone–ethanol (50:50 vol.) saturated with 10% aq. ammonia	Sodium carbonate	Each dye gave several spots, phenol** retarded
3	Water–methyl ethyl ketone–ethanol (50:50:10 vol.)	Untreated	R_F's 0.5
4	Methyl isobutyl ketone–ethanol, saturated with ammonia	Untreated	R_F's 1.00
5	Sodium hydroxide $N/2$ aq. saturated with *n*-butanol[9]	Untreated	*o*,- *m*-, *p***-Cresol not separated
6	Benzene–cyclohexane–dipropylene glycol (30:70:3 vol.)	Untreated	R_F's 1.00
7	Benzene–cyclohexane–dipropylene glycol (30:70:3 vol.)	Sodium carbonate	Spots "comet" badly
8	Benzene–cyclohexane–methanol (50:50:1 vol.)	Formamide	Phenol**, *o*- and *p*-cresol separated
9	Benzene–cyclohexane–dipropylene glycol (30:70:3 vol.)	Formamide	Good separation of most phenols**

* Whatman No. 1.
** Refers to azo dyes.

3. *Preparation of samples*

(*i*) *Phenols uncombined* (*e.g. in kerosine*)

Wherever simple phenols are uncombined *e.g.* in light petroleum distillates they can be extracted with aqueous sodium hydroxide (2 *N*)*. To a suitable quantity of the alkaline extract is added 1 ml of diazotised *p*-nitroaniline solution (soln. A). If a red or yellow colour develops, indicating the formation of an azo dye, the solution is acidified with dilute hydrochloric acid, and extracted with ether. The ethereal solution is dried over anhydrous magnesium sulphate, and then evaporated free of solvent. The solid azo dye is dissolved in 0.1–0.2 ml chloroform.

(*ii*) *Phenols combined* (*e.g. in triaryl phosphates*)

(*a*) *Phosphate esters.* When phenols are combined in the form of esters they can be obtained in the free form by saponification *e.g.* reflux for 30 min to 1 h with alcoholic potassium hydroxide (1 *N*). The saponification liquid is diluted with water, treated with excess solid carbon dioxide, and extracted with ether, or it can be acidified with dilute sulphuric acid and extracted with ether. In either case the ether extract will contain phenols, from which azo dyes are then prepared as in 3(i).

(*b*) *Phosphate esters and/or mineral oil and/or synthetic ester oil.* If other saponifiable material is present with the phosphate esters the saponification liquor (3.ii.a) is diluted with water and extracted with ether or petroleum spirit. This removes alcohols *e.g.* 2-ethylhexanol, unsaponifiable matter such as hydrocarbon oil, and materials such as antioxidants containing nitrogen *e.g.* phenothiazine. Excess solid carbon dioxide is added to the alkaline phase, which is then extracted with ether to remove phenols (acids such as sebacic acid remain in the alkaline phase). The alkaline phase can alternatively be acidified with dilute sulphuric acid and steam distilled, in which case the phenols will be in the distillate.

The azo dyes are then prepared as in 3(i).

4. *Preparation and running of a chromatogram*

A Whatman No. 1 filter paper square 20 cm × 20 cm (Shandon pattern, corner holes) is impregnated with formamide by drawing it once through a 20% vol. solution of formamide in acetone. Small volumes (1–5 μl) of chloroform solutions of the azo dyes are spotted onto the impregnated paper at appropriate positions along the base line. The paper is developed by the ascending technique with a mixture of cyclohexane–benzene (70:30 vol.) containing 3% vol. dipropylene glycol. At 21° the solvent takes some 2 1/2 h to travel a distance of 17.0 cm. The atmosphere in the tank need not be saturated with the vapour of the developing solvent. The paper is dried at 40°.

For the most part the chromatographic zones (spots) are self-indicating. Wherever spots overlap *e.g.*, 3,5-, 2,3-, and 2,5-xylenols, they are not easily distinguished from each other. If, however, a chromatogram of the *p*-nitrophenylazo dyes is exposed to ammonia vapour the spots are changed from pale yellow to intense red or purple. The colours are characteristic, and enable overlapping spots to be identified.

* Although thiophenols and naphthenic acids are also extracted they do not interfere with the separation and identification of phenols from petroleum distillates. Thiophenols give yellow azo dyes which have R_F values of 1.00, and which do not change colour on spraying with alkali. Naphthenic acids cannot be seen on a chromatogram.

The colour changes given in ammonia vapour by the *o*-nitrophenylazo dyes are only slight, *i.e.* from yellow to orange, or orange red.

On standing for a few minutes in air, *i.e.* after the ammonia has evaporated from the paper the colours return to yellow. In order to form permanent red/purple colours from the azo dyes, the papers are sprayed with sodium carbonate solution (1 % aq.) and dried at 40°. The colours and R_F values of the spots are noted.

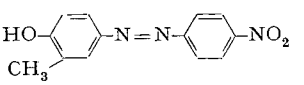
DISCUSSION

The structures of the various phenols which have been examined are of two types:

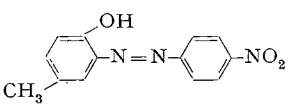
(A) *4 position unsubstituted*	(B) *4 position substituted*
phenol	*p*-cresol
o- and *m*-cresol	*p*-ethylphenol
o- and *m*-ethylphenol	2,4- and 3,4-xylenols
2,3-, 2,5-, 2,6- and 3,5-xylenols	*p*-chlorophenol, 4-chloro-2-methylphenol

On the chromatograms of the *p*-nitrophenylazo dyes and of the *o*-nitrophenylazo dyes, the group A phenols have R_F values < 1.00, whereas group B phenols have R_F values 1.00 only. The *para* (or 4) position of the group B phenols is blocked by either methyl, ethyl or chloro groups, whereas the 4 positions in group A phenols are free. It is therefore postulated that the azo dyes are of two types,

Group A – para coupled
e.g.

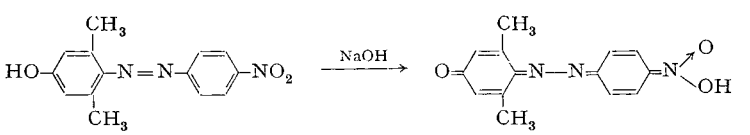


Group B – ortho coupled



Both types of dye are phenolic, and are soluble in sodium hydroxide solution.

The mechanism whereby the neutral yellow azo dyes assume red/purple colours in alkaline media is most probably due to the formation of quinonoid structures[12].



4-*p*-nitrophenylazo-3,5-dimethylphenol aci-nitro form

The instability of the quinonoid form is shown by the rapidity with which the purple-red colours formed in ammonia vapour return to yellow in air.

The R_F values of the *p*-nitro- and *o*-nitro-phenylazo dyes are substantially similar (Table II) with the exception of 2,3-, 2,5- and 2,6-xylenols. Hence if the phenols are run as both types of dye, spots which overlap and have R_F values from 0.5 to 0.65 can be more readily characterized.

TABLE II

R_F VALUES AND IDENTIFICATION OF SIMPLE ALKYLPHENOLS (SINGLE SPOTS)

Phenol	*o-Nitrophenylazo dyes*			*p-Nitrophenylazo dyes*		
		Colours of azo dyes			*Colours of azo dyes*	
	R_F value	*Before ammonia treatment*	*After ammonia treatment*	*R_F value*	*Before ammonia treatment*	*After ammonia treatment*
Phenol	0.14	↑	Yellow-orange	0.13	Yellow	Rose
o-Cresol	0.36		Rose	0.35	Yellow	Mauve
m-Cresol	0.29		Orange	0.27	Yellow	Magenta
p-Cresol	1.00		Red-orange	1.00	Orange	Purple
o-Ethylphenol	0.61		Orange-yellow	0.56	Yellow	Mauve
m-Ethylphenol	0.49		Orange-yellow	0.47	Yellow	Magenta
p-Ethylphenol	1.00		Red-orange	1.00	Orange	Purple
2,3-Xylenol	0.57		Rose	0.43	Yellow	Lilac
2,4-Xylenol	1.00		Red-orange	1.00	Orange	Purple
2,5-Xylenol	0.64		Rose	0.49	Yellow	Lilac
2,6-Xylenol	0.90		Rose	0.73	Yellow	Pale lilac
3,4-Xylenol	1.00		Red-orange	1.00	Orange	Purple
3,5-Xylenol	0.46		Red-orange	0.41	Yellow	Red-brown
p-Chlorophenol	—			1.00	Yellow	Purple
4-Chloro-2-methyl-phenol	—	↓		1.00	Yellow	Purple

Orange-yellow

TABLE

R_F VALUES AND COLOUR REACTIONS OF MIXTURES

Phenols	*A*		*B*		*F*	
	R_F	*Colour**	*R_F*	*Colour**	*R_F*	*Colour**
Phenol	0.16	Rose			0.14	Rose
m-Cresol	0.26	Magenta			0.28	Magenta
o-Cresol	0.35	Mauve			0.38	Mauve
m-Ethylphenol	0.48	Magenta			0.55	Magenta
o-Ethylphenol	0.58	Mauve			0.61	Mauve
p-Cresol	1.00	Purple			1.00	Purple
p-Ethylphenol	1.00	Purple			1.00	Purple
3,5-Xylenol			0.43	Red-brown	0.45	Red-brown
2,3-Xylenol			0.48	Lilac	0.51	Lilac
2,5-Xylenol			0.53	Lilac	0.57	Lilac
2,6-Xylenol			0.73	Pale lilac	0.74	Pale lilac
3,4-Xylenol			1.00	Purple	1.00	Purple
2,4-Xylenol			1.00	Purple	1.00	Purple
Unidentified materials						

* In ammonia vapour.

The R_F values of the azo dye spots are increased by rise in temperature and it is advisable to run chromatograms at 20° ± 2° for best separations. The R_F values of the single spots sometimes differ from those obtained on mixtures.

e.g.:

R_F values of 2,5-xylenol *p*-nitro-phenylazo dye
in mixture of xylenols 0.53 (Fig. 1)

in mixtures of other phenols 0.57 (Fig. 1)
0.53 (Fig. 1)
0.56 (Fig. 1)
} (Table III)

Single spot 0.49 (Table II)

However, this phenol can also be identified by the colour which it forms in ammonia vapour.

ACKNOWLEDGEMENTS

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III

OF SIMPLE ALKYL PHENOLS (SEE FIG. 1)

C		*D*		*E*		*G*	
R_F	*Colour**	R_F	*Colour**	R_F	*Colour**	R_F	*Colour**
0.15	Rose	—	—	0.15	Rose	—	—
0.28	Magenta	0.27	Magenta	0.29	Magenta	0.29	Magenta
0.36	Mauve	0.33	Mauve	0.37	Mauve	0.36	Mauve
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	0.43	Red-brown	0.44	Red-brown	—	—
0.50	Lilac	0.47	Lilac	0.48	Lilac	0.49	Lilac
0.56	Lilac	0.53	Lilac	0.53	Lilac	0.53	Lilac
—	—	—	—	—	—	0.74	Lilac
—	—	—	—	—	—	—	—
1.00	Purple	1.00	Purple	1.00	Purple	1.00	Purple
Extended zone		Extended zone				0.64	Lilac
0.69–0.95	Yellow	0.58–0.95	Yellow			0.64–0.74	Yellow
						0.74–0.92 Extended zones	Yellow
0.08	Yellow	0.08	Yellow	0.08	Yellow	0.07	Yellow

SUMMARY

1. With the paper chromatographic system described, mixtures of simple alkyl phenols in the form of their *o*- or *p*-nitrophenylazo dyes can be separated into recognizable components.

2. The yellow azo dye spots undergo the nitro–aci-nitro reaction in alkaline media to give intense red/purple colours.

3. From the chromatographic behaviour of the dyes it is apparent that two types of dye are present:

(i) *ortho* coupled dyes,

(ii) *para* coupled dyes.

4. The method has been used to identify trace amounts of phenols in gasolines and kerosines, and also to ascertain the nature of the "cresyl" groupings in commercial tritolyl phosphate and trixylenyl phosphate. The method has also helped in the examination of triaryl phosphates in mineral and synthetic ester oils.

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REACTIONS OF CORTICOSTEROIDS WITH TETRAZOLIUM SALTS ON PAPER CHROMATOGRAMS

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INTRODUCTION

BUSH and coworkers[1] observed a 2.5-fold efficiency increase in the reduction of blue tetrazolium (BT) by corticosteroids on paper chromatograms by exposing the paper to one of the less polar solvent systems, in particular petrol ether. This fact, however, was attributed by BUSH to impurities of the reagent and no increase in efficiency was observed in the case of scrupulously pure BT. In our laboratory, methods for the estimation of corticosteroids using tetrazolium salts as spraying reagents have also been elaborated[2] and widely used. We were therefore very much interested in the action of non-polar solvents on the reduction of BT and some investigations have been made in this field. During this work some observations were made, leading to conclusions partially different from those reached by BUSH.

This paper reports some results of our investigations concerning the role of non-polar solvents on the reduction of BT and other tetrazolium salts and the chromatographic behaviour of formazans produced in the reaction.

EXPERIMENTAL

Corticosteroids

Authentic samples of cortisol, cortisone, corticosterone, Reichstein's compound S, and aldosterone were used for which we are indebted to CIBA A.G. (Basel), to N.V. Organon (Oss) and to the Research Institute for Medical Industry (Budapest).

Tetrazolium salts

These were prepared according to the usual methods[3]. The reagents were purified by paper chromatography in the Bush "B5" system (benzene–methanol–water, 2:1:1) and pure reagents were employed in the further investigations. The following tetrazolium salts were used:

2,3,5-Triphenyl-tetrazolium chloride (TTC); 2,3-diphenyl-5-*p*-tolyl-tetrazolium chloride; 2,2′-*p*-(di-*o*-methoxy)-diphenylene-3,3′,5,5′-tetraphenyl-ditetrazolium chlor-

ide (BT)*; 2,2′-*p*-(di-*o*-methoxy)-diphenylene-3,3′-diphenyl-5,5′-di-(*o*-nitrophenyl)-ditetrazolium chloride; 2,2′-*p*-(di-*o*-methoxy)-diphenylene-3,3′-diphenyl-5,5′-di-(*m*-nitrophenyl)-ditetrazolium chloride; 2,2′-*p*-(di-*o*-methoxy)-diphenylene-3,3′-diphenyl-5,5′-di-(*p*-nitrophenyl)-ditetrazolium chloride; 2,2′-*p*-(di-*o*-methoxy)-diphenylene-3,3′-diphenyl-5,5′-di-(*o*-hydroxyphenyl)-ditetrazolium chloride; 2,2′-*p*-(di-*o*-methoxy)-diphenylene-3,3′-diphenyl-5,5′-di-(*m*-hydroxyphenyl)-ditetrazolium chloride.

Chromatographic papers

Whatman No. 1. Whatman No. 2. Macherey-Nagel 214. Schleicher & Schüll 2043a. Schleicher & Schüll 2043b.

Solvents

All the solvents used were "pro analisi".

Method

Corticosteroids were dropped on to the chromatographic paper and run in the Bush "A" solvent system (ligroin–methanol–water; 10:8:2) for 4–12 h. The chromatograms were sprayed with the tetrazolium reagents (1 mg of the tetrazolium salt in 1 ml 5 % NaOH solution). After removing the excess of tetrazolium salt by washing with water, the papers were dried in air at room temperature. The formazans were eluted with ethyl acetate–methanol (7:3 v/v), the solution was made up to 3 ml and optical densities were measured using a Unicam SP 500 spectrophotometer.

Somewhat higher photometric values could be measured if the tetrazolium reaction was interrupted by acid, but the effect of non-polar solvent systems was the same in these cases too.

RESULTS AND DISCUSSION

Fig. 1 shows paper strips on which the formazan spots obtained by the reduction of BT with quantities of 10 μg** of cortisol (F), cortisone (E) and aldosterone (A) respectively are shown. Fig. 1a shows the untreated (control) and Fig. 1b the strips treated previously in the Bush "A" system. A significant increase in efficiency can be seen in the case of previous treatment with a non-polar solvent system in accordance with the quantitative measurements, as shown in Table I.

It is known from the literature[4] and from our own previous work, that on reduction of BT a mixture of formazan-like compounds is formed. Investigations have therefore been made on the chromatographic behaviour of the formazan produced after reaction with the reducing steroids in a non-polar solvent system. Paper strips treated as mentioned above were equilibrated with the Bush "B5" solvent system for 1 h then chromatographed for 45–120 min at 38°. Fig. 2 shows chromatograms obtained by this procedure. It can be seen that both on the untreated (a) and the treated (b) strips two main formazan fractions separate, one of them remaining at the starting point and the other moving along with the developing solvent front. Besides these two fractions a small fraction can also be observed in an intermediate position having a less definite R_F value. The quantity of formazans in these spots depends,

* Several other commercial BT samples including one from BDH were also used.

** The effect discussed here can also be observed in the case of very small quantities of steroids, but to illustrate this in the figure such high concentrations must be employed.

however, on the previous treatment. In the case of untreated paper strips, the quantity of deep blue coloured formazan remaining at the starting point is relatively small and a greater quantity appears at the solvent front which is also deep blue. In the case of the strips treated in the Bush "A" solvent system a greater quantity

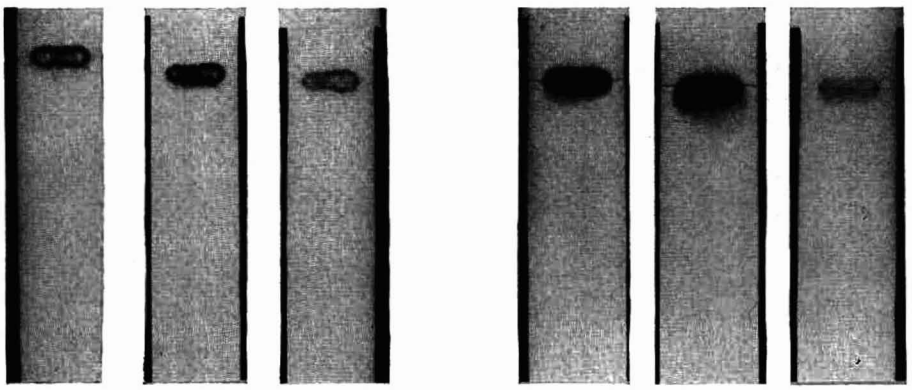

Fig. 1. Efficiency increase in the reduction of BT by treating the paper strips with a non-polar solvent system. (a) Control; (b) treated with the Bush "A" system.

of the more polar fraction remains at the starting point and a fraction of almost unchanged quantity moves along with the developing solvent front.

During investigations on the properties of formazan fractions mentioned above, it was established that the small red coloured fraction in the intermediate position could be easily removed by running the paper with 96 % alcohol just after the spray-

TABLE I

INCREASE IN EFFICIENCY IN THE REDUCTION OF BLUE TETRAZOLIUM BY TREATING THE PAPER STRIP WITH A NON-POLAR SOLVENT SYSTEM

Steroid 10 µg	*Optical densities of formazan solutions*		*Increase %*
	control	*treated*	
Cortisol	0.072	0.156	116
Cortisone	0.050	0.112	124
Aldosterone	0.056	0.112	100

ing of the chromatogram with BT and drying. The chromatogram of the formazan thus produced gave only the two deep blue coloured main fractions. The absence of the red coloured intermediate fraction could also be observed if the tetrazolium salt was dropped on to the chromatographic paper from alcoholic solution, the paper run in the Bush "B5" solvent system, sprayed with alkaline ascorbate solution and the formazan produced chromatographed again in the Bush "B5" system. Furthermore it could be observed that the quantity of the red fraction depended upon the purity

of the tetrazolium salts used. All these facts made it probable that the red coloured fraction in the intermediate position arose from impurities in the tetrazolium salts used and could be removed easily by running the paper with 96 % alcohol.

In addition to the chromatographic behaviour, the two main fractions showed

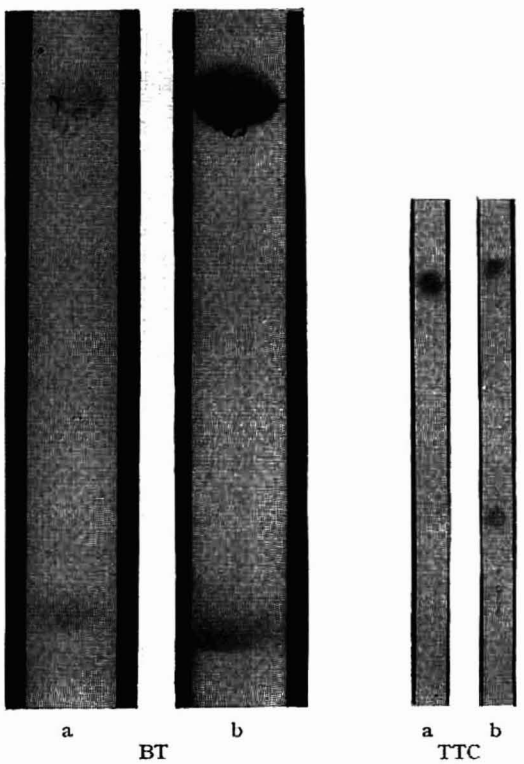

Fig. 2. Chromatograms of the formazans obtained by the reduction of BT and TTC by cortisol. (a) Control; (b) treated with the Bush "A" system.

other differences in their properties. The fraction remaining at the starting point could be dissolved rather slowly in common solvents (ethyl acetate, chloroform), while the fraction at the solvent front dissolved more readily. Absorption spectra of the two fractions were also different, as can be seen in Fig. 3. Moreover the fraction at the solvent front is more sensitive to light than the fraction remaining at the starting point.

These results made it clear that two definite and different substances are formed. The only question is whether they are derived from the same tetrazolium salt or not. To settle this problem both of these formazan fractions were oxidized to tetrazolium salts on the paper strips, then reduced with alkaline ascorbate solution or alkaline steroid solution and chromatographed in the Bush "B5" system. By this procedure, two formazan fractions, a "start" and a "front" fraction could be obtained again from both of the original fractions. This indicates that these fractions are not due to impurities in the tetrazolium salts but are obtained from pure tetra-

zolium salts under the conditions of reduction employed. Normally the so-called "front" fraction is the main product, while a non-polar solvent, such as petrol ether can change the ratio. It is noteworthy that enzymic reduction of blue tetrazolium in rat kidney homogenates *in vitro* gave only the "front" fraction.

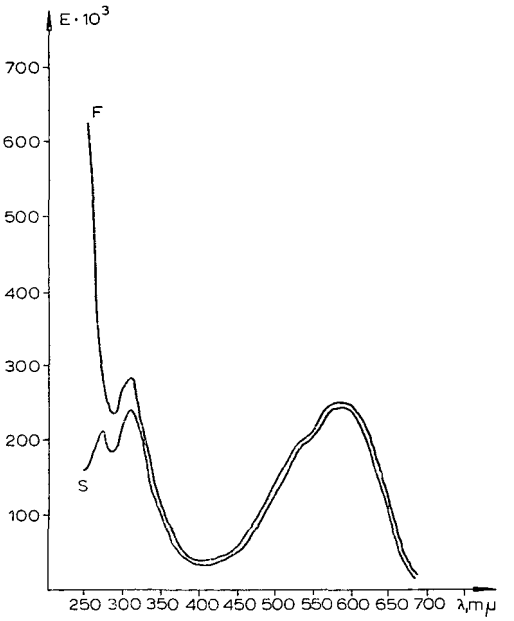

Fig. 3. Absorption spectra of the two formazan fractions in ethyl acetate–methanol (7:3 v/v) solution. S = fraction remaining at the starting point. F = fraction moving along with the solvent front.

To obtain further information, an attempt was made to prepare these different fractions in crystalline form. Paper chromatograms on a preparative scale were developed and the formazan spots eluted with ethyl acetate–methanol (7:3 v/v). After removing the solvent *in vacuo*, a few milligrams of both of the "start" and "front" fractions were obtained. During this operation it was observed that the so-called "start" fraction is unstable both in solution and in the crystalline state, and upon rechromatography in the Bush "B5" system it seemed to be transformed into the "front" substance. On standing in air the "front" substance was partially transformed into a red coloured substance which could be easily dissolved in methanol and was not identical with the red coloured impurity mentioned above. It could be reduced with alkaline ascorbate solution to a blue formazan again. These transformations, however, could not be observed on the paper strips, only in the crystalline state or in solution.

Although the products of the reduction of ditetrazolium salts under various conditions form a rather complex system, we can say that this is certainly not due to impurities. On this point our opinion is different from that of BUSH. The two main fractions could be obtained not only by the reduction of BT, but also by that of other di- and monotetrazolium salts listed in this paper. The appearance of the two

fractions could not be explained either by the presence of partially reduced tetrazolium salts[5] especially when monotetrazolium salts had been employed. It seems probable that these two fractions are isomers of the same compound, one of them (the "start" fraction) showing a great tendency to transform into the other, which can be partially oxidized in air. The absorption spectra of the two fractions (Fig. 3) seem analogous to spectra obtained by HAUSSER and coworkers[6], who examined the *"cis-trans"* isomers of various formazans. A hypothetical reduction pattern of blue tetrazolium and other ditetrazolium salts can be deduced as follows:

$$\text{BT} \underset{\text{ox.}}{\overset{\text{red.}}{\rightleftharpoons}} \text{monoformazan} \underset{\text{ox.}}{\overset{\text{red.}}{\rightleftharpoons}} \text{diformazan ("front")} \rightleftharpoons \text{diformazan ("start")}$$

SUMMARY

Reduction of blue tetrazolium and other tetrazolium salts by corticosteroids on paper chromatograms gave rise to two formazan fractions, the amounts of which depended on the conditions employed. Evidence is presented that these fractions are not due to impurities. A hypothetical scheme for the reduction of blue tetrazolium and other ditetrazolium salts is given.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

I. TANKLESS OR FLAT-BED CHROMATOGRAPHY A METHOD FOR THE ACCURATE DETERMINATION OF R_M VALUES

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INTRODUCTION

The theoretical basis for the relation between R_F values in partition chromatography and chemical structure was first proposed by CONSDEN, GORDON AND MARTIN[1] and later by MARTIN[2], who deduced that, for ideal solutions, the partition coefficient (α) of a substance A between two phases is related to the free energy required to transport one mole of A from one phase to the other, by the expression

$$\ln \alpha = \frac{\Delta\mu_A}{RT}$$

MARTIN showed that addition of a group X to the substance A should change the partition coefficient by a factor depending only on the nature of X and the two phases, but not on A itself. Hence when A is substituted by n groups X, m groups Y etc.

$$RT \ln \alpha = \Delta\mu_A + n\Delta\mu_x + m\Delta\mu_y + \ldots \text{ etc.}$$

and since

$$\alpha = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right)$$

where A_L = cross-sectional area of the mobile phase, A_S = cross-sectional area of the stationary phase,

$$RT \ln \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right) = \Delta\mu_A + n\Delta\mu_x + m\Delta\mu_y + \ldots \text{ etc.}$$

BATE-SMITH AND WESTALL[3] introduced the term

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

and showed experimentally that the relationship predicted by MARTIN was followed for a number of flavones, anthocyanins and some related compounds. However, because of the nature of the substituent groups they studied (for example, hydroxy groups), their data was necessarily restricted to a small range of compounds. Since that

time, several workers have experienced difficulties in demonstrating the experimental validity of MARTIN's theoretical postulates. BATE-SMITH AND WESTALL themselves, from their own findings, were led to question one of the main conclusions that must arise if MARTIN's equation is in fact completely valid; *i.e.*, that the R_M increment (ΔR_M) for any given substituent should be a constant, irrespective of the remainder of the molecule—always providing that other molecular interactions are absent. Thus they found that in several series of anthocyanidin glucosides, the value of ΔR_M(OH) tended to decrease as R_M itself increased and they suggested that the change in chemical potential caused by a substituent might decrease as the polar substitution increased. LEDERER[4] studied several homologous series and concluded in 1957 that sufficient data had already accumulated to show that for these different series, $\Delta R_M(CH_2)$ was in fact a constant. Nevertheless, other workers have questioned this, and HOWE[5], more recently, after a study of several series of compounds, drew two conclusions about $\Delta R_M(CH_2)$: (i) that the majority of series investigated (which included carboxylic acids, dicarboxylic acids, aminocarboxylic acids, etc.) did indeed show a close approximation to linearity, when R_M was plotted against the number of CH_2 groups, and (ii) that $\Delta R_M(CH_2)$ was not a constant in all the series (non-parallelism of slopes). HOWE was therefore led to regard MARTIN's postulates as being an approximation only and was thus prevented from recognizing the importance of certain constitutive effects. Some of these and their theoretical implications will be dealt with in the succeeding paper: this communication is mainly concerned with the technical requirements for the determination of R_F and ΔR_M.

LIMITATIONS OF CONVENTIONAL CHROMATOGRAPHY AND SOME SUGGESTED SOLUTIONS

The accurate determination of R_F values is at the very centre of all attempts to correlate chromatographic behaviour with chemical structure; and, in fact, the practical difficulty of doing this for many series of compounds is still a serious stumbling block to the rigorous examination of MARTIN's postulates. This almost certainly accounts for many of the anomalies in the literature and for at least some of the differing conclusions as to the correctness of MARTIN's postulates. It is important, first, to bear in mind that many of the R_F values reported in the literature have been obtained by workers interested chiefly in the practical aspects of separation rather than theoretical considerations and make, in fact, no pretension to be accurate reflections of chromatographic behaviour. The recent attempt, therefore, by FRANC AND JOKL[6], to use such data to demonstrate the failure of the MARTIN equation and hence of the BATE-SMITH AND WESTALL relationship is open to criticism on these grounds. BATE-SMITH AND WESTALL carefully drew up five conditions, which they considered essential if accurate and reproducible R_F values are to be obtained. These may be briefly summarized as follows: (1) the temperature must be constant, (2) the solvent mixture must be at equilibrium, (3) the paper, after spotting, must be equilibrated for 24 h in the tank, (4) a control substance must be run and, if its R_F value differs by more than 0.02 from standard, the run must be discarded and (5) development must be for at least 30–35 cm. Detailed analysis of the 37 chromatographic runs considered by FRANC AND JOKL reveals that these conditions were in fact only rarely obeyed and that much of the data can be dismissed for various technical reasons, such as the following: (i) the use of

ascending chromatography[7-12], which cannot give accurate R_F values; (ii) inclusion of R_F values outside the acceptable range[13-14]; (iii) solvent interactions—for example, chromatography of acids with solvents containing ammonia[16-18], or bases with solvents containing acid[7,19]; and (iv) pronounced lack of equilibration[20,21], commented on by the authors. Apart from these technical reasons, failure to obtain constant ΔR_M values is, we believe, in a residue of cases due to certain structural characteristics of the compounds used; particularly in the lowest members of homologous series: the significance of these will appear from the succeeding papers in this series.

If it is assumed therefore that, in the absence of recognizable steric or electronic interactions in molecules, departure from the MARTIN equation during the running of homologous series is likely to be due to failure, even under the best chromatographic runs, to obtain ideal conditions, it is essential to examine further the reasons that might account for this. Even if all five conditions adumbrated by BATE-SMITH AND WESTALL are rigorously observed, ideal equilibration can probably only rarely be obtained in a tank. Apart from the practical difficulties, there are theoretical reasons why this might be so. The vapour pressure over a curved surface is not the same as that over a flat surface and this difference is considerable for a meniscus of small radius[22]. Since filter paper consists of a network of capillaries, which may be only partially filled during the chromatographic run, the solvent in the tank cannot be in dynamic equilibrium with that in the capillaries, so there will be a passage of solvent molecules between the paper and the vapour phase in a direction that will depend on the curvature of the capillary menisci. The gross effect will be that the amount of solvent appearing to have passed over the paper will be different from the true amount and the magnitude of the discrepancy will vary according to the length of the run, temperature, types of solvent, etc. Analysis of the literature[6-21] indicates that MARTIN's formula is, even under non-ideal conditions, obeyed better when polar, especially hydroxylic, solvents are used. Such solvents, especially water and alcohols, cause swelling of the paper and close the small capillaries; they also form hydrogen bonds with cellulose, penetrate deep into the structure and consequently both condensation and evaporation are slowed down. Another source of difficulty in conventional paper chromatography is the present impossibility of adequately defining the nature of the stationary phase.

It seems clear that there are some inherent limitations in the accuracy with which R_F values can be determined by conventional paper chromatography and these are, in the main, due to failure to obtain real equilibrium between the paper and vapour phase. If this is so, the following conclusions may be drawn.

(1) It is logical to attempt to remove the vapour phase altogether. This implies removal of the tank *qua* tank—that is, an empty receptable in which papers are suspended.

(2) If the nature of the stationary phase is uncertain and leads to unknown and probably variable effects in partition coefficients, the system should be inverted and reverse-phase chromatography used. If an inert, non-volatile substance is used as stationary phase, its nature is then defined.

(3) The mobile phase can now always be chosen so that no problems concerning changes in its composition need arise. Being neutral, no interactions with acidic or basic functions need occur.

(4) Horizontal chromatography should—ideally—replace vertical chromatography.

TANKLESS CHROMATOGRAPHY

Several attempts were made to put these ideas into practice. After a number of trials, the following system, which we have called "tankless" or "flat-bed" chromatography, was adopted.

The method

Sheets of Whatman No. 1 filter paper, measuring 30 × 60 cm and cut in the same direction from larger sheets, are impregnated with the stationary phase as described below. Each sheet is spotted with the test compounds in the usual manner (about 15 cm from one edge) and then placed between two sheets of thin aluminium foil (as sold for domestic use, about 25 μ thick), carefully smoothed first to remove folds and creases. The aluminium sheets should be about 5 cm wider than the papers and rather longer. The sandwiched paper is then placed horizontally on a pad of filter papers, about 1 cm thick, resting on a flat table-top. The process is repeated with further sheets of paper, each paper being separated from the ones below and above by an aluminium sheet (Fig. 1). As many papers as required can be prepared in this way: we have successfully used up to fifty sheets in one run. When the pile is complete, each paper is sealed at both sides and at the far edge by folding the overlapping sheets of foil. The pile is then compressed evenly by placing a flexible polythene sack of sand, weighing about 50 kg, on top. It is important to distribute the weight evenly, as spot migration is affected by pressure differences. The ends of the papers that protrude from the pile are sealed at their lateral edges by folding the overlapping aluminium foil, and then dipped into a tray of solvent. The system is then rendered vapour-tight with aluminium foil as shown in Fig. 1.

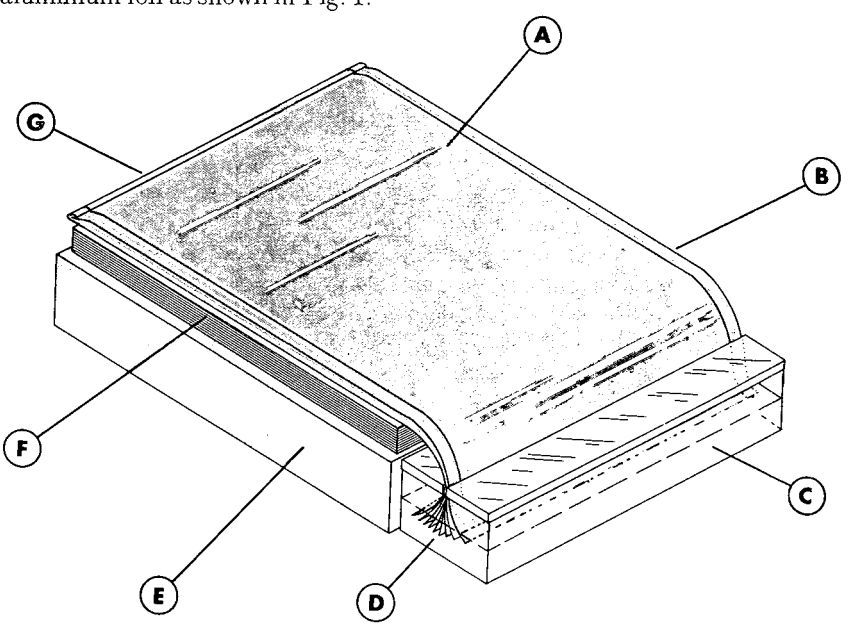

Fig. 1. Flat-bed or tankless chromatography. A. Sheets of paper alternating with aluminium foil. B. Edge of aluminium folded over. C. Solvent trough. D. Papers separated in solvent. E. Rigid support. F. Pile of filter papers. G. Ends of aluminium sheets folded over.

With the system described above and with aqueous ethanol as solvent, chromatography runs of about 40 cm take about 16 h, but the time can be varied by altering the weight used to compress the papers. It is important that sufficient compression be used to eliminate all micro-channels between the aluminium and paper sheets. If folds are present in the foil or if for some other reason contact is faulty, channelling occurs. The aluminium sheets can be used over and over again, but each time they must be carefully smoothed to remove creases and folds. Glass plates should not be used in the pile to give even contact: they are flexible enough to produce local pressure differences in the pile.

Preparation of papers

When papers are normally prepared for chromatography by impregnating them with a stationary phase, this is usually carried out by dipping them in a solution of the latter and allowing the solvent to evaporate. This method is not suitable for the determination of R_F values with the accuracy required for testing MARTIN's equation. It can easily be shown by comparing chromatographic runs on such papers in different directions across the paper that a gradient of stationary phase is produced by gravity in the direction that the paper is hung for drying. This phenomenon is normally undetectable when papers are run in the same direction, but its effect is quite sufficient to account for a severe departure of observed R_F values from true R_F values. The following technique was found suitable for the preparation of impregnated papers and should be used for the accurate determination of R_F values.

A pad of 50 sheets of chromatographic paper is immersed in a bath containing the solution of stationary phase (such as liquid paraffin) in a very light solvent such as diethyl ether. After a few minutes, the pad is removed, drained rapidly and then placed horizontally on another pad of paper. The impregnated papers are then covered with a third pad of dry papers and the pile compressed with the bag of sand. Under these conditions, the papers in the middle of the pile dry out from the edges and are quite dry from the light solvent in about an hour. They are then removed and about 2 cm of paper is cut from each edge, where there is usually a slight build-up of stationary phase. Papers prepared in this fashion have been checked several times and do not show a concentration gradient in any direction.

Advantages and disadvantages of the method

1. Tankless chromatography ensures that as many compounds as required can be run at one time under virtually identical conditions. Duplicate and triplicate papers can be used *ad lib.* throughout the pile to check running conditions and provide statistical data on R_F deviations. It is usually never possible to do this in a tank, for here R_F values vary according to the dimensions of the tank, degree of saturation, and particularly, the actual position of the papers relative to each other and the walls of the tank. In experiments to be described in succeeding papers we have sometimes obtained data on as many as 200 spots on the same day.

2. The run can be adjusted so that in the required time (usually overnight) the solvent front traverses the paper completely. Because there is no vapour and the system is sealed, there occurs at this point virtually a dead stop to chromatography; in tank chromatography this does not happen and, as the front nears the end of the paper, considerable distortion of the R_F values is produced. For accurate calculation

of R_F values, of course, the front must not be allowed to "run out", even in tankless chromatography.

3. Reproducible R_F values varying by less than 0.01 can be produced over the range 0.12–0.90.

4. It is not suggested that tankless chromatography is a substitute for conventional descending chromatography, which for practical purposes is more convenient. It is, however, the only means we have found of studying R_M values over a large range of compounds accurately enough to test MARTIN's equation exhaustively. For much work of this nature, providing accurate R_F values of key compounds have been found under tankless conditions, comparison with unknowns *of similar R_F range* can be carried out in tanks with sufficient accuracy. When this is done, two precautions are necessary. (1) R_F values must be chosen to lie between 0.20 and 0.80 under the normal descending conditions, (2) the time of development must not exceed about 5 h. It is important to bear in mind that R_F values by tank chromatography are usually somewhat different from those obtained under tankless conditions and they *cannot be directly compared with each other*: but the ΔR_M values are nearly identical under both sets of conditions, when the tank chromatography is carried out as nearly ideally as possible.

5. The use of reversed phase chromatography covers a wide range of organic compounds. There are obvious limitations, however. It is not easy to obtain data directly from compounds such as amino acids or carbohydrates, which have little organic-phase solubility. However, as will be shown in a succeeding paper, this is only a practical and not a theoretical limitation, since the required data on compounds can usually be obtained from suitable derivatives.

EXPERIMENTAL

Much detailed experimental work with the aid of the new method is described in the succeeding paper and it is sufficient here to give a single example. We have chosen for this purpose the chromatography of straight-chain alkyl 3,5-dinitrobenzoates. This is a readily available series of compounds that has been studied by several other workers. It is of particular interest since it appears as one of the worst examples from the

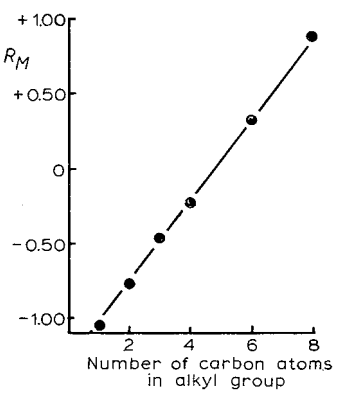

Fig. 2. Relationship between R_M and number of carbon atoms in *n*-alkyl dinitrobenzoates.

point of view of the MARTIN equation in the survey by FRANC AND JOKL[6]: the three plots of ΔR_M against number of carbon atoms given by these authors are all non-linear. Fig. 2 illustrates the chromatography of methanol, ethanol, *n*-propanol, *n*-butanol, *n*-hexanol, *n*-octanol, all as dinitrobenzoates, under tankless conditions. The stationary phase was liquid paraffin (papers impregnated with a 5 % solution of liquid paraffin, B.P., in ether); the mobile phase was 50 % aqueous ethanol. The plot of R_M against alcohol carbon number is seen to be completely linear.

SUMMARY

Attempts to correlate the chromatographic behaviour of substances with their chemical structure must be based on accurate knowledge of R_F values and a survey of published work indicates that these may often be in error because of the practical difficulties of carrying out chromatography under ideal conditions. The theoretical limitations of chromatography in tanks are also discussed. In order to overcome certain of these difficulties, a new method for the accurate determination of R_F values, tankless or flat-bed chromatography, has been studied and its advantages examined. Chief among these are its extreme reproducibility, the possibility of running very large numbers of compounds together under near-ideal conditions and the precision of the experimental R_F values. A series of alkyl dinitrobenzoates was shown, by this method, to obey MARTIN's equation with respect to $\Delta R_M(CH_2)$.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

II. THE CHROMATOGRAPHY OF PHENOLS, ALKOXYPHENOLS, COUMARANOLS AND CHROMANOLS. THE USE OF GROUP AND ATOMIC ΔR_M VALUES. STERIC AND ELECTRONIC EFFECTS IN CHROMATOGRAPHY

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INTRODUCTION

The relation between chemical structure and chromatographic behaviour has, in recent years, received the attention of several workers. Attempts to correlate structure with chromatography has so far been restricted to the study of relatively simple chemical or homologous series and they have met with varying degrees of success. In the preceding paper[1], we have discussed some of this work, and advanced reasons for believing that many of the difficulties experienced in demonstrating the validity of MARTIN's equation are largely caused by the practical difficulty of determining R_M values accurately enough over a wide enough range of compounds. It was shown that, by using reversed phase chromatography and "tankless" conditions, the experimental obstacles could probably be frequently overcome.

The conclusions of previous workers fall into two groups: some authors in general confirm MARTIN's postulates or, at any rate, use a theoretical approach based on them, whilst others differ in some respect and have criticisms of the validity of MARTIN's predictions. The first category includes OPIEŃSKA-BLAUTH, SAKŁAWSKA-SZYMONOWA AND KAŃSKI[2], who studied organic acids and concluded (incorrectly) that R_F values were additive; REICHL[3,4] and SCHAUER AND BULIRSH[5] who calculated average ΔR_M values for several substituent groups occurring in organic acids and amino acids and found them to be constant; and LEDERER[6] who has summarized a considerable body of work demonstrating the constancy of ΔR_M (CH_2) in several series of compounds. On the other hand, FRANC AND JOKL[7,8] have taken the opposite view: they consider MARTIN's equation to be invalid and have proposed a logarithmic relationship between ΔR_M and the homologous increment in homologous series. We have already criticized this point of view[1]. More recently HOWE[9], who studied 111 organic acids, including nine homologous series, was unable to find that ΔR_M was constant even for the well-studied CH_2 group. HOWE's conclusions, in view of his careful study, must be considered to illuminate the serious technical difficulties involved in the accurate determination of ΔR_M values by tank chromatography.

We were led to a study of the relation between structure and chromatographic behaviour through a series of investigations[10–16] on tocopherols, ubiquinones and ubichromenols. Some years ago GREEN AND MARCINKIEWICZ[17] posed the problem of

relating the chromatographic behaviour of a complex molecule like α-tocopherol, through a series of chromatograms, with that of a simple molecule such as phenol. In this study we have investigated the chromatography of several series of compounds necessary for the solution of this problem; these included phenols, hydroquinone mono-ethers (*p*-alkoxyphenols), fused-ring phenols, and fused-ring heterocycles such as 5-coumaranols and 6-chromanols.

CHROMATOGRAPHIC METHODS

Whatman No. 1 paper was used throughout. Sheets were impregnated with ethyl oleate by immersing them in a solution in diethyl ether and drying them by the "pile" technique as described in the preceding paper. The mobile phase was 25% aqueous ethanol. This system is referred to in this and in succeeding papers as System 1. Ethyl oleate is sufficiently polar to be suitable for the chromatography of low molecular weight phenols and ethers containing a single hydroxy function. It can be used with strengths of ethanol up to about 40% v/v, after which it becomes too soluble in the mobile phase.

Each substance was spotted as a line about 3 cm long, a technique that produces fairly narrow spots. Chromatography was, in the main, carried out under tankless conditions, but many additional and confirmatory chromatograms were run by conventional descending methods, particularly when fine *differences* in running (as opposed to accurate R_M determination) were being examined between a limited number of related compounds. The R_M value of each substance was determined from at least three separate chromatograms, often more. A run under tankless conditions might include 20–200 spots, always with internal controls. Key control substances of known R_F value were distributed on several papers at random throughout the pile, each paper normally containing a substance whose R_F had been determined in a previous run. "Edge" effects were carefully looked for, as, if for some reason a paper has been irregularly impregnated, distorted R_F values may be found near the edges of the paper. To obviate such discrepancies, no substance was run nearer than 2 cm to each lateral edge of the paper. By these means, great constancy was obtained, and any anomalous behaviour could be readily ascertained and discounted. R_F values determined on the same compound in different runs agreed to within 0.01.

Substances were visualised in two ways. Papers were pre-impregnated with zinc carbonate containing 10 p.p.m. of sodium fluorescein, according to our previously described techniques[10–17], and the substances were then observed under ultra-violet light (Hanovia "Chromatolite") as dark spots. For record purposes, papers were also sprayed with ferric chloride–dipyridyl solution or diazotised *o*-dianisidine solution, as previously described. Chromatography is virtually identical on papers with and without zinc carbonate, adsorption playing no part in this reversed phase system.

PREPARATION OF COMPOUNDS

Seventy-seven compounds were used in this study. They were chosen with specific structural features in view. Furthermore, their general chemical nature was such as to bear close relationships to the series of higher molecular weight compounds discussed in the succeeding paper of this series. A number of new compounds had to be prepared. Their preparation and properties are described below. Other compounds

listed in Table I were either obtained commercially or synthesised according to a previous literature description, which we have annotated below.

Phenols (compounds 1-24)

(i) Compounds 1–7, 9, 10, 11, 17, 19, 20, 21, 22 and 24 were obtained commercially. The Aldrich Chemical Co. lists many relatively inaccessible phenols.

(ii) The following substances were prepared by methods described in the literature: *p*-*n*-propylphenol[18], b.p. 230–232°; *p*-cyclopentylphenol[19]; *p*-cyclohexylphenol[20], m.p. 130°; 3-methyl-4-*n*-propylphenol[21]; 3-methyl-4-isopropylphenol[22], m.p. 110–111°; *p*-isopropylphenol[23], m.p. 61°; *p-tert.*-amylphenol[24], m.p. 91–92°; *p*-*n*-propenylphenol[25], m.p. 93-94°.

Fused-ring phenols (compounds 25–33)

(i) Compounds 25, 26, 28 and 29 were obtained commercially.

(ii) 1-Tetralol was prepared by reduction of 1-naphthol, m.p. 74°; ref. [26] 71°. 2-Phenanthrol was prepared according to FIESER[27], and 9-phenanthrol was obtained in good yield according to the improved method of SOLOMON AND HENNESSY[28]. 1-Anthrol and 2-anthrol were prepared according to BATTEGAY AND BRANDT[29].

Ortho-substituted phenols (compounds 34–44)

(i) Compounds 34, 35, 36, 37, 38, 43, and 44 were obtained commercially.

(ii) *o*-Propylphenol[30] was prepared by hydrogenation of *o*-allylphenol. *o*-Propenylphenol[31] had m.p. 34°. *o*-Allylphenol[32] had b.p. 219–221°. *o*-α-Methylallylphenol[33] was prepared by thermal rearrangement of phenyl crotyl ether; it had b.p. 229–235°.

Mono-ethers of hydroquinones (compounds 45–69)

(i) *General method of preparation.* Compounds 45–57 and 59-61 were prepared by Williamson synthesis. Equimolecular quantities of the alkyl bromide, the quinol and sodium ethoxide were heated under reflux in ethanol for 1–3 h. Unchanged quinol and any di-ether formed during the reaction were separated from the required mono-ether by chromatography on alumina (Peter Spence, Type O). The di-ether could always be easily eluted with benzene, and then the pure mono-ether was eluted with 5 % v/v ethanol–benzene leaving unchanged quinol on the column. Final purification was by distillation or crystallization. Any departures from the general method are described below, under the appropriate compound.

(ii) *p*-Phenoxyphenol (No. 58) cannot be prepared by Williamson synthesis and was prepared by the method of KLARMAN, GATYAS AND SHTERNOV[34]. Compounds 45, 46, 47, 48, 49, 50, 51, 53, 58, 60, 61, 62, 63, 64 and 65 have all been described previously.

(iii) The following ethers are new compounds. They all analysed correctly (results not shown) and had infra-red spectra in accordance with their structures.

p-sec.-Butoxyphenol, b.p. 158°/20 mm; n_D^{27} 1.5149; *p-tert.-butoxyphenol*, m.p. 152–154°; *p-isoamyloxyphenol*, m.p. 97–98°; *p-pent-4-enyloxyphenol*, m.p. 51–52°; *p-cyclohexyloxyphenol*, m.p. 62–63°; and *p-cyclopentyloxyphenol*, m.p. 52° were all prepared by the general method. In the case of the *tert.*-butyl ether, the reaction was carried out for 48 h at room temperature because of the tendency of the product to cleave at elevated temperatures.

2-Crotyl-4-methoxyphenol was prepared by heating the sodium salt of *p*-methoxy-

phenol and crotyl bromide in benzene for several hours. The product was distilled in a short-path still [80° (bath)/0.2 mm] as a pale yellow oil, n_D^{21} 1.5421.

4-Methoxy-2-(α-methylallyl)-phenol was prepared by heating *p*-methoxyphenol (6.2 g), crotyl bromide (8.9 g), potassium carbonate (6.9 g) and acetone (40 ml) under reflux for 3 h. The product, *p*-methoxyphenyl crotyl ether, was obtained as a colourless oil [40° (bath)/$5 \cdot 10^{-3}$ mm], m.p. 17–18°. When this ether was heated at 220° under nitrogen for 1 h, it gave, by thermal rearrangement, the required mono-ether as a colourless oil, b.p. 80°/0.2 mm.

4-Methoxy-2-propenylphenol. 2 Allyl-4-methoxyphenol (1.7 g) was heated in methanol (12 ml) with KOH (3.0 g) until the distillate temperature reached 110°. After 5 h reflux, the product was worked up and distilled in a short-path still as a colourless oil [110–120° (bath)/0.5 mm], n_D^{23} 1.5788, λ_{max} 294 mμ, $E_{1cm}^{1\%}$ 211 (in ethanol).

4-Methoxy-2-propylphenol. 2-Allyl-4-methoxyphenol was hydrogenated over palladised charcoal. The product was a colourless oil, b.p. 142°/15 mm, n_D^{20} 1.5313.

Coumaranols and chromanols (compounds 70–77)

All these, except compound 70, have been previously described[35,36].

2,2-Dimethyl-6-chromanol. p-Methoxyphenol (8.2 g), zinc chloride (1.0 g) and acetic acid (100 ml) were heated on a steam bath while isoprene (10 g) was slowly added. After 2 h, one drop of sulphuric acid was added and heating continued for 1 h. The cooled mixture was poured into water and the oil extracted with ether. Distillation gave crude 2,2-dimethyl-6-methoxychroman as a pale yellow oil (2.4 g), b.p. 140–160°/16 mm, n_D^{19} 1.5248. The oil was refluxed with hydrogen bromide in acetic acid (40 ml, 20 %) for 5 h, then evaporated. The residual oil was dissolved in ether and extracted with aqueous *N* sodium hydroxide. Acidification gave an oil, which was distilled, b.p. 85–90°/0.1 mm (1.0 g). The oil solidified and the chromanol crystallised from light petroleum as needles, m.p. 73–74°, and analysed correctly.

PAPER CHROMATOGRAPHY AND RESULTS

The chromatographic results are given in Table I. Each R_M value is the mean from at least three runs. In each case, it was calculated *directly* from the measured migration of spot and solvent front, without the intervening calculation of the R_F value, and the third figure is usually significant. The next column indicates the maximum experimental deviation in any run from the mean (in most cases it was negligibly small). R_F values are of no interest from the structural point of view, but are given in the next column because of their more practical applications. They were, however, calculated *from* the R_M values and are—as is usual—quoted to two significant figures only. The last column in Table I gives the calculated R_M values for most of the compounds. They were obtained by the methods described below.

GROUP ΔR_M PARAMETERS

The $\Delta R_M(CH_2)$ parameter

The mean value of $\Delta R_M(CH_2)$ for the homologous increment CH_2 was calculated from two series of compounds, (1) *p*-ethylphenol to *p*-*n*-amylphenol, and (2) *p*-ethoxyphenol to *p*-*n*-heptyloxyphenol. The values were respectively, + 0.462 and + 0.448, giving a mean value of + 0.455. The maximum deviation in either series was only

TABLE I

CHROMATOGRAPHY OF PHENOLS, HYDROQUINONE MONO-ETHERS, CHROMANOLS AND COUMARANOLS IN SYSTEM I

Stationary phase: ethyl oleate; mobile phase: 25 % aqueous ethanol.

No.	*Compound*	R_F	*Mean* R_M	*Max. deviation of* R_M *from mean* R_M	*Calculated* R_M
	Phenols without ortho-substituents				
1	Phenol	0.92	−1.063	0.007	
2	*m*-Cresol	0.85	−0.767	0.003	
3	*p*-Cresol	0.85	−0.767	0.003	
4	3,4-Xylenol	0.735	−0.444	0	−0.473
5	3,5-Xylenol	0.735	−0.444	0	−0.473
6	3,4,5-Trimethylphenol	0.585	−0.149	0.006	−0.178
7	*p*-Ethylphenol	0.70	−0.376	0.010	
8	*p*-*n*-Propylphenol	0.45	+0.087	0.001	
9	*p*-*n*-Butylphenol	0.22	+0.556	0	
10	*p*-*n*-Amylphenol	0.09	+1.010	0.010	
11	*p*-(3-Methylbutyl)-phenol	0.09	+1.000	0.016	+1.024
12	*p*-Cyclopentylphenol	0.16	+0.724	0	+0.776
13	*p*-Cyclohexylphenol	0.055	+1.230	0.009	+1.231
14	3-Methyl-4-*n*-propylphenol	0.285	+0.396	0.017	+0.381
15	4-Isopropyl-5-methylphenol	0.34	+0.289	0.006	+0.313
16	*p*-Isopropylphenol	0.49	+0.013	0.013	+0.018
17	*p*-*tert.*-Butylphenol	0.285	+0.395	0.008	+0.411
18	*p*-*tert.*-Amylphenol	0.12	+0.857	0.008	+0.872
19	*p*-Crotylphenol	0.32	+0.325	0.006	+0.425
20	*p*-(3-Methylbut-2-enyl)-phenol	0.16	+0.724	0.012	+0.894
21	*p*-Benzylphenol	0.23	+0.530	0.004	+0.846
22	*p*-Cyclopent-2-enylphenol	0.30	+0.369	0	+0.646
23	*p*-*n*-Propenylphenol	0.47	+0.054	0.005	+0.024
24	*p*-Phenylphenol	0.22	+0.556	0.008	
	Phenols containing a fused-ring structure				
25	2-Indanol	0.595	−0.168	0.001	−0.142
26	2-Tetralol	0.44	+0.111	0.009	+0.217
27	1-Tetralol	0.39	+0.197	0.009	+0.343
28	1-Naphthol	0.41	+0.160	0.013	+0.136
29	2-Naphthol	0.495	+0.010	0	
30	2-Phenanthrol	0.09	+1.000	0	+1.081
31	9-Phenanthrol	0.07	+1.130	0.020	+1.207
32	1-Anthrol	0.07	+1.130	0.020	+1.207
33	2-Anthrol	0.09	+1.000	0	+1.081
	Phenols with ortho-substituents				
34	*o*-Cresol	0.78	−0.547	0.002	−0.641
35	2,3-Xylenol	0.64	−0.250	0.002	−0.347
36	2,4-Xylenol	0.64	−0.250	0.002	−0.347
37	2,5-Xylenol	0.64	−0.250	0.002	−0.347
38	2,6-Xylenol	0.63	−0.240	0.003	−0.221
39	*o*-Propylphenol	0.36	+0.245	0.003	+0.213
40	*o*-Propenylphenol	0.43	+0.136	0.006	+0.151
41	*o*-Allylphenol	0.53	−0.023	0	+0.082
42	*o*-α-Methylallylphenol	0.26	+0.458	0.004	+0.551
43	2,3,5-Trimethylphenol	0.44	+0.111	0.004	−0.052
44	2-Isopropyl-5-methylphenol	0.24	+0.495	0	+0.439

(continued on p. 47)

TABLE I (*continued*)

No.	Compound	R_F	Mean R_M	Max. deviation of R_M from mean R_M	Calculated R_M
	Mono-ethers of hydroquinones without ortho-substituents				
45	*p*-Methoxyphenol	0.92	−1.070	0	
46	*p*-Ethoxyphenol	0.88	−0.842	0.020	−0.911
47	*p*-*n*-Propoxyphenol	0.73	−0.420	0	−0.456
48	*p*-*n*-Butoxyphenol	0.50	−0.001	0.003	
49	*p*-*n*-Hexyloxyphenol	0.105	+0.933	0.011	+0.909
50	*p*-*n*-Heptyloxyphenol	0.04	+1.400	0	+1.364
51	*p*-Isopropoxyphenol	0.83	−0.688	0.020	
52	*p*-Isoamyloxyphenol	0.24	+0.501	0	+0.454
53	*p*-Allyloxyphenol	0.80	−0.602	0	−0.648
54	*p*-Pent-4-enyloxyphenol	0.35	+0.269	0.019	+0.262
55	*p*-Cyclohexyloxyphenol	0.35	+0.266	0.011	+0.485
56	*p*-*tert.*-Butoxyphenol	0.80	−0.602	0	
57	*p*-*sec.*-Butoxyphenol	0.64	−0.241	0.008	−0.233
58	*p*-Phenoxyphenol	0.33	+0.311	0.016	
59	*p*-Cyclopentyloxyphenol	0.55	−0.080	0.004	+0.031
60	*p*-Benzyloxyphenol	0.40	+0.185	0.007	+0.002
61	4-Methoxy-5-methylphenol	0.85	−0.772	0.003	
	Mono-ethers of hydroquinones with ortho-substituents				
62	2,3-Dimethyl-4-methoxyphenol	0.71	−0.381	0.001	−0.354
63	2,5-Dimethyl-4-methoxyphenol	0.71	−0.381	0.001	−0.354
64	4-Methoxy-2-methylphenol	0.835	−0.710	0	−0.649
65	2-Allyl-4-methoxyphenol	0.635	−0.236	0.014	−0.075
66	2-Crotyl-4-methoxyphenol	0.405	+0.167	0.026	+0.544
67	4-Methoxy-2-(α-methylallyl)-phenol	0.42	+0.137	0.006	+0.468
68	4-Methoxy-2-propenylphenol	0.48	+0.031	0.001	+0.143
69	4-Methoxy-2-propylphenol	0.50	+0.003	0.007	0.205
	Chromanols and coumaranols				
70	2,2-Dimethyl-6-chromanol	0.78	−0.547	0.002	−0.513
71	2,5,8-Trimethyl-6-chromanol	0.36	+0.245	0.004	+0.103
72	2,5,7,8-Tetramethyl-6-chromanol	0.225	+0.534	0.004	+0.524
73	2,2,5,7,8-Pentamethyl-6-chromanol	0.175	+0.676	0.002	+0.624
74	4-Methyl-5-coumaranol	0.84	−0.730	0.012	−0.856
75	6-Methyl-5-coumaranol	0.84	−0.730	0.012	−0.856
76	2,4,7-Trimethyl-5-coumaranol	0.67	−0.296	0.009	−0.332
77	2,4,6,7-Tetramethyl-5-coumaranol	0.51	−0.015	0.015	+0.089

0.032. Thus $\Delta R_M(CH_2)$ is constant, in agreement with the findings of LEDERER[6].

It must be stressed that $\Delta R_M(CH_2)$ is constant only if each successive CH_2 group is added sufficiently far removed from any functional group as to be regarded as free of any constitutive interaction with it (such an interaction could be steric or electronic in character or could involve an increased measure of internal hydrogen bonding). If this requirement is neglected, certain compounds may then appear to have "anomalous" R_F values—especially the first (and sometimes second) member of homologous series[6–9]. These "anomalous" R_F values are *real* deviations from MARTIN's equation. They are not to be confused with the experimental deviations that are also especially likely when first and second members of a series are run in the same system

as higher members. Under such conditions their R_F values are often either too high or too low to be determined accurately, and can be distorted by non-equilibrium. Even under ideal conditions, however, the R_M value of the first (and sometimes second) member of a series may be "anomalous" because in these compounds constitutive interaction of the first CH_2 group with the functional group exists. The chromatographic deviations are consistent with the fact that in a homologous series the first member is structurally unique and homology does not strictly begin until the second member of the series is reached. Whether the R_F "anomaly" of the first member is large enough to be observable would appear to depend on the nature of the series and of the system. Thus, although it is clear from the summary of FRANC AND JOKL[7] that some workers found a first member "anomaly" in the series of alkyl dinitrobenzoates, we did not do so, using a reversed phase system[1]. LEDERER[6] also shows that the first member "anomaly" can exist in some series but not in others. It is most frequently observed in series of organic acids[6,7]. HOWE[9], for example, found benzoic acid to be "anomalous" in a series of ω-phenyl-substituted monocarboxylic acids. HOWE[9], LONG, QUAYLE AND STEDMAN[37] and SEHER[38] all observed oxalic acid to be anomalous in series of dicarboxylic acids.

The ΔR_M(ring-attached CH_2) parameter

It will have been noticed that, for the calculation of $\Delta R_M(CH_2)$, the lowest member of the phenol series used was the third member, *p*-ethylphenol. It is clear that in the second member, *p*-cresol, the CH_2 group, being directly attached to an aromatic ring and under its electronic influence, is constitutively different from a homologous CH_2 group. In accordance with the views already expressed, *p*-cresol can be regarded as a special case of a lower member "anomaly". It is not unexpected therefore to find that ΔR_M(ring-attached CH_2) is different from $\Delta R_M(CH_2)$ itself. FRANC AND JOKL[8] studied several substituted phenols and observed such a difference. However, they were unable to draw the correct conclusions from this as they did not take into account the *steric* effects of substitution in their compounds.

ΔR_M(ring-attached CH_2) has been calculated from the data in Table I by comparing phenol with its mono-, di- and tri-methylated derivatives, but restricting the calculations to those phenols that do not contain substituents *ortho* to the hydroxy group (see discussion on the *ortho*-effect below). These were *m*- and *p*-cresol, 3,4-xylenol, 3,5-xylenol and 3,4,5-trimethylphenol. ΔR_M(ring-attached CH_2) was found to be $+ 0.305 \pm 0.018$, significantly different from the value of $\Delta R_M(CH_2)$.

The $\Delta R_M(OCH_3)$ parameter

The increment in R_M produced by the addition of an OCH_3 group to an aromatic ring can be derived from the R_M values of phenol and *p*-methoxyphenol. Its value in System 1 is -0.007. This parameter is compounded of two opposing effects, one due to the oxygen atom, the other due to the alkyl group. It is analysed further by the method of atomic parameters (see below).

The ortho-effect

If two substituent groups in a molecule are close to each other there may be an interaction between them. Such effects are familiar in several fields. They not only influence chemical reactivity by affecting the stability of the transition state, but they

can affect a number of the physical constants of substances. Since group interaction can affect both the molar volume and the cohesion energy of a molecule, it is therefore to be expected that it will affect R_M (this follows from DIKSTEIN's[39] thermodynamic derivation of R_F). We have only studied one such effect, the *ortho*-effect in phenols. If an alkyl group is substituted *ortho* to the OH group, some screening of the functional group occurs. This might be purely spatial (so-called steric hindrance), but, as in other aspects of organic chemistry, it is not always possible to distinguish the strictly steric nature of the screening from the electronic effects (if any) of the substituent on the functional group. Thus, when both groups are *ortho* to each other on an aromatic ring, interactions might include polar effects ranging from small inductive effects to permanent polarizations and major intramolecular disturbances could arise through the possibilities of internal hydrogen bonding or even tautomerism. Although in the series of compounds studied here (where the *ortho*-effect is limited to that produced by simple alkyl groups) polar effects can probably be regarded as minimal, the complex nature of the origin of the effect makes it hardly likely that ΔR_M (*ortho*-effect) would be constant. Its magnitude will depend not only on the size and electronic character of the alkyl group, but, as ROUBALOVA's work on aurones has shown[40], will be determined by the total structure of the molecule in which it occurs. It follows that R_M(*ortho*-effect)—even for the same *ortho*-group—will not be constant, and, at present, we have only found it possible to treat the *ortho*-effect empirically.

ΔR_M(*ortho*-effect)—that is, the *additional* R_M increment produced by an *o*-alkyl group over and above the normal ΔR_M value of the same alkyl group when substituted elsewhere in the molecule—was calculated from three pairs of phenols whose R_M values are given in Table I. From *o*- and *m*-cresol the value is + 0.220; from 2,3-xylenol and 3,4-xylenol, + 0.194; from *o*- and *p*-propylphenol, + 0.158. The mean value is thus + 0.161, about a third of $\Delta R_M(CH_2)$. When two *ortho*-groups are present in the same compound, as in 2,6-xylenol (Table I), it appears that the effect of the second group may be slightly less than that of the first. (Because of the large variation in the magnitude of ΔR_M (*ortho*-effect), however, data from one compound may be misleading. In all subsequent calculations of R_M values (see below) we have arbitrarily taken the effect of two *ortho*-groups as twice that of one. Any error involved is, in any case, small.)

In the hydroquinone mono-ether series, ΔR_M(*ortho*-effect) is different again. From a comparison of *p*-methoxy-2-methylphenol and *p*-methoxy-5-methylphenol, it is found to be only + 0.062. This may be due to some electronic interaction between the *p*-methoxy and the hydroxy group altering the steric effect of the methyl group, and is in accord with ROUBALOVA's work[40]. The mean value of ΔR_M(*ortho*-effect) in all compounds listed in Table I is + 0.126 and this has been used (see below) in all the calculations in which it occurs.

The ΔR_M parameter for ether oxygen

The two main series of compounds in Table I, phenols and hydroquinone mono-ethers, are related to each other by the introduction of a *p*-alkoxy group into the phenolic nucleus. The value of ΔR_M(O) due to the introduction of the new oxygen atom can be calculated in the usual way by comparing the R_M values of a series of *p*-*n*-alkylphenols with those of the corresponding *p*-*n*-alkoxyphenols. If this is done, the value is in fact found to be fairly constant between the ethyl and butyl compounds, being

— 0.510 ± 0.047. However, closer inspection reveals that there is a steady variation in the value of ΔR_M(O) over this range of compounds, the actual values being — 0.557, — 0.507 and — 0.466 for the pairs of butyl, propyl and ethyl compounds respectively. Whereas, if the R_M values of the pairs of amyl, hexyl and heptyl compounds (which can be easily found by extrapolation) are compared, it is found that over the range butyl to heptyl, ΔR_M(O) is much more constant at + 0.541 ± 0.023. If, on the other hand, *p*-methoxyphenol is compared with *p*-cresol, ΔR_M(O) is found to be only — 0.303, significantly different from any of the above values. The variation in ΔR_M(O) is not restricted to primary ethers. If the value of ΔR_M(O) is calculated for a typical secondary ether by comparing the R_M values of *p*-isopropoxyphenol and *p*-isopropylphenol, it is found to be — 0.801. The value of ΔR_M(O) is thus profoundly affected by the nature of the alkyl substituent attached to the oxygen atom. This phenomenon is dealt with in more detail below, in the discussion on atomic ΔR_M parameters.

The ΔR_M(double bond) parameter

The presence of a double bond in a molecule always (with the one exception to be noted later) increases its R_F value in reversed phase systems such as System 1. This agrees with the observation that in direct phase systems, unsaturation usually leads to a decrease in R_F. Thus SUNDT AND WINTER[41] observed that hexen-1-al migrated more slowly than hexan-1-al in dimethylformamide–decalin. We have shown else-

TABLE II

ΔR_M(DOUBLE BOND) VALUES CALCULATED FROM EIGHT PAIRS OF COMPOUNDS CHROMATOGRAPHED IN SYSTEM 1

For R_M data, see Table I.

No.	*Compound*	*ΔR_M(double bond)*
9	*p*-*n*-Butylphenol	—0.231
19	*p*-*n*-Crotylphenol	
11	*p*-(3-Methylbutyl)-phenol	—0.276
20	*p*-(3-Methylbut-2-enyl)-phenol	
12	*p*-Cyclopentylphenol	—0.355
22	*p*-Cyclopent-2-enylphenol	
8	*p*-*n*-Propylphenol	—0.033
23	*p*-*n*-Propenylphenol	
39	*o*-Propylphenol	—0.109
40	*o*-Propenylphenol	
39	*o*-Propylphenol	—0.268
41	*o*-Allylphenol	
47	*p*-*n*-Propoxyphenol	—0.182
53	*p*-Allyloxyphenol	
*	*p*-*n*-Pentyloxyphenol	—0.198
54	*p*-Pent-4-enyloxyphenol	

* This compound was not run in System 1, but its R_M value can be calculated with a high degree of accuracy from the mean of the R_M values for *p*-*n*-butoxyphenol and *p*-*n*-hexyloxyphenol.

where[42] that reversed phase systems are especially suitable for measuring olefinic unsaturation.

If ΔR_M(double bond) were a constant chromatographic parameter in System 1, it could be calculated in the usual way by comparing pairs of compounds differing in their structures only by one double bond. This has been done for eight such pairs of compounds and the results are shown in Table II. It will be observed that, for the six pairs of substituted phenols, the value is not constant. This is perhaps to be expected. The "constitutive" surroundings of the double bond in these compounds with respect to the aromatic ring vary widely and there is thus no reason to suppose that the double bond will behave as a regular "additive group". In the hydroquinone half-ether series, however, which is represented by two pairs of compounds (*p*-allyloxyphenol and *p*-propoxyphenol; *p*-*n*-pentyloxyphenol and *p*-pent-4-enyloxyphenol) where the double bond is separated from the neighbourhood of the aromatic ring by an oxygen atom, the agreement is good, and it will be shown later that the calculated R_M values for these compounds agree well with the experimental values. Table II suggests that the variation in ΔR_M(double bond) may be due to interaction of the double bond with the aromatic ring. Thus if ΔR_M(double bond) calculated from the two pairs of *p*-alkoxyphenols is taken as — 0.190, then the value is clearly less for the two propenyl compounds and more for the four allyl-type compounds, being particularly high in the case of *p*-cyclopent-2-enylphenol, which is a cyclic allyl compound. The nature of these variations and an explanation of their origin is discussed below.

ATOMIC ΔR_M PARAMETERS

MARTIN's equation can only be applied to every group and atom in a molecule if all their constitutive relationships are considered. The correlation of R_M values of compounds varying in a more complex way than do members of a homologous series must therefore involve an analysis of these relationships. It is the complexity of this task, even in relatively simple compounds, that prevents the universal application of MARTIN's equation to problems of structure. For example, the R_M value of *p*-cyclopentylphenol cannot be calculated with any degree of accuracy from that of the straight-chain *p*-*n*-amylphenol, or the R_M values of the fused-ring structures, 2-indanol and 2-tetralol, from the analogous *p*-*n*-propylphenol and *p*-*n*-butylphenol, by simply using the value for $\Delta R_M(CH_2)$. In addition, it is essential to know the ΔR_M increments for CH groups and quaternary C atoms and something about the additive properties of such groups when they occur in rings. From the data in Table I it would be possible, as a first approximation, to devise a correction parameter for these ring compounds, as follows: ΔR_M(saturated ring) = — 0.305, but the maximum deviation of such a correction is large, about ± 0.140. In any case, the use of a correction does not solve the problem, which arises anew with every series of compounds. Thus, the R_M values of compounds containing fused aromatic rings—naphthols, phenanthrols and anthrols —cannot be calculated from phenol by the addition of increments for CH_2 even if a ring correction factor is used: for the R_M value of 2-naphthol (+ 0.010) is much less in System 1 than that of the analogous C_{10} compound, *p*-*n*-butylphenol (+ 0.556); and the R_M value of 9-phenanthrol (+ 1.130) departs even more from the calculated R_M value (+ 2.372) for *p*-*n*-octylphenol.

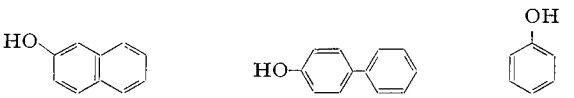
The difference between $\Delta R_M(CH_2)$ and ΔR_M(ring-attached CH_2), which has al-

ready been discussed, provides another simple but important example. In System 1, this difference is appreciable, amounting to about one-third of $\Delta R_M(CH_2)$ itself. When more than one ring-attached CH_2 group is present the effect is multiplied accordingly and may in fact lead to a clear-cut chromatographic separation between isomers. Thus, 3,4,5-trimethylphenol and *p*-*n*-propylphenol are readily separated in System 1, where their R_M values differ by 0.236. Indeed, the difference between the ΔR_M increments produced by constitutively different CH_2 groups is not confined to the ring-attached CH_2 group alone. As is shown below the difference extends, on a diminishing scale, to CH_2 groups α and β to the aromatic system as well. This is why FRANC AND JOKL[8] were able to observe a significant difference in the R_F values of 3,4-dimethylphenol and *p*-ethylphenol. (They were prevented from observing the larger difference between a trimethylphenol and *p*-propylphenol by the fact that their compounds were *ortho*-substituted. Since ΔR_M(*ortho*-effect) may be approximately equal in magnitude and opposite in direction to the difference between $\Delta R_M(CH_2)$ and ΔR_M(ring-attached CH_2) in this series of compounds, this is to be expected.)

All these differences derive from the differing constitutive relationships of the carbon and hydrogen atoms in these compounds, compared to those existing in the homologous series. The method of atomic ΔR_M parameters now to be discussed provides a way of dealing with such constitutive effects in molecules, and its use resolves many of the problems inherent in the use of group ΔR_M values. It consists, in principle, of extending MARTIN's equation by considering carbon and hydrogen atoms separately and assigning to each a ΔR_M value that is determined by both the normal "additive" quantity and also by a "constitutive" quantity depending on the structural or constitutive relation of the atom to the rest of the molecule. Although the experimental determination of such ΔR_M values for carbon and hydrogen is theoretically possible, in practice it would involve the greatest difficulties, both with regard to the amount of chromatographic data required and also the tedious mathematical treatment that would be necessary. Thus, in all but the simplest molecules, there would be many types of carbon and hydrogen atom all with different "constitutive" relationships and hence all necessitating the assignment of different ΔR_M increments. This otherwise complex treatment can be simplified by the use of the following mathematical device. In this treatment, all carbon atoms are assigned the *same* ΔR_M value, whatever their position or structural relationship in a molecule, *and all other variations in the* ΔR_M *values of groups containing only carbon and hydrogen are considered to be due solely to variations in the* ΔR_M *contributions of structurally different hydrogen atoms*. This reduces the experimental requirements at once. Consider, for example, an alkylated phenol, such as one given in Table I. All the carbon atoms are assumed to have one ΔR_M value, while all the hydrogen atoms—regardless of whether they are in side chains, the aromatic nucleus, fused ring or in ether groupings—can be considered as being α, β, γ, etc. to the aromatic nucleus. The experimental problem is reduced to determining the ΔR_M values for these different types of hydrogen atoms. If this is done, the atomic ΔR_M values can be used in MARTIN's equation. (This is theoretically desirable in any case. The R_F value of a substance is partly related to its molecular volume and the latter is in principle determined by the sum of independent carbon and hydrogen contributions. The fact that, for the reasons outlined we have included the unknown ΔR_M(carbon) variation into the experimental values for ΔR_M(H) in no way invalidates this.)

The calculation of ΔR_M values for carbon and hydrogen

It is first necessary to calculate the "constant" ΔR_M parameter for the carbon atom. This is done by the following process, in which the structures of 2-naphthol (I), *p*-phenylphenol (II) and phenol (III) are compared and their R_M values correlated.



(I) (II) (III)

2-Naphthol contains seven CH groups and three quaternary C atoms. *p*-Phenylphenol, on the other hand, contains nine CH groups and three quaternary C atoms. The R_M difference between the two compounds, 0.546, can thus be considered to be formally due to a difference in two CH groups. Therefore, ΔR_M(CH) is + 0.273. (This treatment ignores any differences of bond order, resonance energy or other manifestations of "aromaticity" between the two compounds (see later).) Phenol and 2-naphthol differ formally by two CH groups plus two quaternary carbon atoms, and they differ in R_M by 1.073.

$$2 \times \Delta R_M(\mathrm{C}) = R_M(\text{naphthol}) - R_M(\text{phenol}) - 2 \times \Delta R_M(\mathrm{CH})$$

Then,

$$\Delta R_M(\mathrm{C}) = + 0.263$$

The next stage is to calculate the various ΔR_M values for hydrogen, as follows:

(*i*) ΔR_M(*aromatic H*).

$$\Delta R_M(\text{aromatic H}) = \Delta R_M(\mathrm{CH}) - \Delta R_M(\mathrm{C}) = + 0.010$$

(*ii*) ΔR_M(*α-hydrogen*). The R_M values of phenol and cresol are compared.

$$R_M(\text{cresol}) - R_M(\text{phenol}) = \Delta R_M(\mathrm{C}) + 3 \times \Delta R_M(\alpha\text{-hydrogen}) - \Delta R_M(\text{aromatic H})$$

Then,

$$\Delta R_M(\alpha\text{-hydrogen}) = + 0.014$$

(*iii*) ΔR_M(*β-hydrogen*). Similarly,

$$R_M(p\text{-ethylphenol}) = R_M(\text{phenol}) - \Delta R_M(\text{aromatic H}) + 2 \times \Delta R_M(\mathrm{C}) + 2 \times \Delta R_M(\alpha\text{-hydrogen}) + 3 \times \Delta R_M(\beta\text{-hydrogen})$$

Then,

$$\Delta R_M(\beta\text{-hydrogen}) = + 0.048$$

By analogous methods, the ΔR_M parameters for γ-, δ-, and ε-hydrogen atoms can be calculated by comparing the higher alkylated phenols with phenol itself. Their values are + 0.082, + 0.096 and + 0.096 respectively.

The ΔR_M(H) values thus increase with the distance of the atom from the ring up to the δ-hydrogen, after which they remain constant.

Atomic ΔR_M parameters for oxygen

It has already been shown that, if the "group" ΔR_M parameter for oxygen is calculated from a comparison of alkylphenols with corresponding alkoxyphenols, the

value varies markedly depending on the pair of substances chosen. As a result, if an attempt is made to calculate, say, the R_M values of alkoxyphenols from the chromatographic data obtained from the alkylphenols, MARTIN's equation cannot be used because the "group" ΔR_M parameter is not constant. This situation can now be considered afresh.

(i) ΔR_M*(O in* OCH_2R*)*. Consider first the comparison of straight-chain primary alkylphenols with straight-chain primary alkoxyphenols. In *p-n*-propylphenol, there are two α-hydrogens, two β-hydrogens and three γ-hydrogens, and these have different ΔR_M values. It is not known, however, whether the same values should apply to the corresponding hydrogen atoms of the alkoxy group in *p-n*-propoxyphenol. Since these hydrogen atoms are no longer influenced electronically by the aromatic ring, it is probable that the variation in ΔR_M no longer applies. In any case, since the value for ΔR_M(O) is affected so much by substitution at the oxygen atom, variations in the value for ΔR_M(H) are relatively insignificant. We therefore assume that in the case of hydrogen atoms in the alkoxy radical, ΔR_M(H) is constant and equal to + 0.096, the same value as for hydrogen atoms remote from the aromatic ring in alkylphenols. The value of ΔR_M(O in OCH_2R) can now be calculated as follows, choosing *p*-butoxyphenol as a suitable compound.

$$R_M(p\text{-butoxyphenol}) = R_M(\text{phenol}) - \Delta R_M(\text{aromatic H}) + 4 \times \Delta R_M(\text{C}) + 9 \times \Delta R_M(\text{H}) + \Delta R_M(\text{O in } OCH_2R)$$

Then

$$\Delta R_M(\text{O in } OCH_2R) = -0.844$$

If similar calculations are made for the whole series of alkoxyphenols from the *p*-propoxy to the *p*-heptyloxy compound, the value of this parameter is found to be virtually constant. If, however, in an exactly similar manner, ΔR_M(O in OCH_2R) is calculated from a comparison of *p*-ethoxyphenol and phenol, it is already somewhat different and has a value of only — 0.775. As will be seen from study of the branched ethers, ΔR_M(O) is profoundly influenced by vicinal branching. The ethyl group itself can be considered as a limiting case of "branching" at the carbon atom attached to oxygen, for this carbon is uniquely substituted by two hydrogen atoms and one CH_3 group. It thus provides another example of a "lower member" anomaly and one that is extraordinarily sensitive to constitutive effects. Subsequent CH_2 group addition in the alkoxy radical no longer affects the α-carbon atom and ΔR_M(O) is constant for all higher alkoxy groups.

It will be observed that the value of ΔR_M(O in OCH_2R) found by the method of atomic parameters differs considerably from the previously-calculated "group" parameter for OCH_2R, —0.557. This is, of course, simply due to the fact that the "group" parameter is calculated from a direct comparison of an alkylphenol and an alkoxyphenol and therefore integrates all the variations of ΔR_M(H) in the R_M value of the former. The atomic ΔR_M(O) parameter is calculated *ab initio* from phenol and does not include variable ΔR_M parameters for hydrogen atoms in the alkoxy group: if these exist they are included in the atomic ΔR_M(O) parameter. It follows that, in calculating the R_M values of compounds containing ether groupings from R_M(phenol), the atomic ΔR_M parameter must be used, as shown below.

(ii) ΔR_M*(O in* OCH_3*)*. It has already been shown that the "group" ΔR_M value for OCH_3 is considerably less negative than that of other alkoxy groups. This difference

can be considered as arising from the unique nature of the substitution at the carbon atom attached to oxygen in a methyl ether. This carbon atom bears three hydrogen atoms, whereas in other primary ethers it is attached to only two. The atomic ΔR_M parameter for oxygen in OCH_3 is calculated as follows:

$$R_M(p\text{-methoxyphenol}) = R_M(\text{phenol}) - \Delta R_M(\text{aromatic H}) + \Delta R_M(\text{C}) + 3 \times \Delta R_M(\text{H}) + R_M(\text{O in } OCH_3)$$

Then,

$$\Delta R_M(\text{O in } OCH_3) = -0.558$$

(iii) $\Delta R_M(O\ in\ OCHR_2)$. Since the value of ΔR_M(O) in primary ethers is so markedly affected by changes in the substitution at the carbon atom attached to oxygen, it is not surprising that its value should prove to be different for secondary ethers. Table I shows this is so. *p*-Isopropoxyphenol runs faster than *p*-*n*-propoxyphenol and *p*-*sec*.-butoxyphenol faster than *p*-*n*-butoxyphenol. Therefore, a new atomic ΔR_M(O) parameter must be calculated for secondary ethers. By similar methods to those already shown, comparing R_M(*p*-isopropoxyphenol) with R_M(phenol), ΔR_M(O in $OCHR_2$) is found to be — 1.076.

(iv) $\Delta R_M(O\ in\ OCR_3)$. Table I shows that *p*-*tert*.-butoxyphenol runs much faster than *p*-*n*-butoxyphenol. Indeed, the former compound runs almost as fast as *p*-ethoxyphenol. This indicates that the atomic ΔR_M parameter for oxygen in tertiary ethers must be even more negative than in any other type of ether. Comparison of R_M (*p*-*tert*.-butoxyphenol) with R_M(phenol) and calculating as above gives ΔR_M(O in OCR_3) as — 1.445.

(v) $\Delta R_M(O\ in\ OPh)$. Replacement of the alkyl group in alkoxyphenols by an aromatic radical can also be expected to introduce a pronounced new constitutive effect on the value of ΔR_M(O). This is so. As Table I shows, *p*-phenoxyphenol runs much slower than *p*-hexyloxyphenol, even though it contains fewer (and only aromatic) hydrogen atoms. This implies that ΔR_M(O in OPh)must be much more positive than ΔR_M(O in OCH_2R). (In the calculation of the former parameter, the five hydrogen atoms in the phenoxy group are given their aromatic ΔR_M values of + 0.010, since they are themselves part of an aromatic system.) Thus,

$$R_M(\text{O in OPh}) = R_M(p\text{-phenoxyphenol}) - R_M(\text{phenol}) - 6 \times R_M(\text{C}) - 4 \times \Delta R_M(\text{aromatic H}) = -0.244$$

CALCULATION OF R_M VALUES

Table III summarizes the values of the atomic ΔR_M parameters for carbon, hydrogen and oxygen, which have been obtained by the methods described above. By using these values, it is now possible to calculate the R_M values of the compounds in Table I from the R_M value of phenol. The calculated values are shown in Table I. The same atomic ΔR_M parameters were used, irrespective of whether the atoms concerned were in a ring or a chain. (Calculated values are not given for those compounds that were used for the derivation of the various ΔR_M parameters.)

Phenols without ortho-substituents

In this group (compounds 1–24) the agreement between calculated and experimental

TABLE III

ΔR_M PARAMETERS FOR CARBON, HYDROGEN AND OXYGEN IN SYSTEM I

Substituent	ΔR_M
C	+0.263
Aromatic hydrogen	+0.010
α-Hydrogen	+0.014
β-Hydrogen	+0.048
γ-Hydrogen	+0.084
δ-Hydrogen	+0.096
ε-Hydrogen	+0.096
O in OCH_2R	—0.844
O in $OCHR_2$	—1.076
O in OCR_3	—1.445
O in OCH_3	—0.558
O in OPh	—0.242

R_M values is good. A typical calculation is for *p*-(3-methylbutyl)-phenol (Table IV).

Only four compounds in this group have an R_M value that differs from the calculated value by more than $\pm$ 0.052. The discrepancies are thus less than can be attributed to one-fifth of a carbon atom. The four compounds (19–22) that show larger discrepancies are *p*-crotyl, *p*-(3-methylbut-2-enyl)-, *p*-benzyl- and *p*-cyclopent-2-enylphenol. These discrepancies can be attributed to the special structures of these substances, which are all "allyl"-type compounds. The nature and origin of the "allyl" effect is discussed below. It may be noted here, however, that the R_M value of *p*-propenylphenol, in which the side-chain double bond is conjugated with the ring, is in excellent agreement with the calculated value, and *p*-cyclopentylphenol, which has a saturated cyclic side-chain, also shows no R_M anomaly. Attention is drawn to the calculation for the ring-containing phenol, *p*-cyclohexylphenol. In this molecule, there is only *one* α-hydrogen, four β-hydrogens, four γ-hydrogens and two δ-hydrogens. The calculated R_M value is in excellent agreement with the experimental value.

TABLE IV

CALCULATION OF R_M FOR *p*-(3-METHYLBUTYL)-PHENOL

Constituent	*Increment*	
	+	—
R_M(phenol)		1.063
$-\Delta R_M$(aromatic H)		0.010
$+ 5 \times \Delta R_M$(C)	1.315	
$+ 2 \times \Delta R_M$(α-hydrogen)	0.028	
$+ 2 \times \Delta R_M$(β-hydrogen)	0.096	
$+ \Delta R_M$(γ-hydrogen)	0.082	
$+ 6 \times \Delta R_M$(δ-hydrogen)	0.576	
Sum of R_M increments	2.097	1.073

Calculated R_M = + 1.024
Experimental R_M = + 1.000

Phenols containing fused rings

The calculated R_M values for these compounds (25–33) are in good agreement with the experimental values. The two tetralols run a little faster than required by theory (by about one-third to one-half of a carbon atom), although 2-indanol runs correctly.

TABLE V

CALCULATION OF R_M FOR *p*-PENT-4-ENYLOXYPHENOL

Constituent	*Increment*	
	+	—
R_M(phenol)		1.063
+ 9 × ΔR_M(hydrogen)	0.864	
+ 5 × ΔR_M(C)	1.315	
+ ΔR_M(O in OCH_2R)		0.844
— ΔR_M(aromatic H)		0.010
Sum of R_M increments	2.179	1.917

Calculated R_M = + 0.262
Experimental R_M = + 0.185

In the calculation of 1-tetralol, 1-naphthol, 1-anthrol and 9-phenanthrol, an increment for ΔR_M(*ortho*-effect) was included. (There is clear chromatographic evidence that an *ortho*-effect does exist in fused-ring compounds, since 1- and 2-naphthol, 1- and 2-tetralol, and 1- and 2-anthrol can all be separated in System 1. The *ortho*-effect is due to some undefined interaction of the hydroxy group with the *peri* CH_2 or CH group of the second ring. There is considerable evidence, both chemical and physical, that confirms this. Thus ARNOLD and his co-workers[43–45] have demonstrated steric hindrance due to the *peri* methylene group in a variety of chemical reactions and also by a study of Raman spectra, while HUNSBERGER *et al.*[46] have demonstrated a similar effect by a study of infra-red spectra.) A typical calculation in this group is for 1-anthrol: R_M(1-anthrol), calculated from R_M(phenol) by adding 8 × ΔR_M(C), 4 × ΔR_M (aromatic H) and ΔR_M(*ortho*-effect), is found to be + 1.207, in excellent agreement with the experimental R_M value of + 1.130.

Mono-ethers of hydroquinones, without ortho-substituents

Calculated R_M values for eleven compounds are given in Table I. The appropriate ΔR_M(O) values, which are given in Table III, were used in each case, depending on whether the ether was primary, secondary, etc. The experimental R_M values of all the ethers that do not contain a ring-containing alkoxyl group agree excellently with the calculated values. The calculation for *p*-pent-4-enyloxyphenol is shown in Table V.

p-Cyclopentyloxyphenol and *p*-cyclohexyloxyphenol, however, run rather faster than required, by an amount equivalent to about one quarter to one half of ΔR_M(C). This may be due to the fact that the use of ΔR_M(O in $OCHR_2$), which is derived from R_M(isopropoxyphenol), is probably not entirely justified when calculating the R_M values of secondary ethers containing cyclic alkoxy groups. It should be noted that the R_M values of *p*-allyloxyphenol and *p*-pent-4-enyloxyphenol are in good agreement

with their calculated values (*cf.* the corresponding alkylated phenols, which show anomalies, and DISCUSSION).

The calculation for *p*-benzyloxyphenol is of some interest as it involves a special feature. The five ring hydrogens of the benzyloxy group must be evaluated as aromatic hydrogens ($\Delta R_M = 0.010$); and the two hydrogen atoms of the methylene moiety must be evaluated as α to an aromatic ring. Thus,

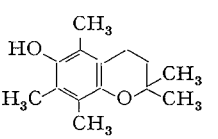
$$R_M(p\text{-benzyloxyphenol}) = R_M(\text{phenol}) + 7 \times \Delta R_M(\text{C}) + 2 \times \Delta R_M(\alpha\text{-hydrogen}) + 4 \times \Delta R_M(\text{aromatic H}) + \Delta R_M(\text{O in } OCH_2R)$$

The calculated value (Table I) is in fair agreement with the experimental value. The chromatography of *p*-phenoxyphenol is of especial interest. The value for ΔR_M(O) in this compound is much more positive than in any other ether (— 0.244). In *p*-cyclohexyloxyphenol, on the other hand, ΔR_M(O) is at least — 1.076, and probably even somewhat more negative. As a result, formal reduction of *p*-phenoxyphenol to *p*-cyclohexyloxyphenol leads to chromatographic *acceleration*. As already noted, reduction of all other types of carbon unsaturated compound leads to a decrease in R_F values.

The unique case of *p*-phenoxyphenol is, of course, due to the destruction of the aromatic character of the aryloxy group on formal reduction.

Chromanols and coumaranols

The eight compounds in this series are structurally the most complex used in this study. For R_M calculations the appropriate oxygen parameters were used, depending on whether the compound contained a cyclic secondary or tertiary ether grouping, and where necessary (as in all compounds except No. 70) the increment for the *ortho*-substituent (0.126) was added. The agreement is in general good, the maximum de-



(IV)

parture from theory being found in 2,5,8-trimethyl-6-chromanol, which ran slower than required by an amount due to about one-third of a CH_2 group.

The calculation for compound 73 (IV), which illustrates several points, is given in Table VI.

Phenols and hydroquinone mono-ethers, with ortho substituents

The calculations of the two groups of compounds with *ortho*-substituents include the increment due to ΔR_M(*ortho*-effect) and we have used throughout the mean value of + 0.126, although, as already shown, the value of this parameter is not very constant. The calculated R_M values are, in spite of this, in moderate agreement with experimental values. In the phenol series, the maximum deviation is only about one-quarter of $\Delta R_M(CH_2)$. In the hydroquinone mono-ether series, it is clear that ΔR_M(*ortho*-substituent) is much more markedly influenced by the size of the *ortho*-group. Thus, compounds 65, 66 and 67, where the groups are large, show deviations from theory by amounts almost equivalent to one CH_2 group.

TABLE VI

CALCULATION OF R_M FOR 2,2,5,7,8-PENTAMETHYL-6-CHROMANOL (IV)

Constituent	*Increment*	
	+	—
R_M(phenol)		1.063
$-5 \times \Delta R_M$(aromatic H)		0.050
$+8 \times \Delta R_M$(C)	2.104	
$+11 \times \Delta R_M$(α-hydrogen)	0.154	
$+2 \times \Delta R_M$(β-hydrogen)	0.096	
$+6 \times \Delta R_M$(δ-hydrogen)	0.576	
$+\Delta R_M$(O in OCR_3)		1.445
$+2 \times \Delta R_M$(*ortho*-effect)	0.252	
Sum of R_M increments	3.182	2.558
Calculated R_M = + 0.624		
Experimental R_M = + 0.676		

o-Allylphenol and *o*-propenylphenol are just separable chromatographically: this is another example of the "allyl" effect.

DISCUSSION

The results of this study, in which the R_M values of seventy-seven derivatives of phenol have been correlated with their structure, demonstrate that MARTIN's equation is rigorously obeyed for several group and atomic ΔR_M parameters. They support the assumption that MARTIN's equation is probably obeyed for all groups and that, where deviations are observed, they are likely to be caused either by experimental difficulties in obtaining ideal conditions or, if these can be satisfactorily discounted, by constitutive effects in molecules.

With regard to the former, the technical difficulties in measuring R_M values accurately must certainly not be underestimated. Failure to distinguish between experimental and constitutive deviations from MARTIN's equation would still appear to be one of the most serious obstacles preventing further advances in structural analysis by chromatography. (Thus, although BUSH[47], in his extensive treatise, clearly illuminates the nature and origin of experimental deviations, he sometimes fails to distinguish them from constitutive effects: the pronounced anomalies he has noted in the value of $\Delta R_M(CH_2)$ in the first five members of a series (C_1–C_{25}) of fatty acid dinitrophenylhydrazides[48] are probably not due to aberrant solute–solvent interactions as suggested by BUSH, but appear—from the R_F data— to be attributable to non-ideal conditions, perhaps coupled, in the case of the first and second members, with a true constitutive interaction of the CH_2 group with the hydrazide function.) Providing experimental effects are satisfactorily eliminated, structural correlation must depend on the accurate calculation of ΔR_M parameters for constitutive effects. The method of atomic parameters described here illustrates a convenient way of doing this and, as we have shown, such parameters are additive as are other group ΔR_M values. Our approach is similar to and extends the work of REICHL[3,4] and SCHAUER AND BULIRSH[5], who calculated several ΔR_M parameters for amino acids. However, as BARK AND GRAHAM[49]

have said, these workers did not deal adequately with the constitutive effects in their compounds.

Constitutive interactions in molecules can be of various kinds. They can be polar (inductive, hyperconjugative or due to ionic bond formation) or steric. In addition, they can affect internal hydrogen bonding (chelation) and they can introduce tautomeric possibilities into the molecule. Any of these effects can affect R_M, but with the exception of steric factors, none of them has been adequately studied. As a result, the influence of steric effects on R_M has sometimes been over-emphasized. MARTIN[50] first suggested that deviations from group additivity would be mainly due to steric effects, and BUSH[47], in his comprehensive study of steroids, considers most ΔR_M variations in these molecules as being stereochemical in origin. BARK AND GRAHAM[49] also considered only steric factors as affecting the chromatography of their series of nuclear-substituted phenoxyacetic acids and did not include the possibility of polar interactions between the substituent groups. As we have already suggested, the term "steric" is itself misleading, since many so-called steric effects are only partly spatial in character and, in fact, may include polar contributions. TAFT[51], for example, considers the *ortho*-effect in benzenoid compounds as a clear example of the dual nature of a "steric" effect, and our findings on the chromatography of *o*-substituted phenols confirm this view. Certain observations by other workers are also revealing in this connection. BATE-SMITH AND WESTALL[52], for example, found no difference between the R_F values of either catechol and resorcinol or pyrogallol and phloroglucinol in an acetic acid–butanol system, but observed a strong *ortho*-effect in catechol and pyrogallol in an acetic acid–cresol system. This marked dependence on solvent is difficult to correlate with a purely spatial effect. Furthermore, these authors found, in the same acid–butanol system that gave no *ortho*-effect with the hydroxy compounds, a pronounced *ortho*-effect when *o*-hydroxybenzoic acid was compared with the *m*- and *p*-compound. These results show that the nature of any internal hydrogen bonding between two vicinal groups (and this is partly polar in character) must affect ΔR_M-(*ortho*-effect).

Polar effects on ΔR_M values are caused by electronic interactions between the atoms and groups in a molecule, which may arise by a variety of mechanisms. We regard the variation in ΔR_M(H) that we have found in alkylated phenols as being primarily due to the electronic effects in these molecules. (It will be apparent that, although we discuss a variation in ΔR_M(H), in physical reality the variation must lie in the nature of the CH groups themselves. The fact that we have arbitrarily made ΔR_M(C) constant merely transforms a real variation in ΔR_M(CH) into a variation in ΔR_M(H).)

Consider first what may be the origin of the exceptionally small value of ΔR_M-(aromatic H) compared to ΔR_M(δ-hydrogen), which is the normal increment for hydrogen in a long alkyl chain or in a cyclohexane ring. The ΔR_M(aromatic H) parameter is derived from a study of five compounds: phenol, 2-naphthol, *p*-phenylphenol, 2-phenanthrol and 2-anthrol. If the R_M values of these compounds are plotted against the number of carbon atoms in each (Fig. 1) the relationship is found to be linear. Since the four polynuclear compounds are formally derived from phenol by removal of two hydrogens and adding n CH groups, and since by definition ΔR_M(C) is constant, this demonstrates that ΔR_M(aromatic H) is also constant for all five compounds. It follows, therefore, that since the conjugative displacements in the five molecules are

different, *polarization* effects cannot account for the low value of ΔR_M(aromatic H). It further follows that any differences in the incipient ionization of the phenolic OH group in these five phenols can certainly be neglected. We regard the low value of ΔR_M(aromatic H) as being primarily due to the large molar volume difference between benzene and cyclohexane, resulting in a relative compression of aromatic CH groups

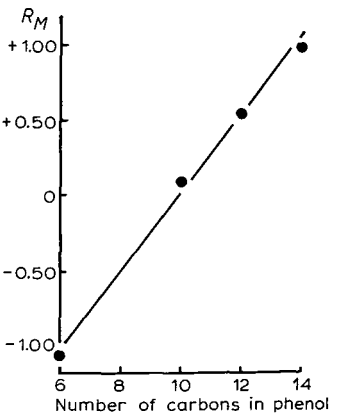


Fig. 1. Relationship between R_M and number of carbon atoms in polynuclear phenols.

compared to aliphatic CH_2 groups. As DIKSTEIN has shown[39], this can be expected to affect the free energy of transfer, and in System 1 this produces a decrease in R_M. This may not be the only effect, however. It is known that aromatic rings can directly partake in hydrogen bonding by means of their π-bonds. Thus, aromatic bonds may well affect solute–solvent interactions differently from normal C–C bonds, in a manner not dealt with by DIKSTEIN's equation (see later).

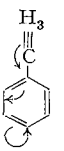
The sequential order of variation in ΔR_M(H) from α- to δ-hydrogen is considered to be due to other factors. Any difference between the molar volume effects of CH_2 groups α and β to the ring must be minute compared to the effect of "aromatization", and there is, in fact, no evidence that substitution of an aromatic hydrogen atom by any alkyl group, however branched, leads to a shortening of the C–C bond between the ring and the alkyl group[53]. We regard this variation, therefore, as a consequence of the electronic interactions of alkyl groups with the aromatic ring. In order to understand how these might affect chromatography, it is necessary to discuss such interactions in some detail.

Although any alkyl group is, of necessity, neutral when attached to hydrogen or aliphatic carbon, it is subject to a polarization when attached to a conjugated or aromatic system. This usually manifests itself in the direction of the ring (vinyl groups are an exception), and thus alkyl groups, compared to hydrogen, are considered to repel electrons into the ring. Such a polarization affects certain physical properties of the molecule, such as its dipole moment, and also its chemical reactivity. Study of the various alkylated derivatives of benzene, however, by chemical and physical means, leads to the observation that there are in fact *two* orders of electron release by alkyl groups and hence to the concept that two mechanisms are involved. One of

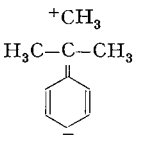
these is the general inductive effect (+ I)[53], the magnitude of which varies in the order,

$$C(CH_3)_3 > CH(CH_3)_2 > C_2H_5 > CH_3$$

The other is hyperconjugation[54], which is usually regarded as being due to the conjugation of single C–H bonds with an aromatic ring (V).



(V)



(VI)

This hyperconjugative mechanism, depending as it does on the availability of α-hydrogen atoms, means that the electron-releasing effect of the CH_3 group must be greater than that of a *tert.*-butyl group: thus the magnitude of the hyperconjugative effect is observed to vary inversely to that of the inductive effect—the so-called BAKER-NATHAN order. Hyperconjugation is essentially a resonance phenomenon; or, in INGOLD's terms[53], since it involves an electronic displacement, includes a mesomeric effect (+ M). Hyperconjugation therefore results in a permanent polarization in alkylbenzenes, and, as shown by the evidence of dipole moments and spectra, is undoubtedly operative in the ground state. The inductive effect of alkyl groups attached to a benzene ring also exists in the ground state, but is not stabilized by a mesomeric interaction[53]. WHELAND[55] regards the inductive effect as a permanent resonance effect, initiated by carbon–hydrogen hyperconjugation (this, however, fails to explain the existence of two different orders of electron release).

There are thus two mechanisms of electronic displacement that may be concerned with the variation in $\Delta R_M(H)$. The approach of BERLINER AND BONDHUS[56] has been found rewarding. They unify the two mechanisms by considering them both as resonance effects. Unlike WHELAND, however, they propose that while the BAKER-NATHAN order is due to carbon–hydrogen hyperconjugation (V), the inductive effect is due to carbon–carbon hyperconjugation. Thus, the *tert.*-butyl group is considered to release electrons and partake in resonance through the contribution of "no-bonded" structures such as (VI). Since this type of resonance does not depend on α-hydrogens, the order is the inductive order. It also implies stabilization in the ground state.

It does not appear possible to associate the sequential variation in $\Delta R_M(H)$ exclusively with either mechanism of electron release. If the chromatographic effect of adding an alkyl group to the ring is compared with the effect of adding the same group remote from the ring, then there is clearly a larger deviation in the case of the *tert.*-butyl group than with the *n*-butyl group. This supports an inductive order of polarization and hence an effect due to carbon–carbon hyperconjugation. However, this comparison involves a change in the relative numbers of α- and β-hydrogen atoms, and consideration of the replacement of α-hydrogens in *p*-cresol by successive CH_3 groups leads to the conclusion that carbon–hydrogen hyperconjugation may also be involved. To take the matter further it is necessary to consider now how these effects of resonance (by whatever mechanism they are produced) affect R_M. It would seem

that every polarization involving alkyl groups and the ring, being the result of hyperconjugation, results in a relative loosening of the hydrogen atoms attached to the carbon atoms involved in the delocalization. This appears to be true whether carbon–hydrogen or carbon–carbon hyperconjugation is involved (a study of "no-bonded" structure VI shows that the hydrogen attached to the charged carbon atom will be held more loosely than in the unperturbed molecule). As a result, all alkyl group polarizations lead to an increased measure of hydrogen bonding with solvent molecules (usually water or an alcohol). The chromatographic effect, therefore, will be one of increased solubility in the more polar phase: in reversed phase systems, such as System 1, this means that R_M will be decreased. The diminishing values of $\Delta R_M(H)$ therefore, are a measure of this loosening of hydrogen atoms, which increases nearer the ring. The large chromatographic deviation of the *tert.*-butyl group can thus be regarded as due to the loosening of the bonds attached to the nine β-hydrogen atoms of this alkyl group. It must be noted that we do not regard the polarization itself—that is, the existence of a finite separation of charges due to either the inductive effect or the BAKER-NATHAN effect— as *directly* affecting the partition coefficient. Indeed there is some evidence from Table I that—as expected, if the above hypothesis approximates to the truth —even the *direction* of the polarization with respect to the ring is unimportant. Thus, the R_M value of *p*-propenylphenol is in good agreement with the value calculated by the use of atomic parameters, although the propenyl group, unlike the other alkyl groups, is electron-attractive[53]. In a subsequent paper we shall present further evidence that the charges on carbon due to the inductive effect play little part in affecting R_M. Our concept of the way in which the polar effects of alkyl groups affect R_M, therefore, places the constitutive change *in the substituent alkyl group itself*. We do not regard the effects on R_M as being, in any way, due to a change in the phenolic OH function (*cf.* SUNDT[57]).

To examine these views further, we have compared the effects of alkyl group resonance on R_M with two other physical and chemical phenomena in alkylated benzenes,

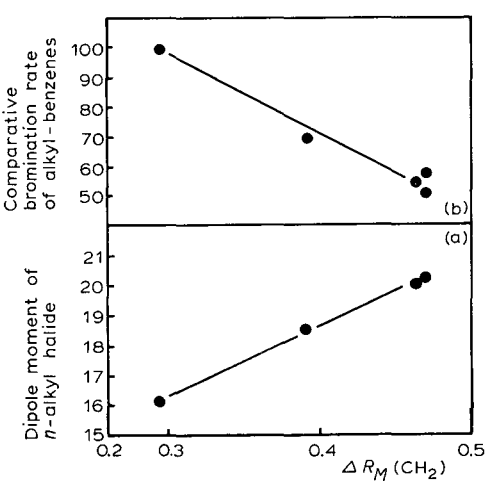


Fig. 2. Relationship between $\Delta R_M(CH_2)$ in *p*-methyl-, *p*-ethyl-, *p*-propyl-, and *p*-butylphenol and (a) dipole moments of the *n*-alkyl halides and (b) comparative rates of bromination of methyl-, ethyl-, propyl-, butyl-, and amylbenzene.

which are also generally ascribed to resonance. We have plotted in Fig. 2 the four successive values of $\Delta R_M(CH_2)$ for the series, phenol to *p*-butylphenol, against (a) the dipole moments of *n*-alkyl halides (methyl to butyl)[58], and (b) the rates of bromination of *n*-alkylbenzenes (toluene to *n*-amylbenzene)[59]. Both curves are approximately linear. (Although in (b) the point for *n*-butylbenzene is slightly anomalous, BERLINER AND BERLINER[59] state that the rate of bromination of this compound is in fact abnormal, because of an exceptional hyperconjugation effect involving the δ-hydrogen and the ring.) The dipole moment order is usually attributed to the inductive effect, whilst the affect on bromination rates is due to carbon–hydrogen hyperconjugation.

It is now possible to consider in more detail the origin of the "allyl" effect on R_M. As described in the experimental section, alkylated phenols that contain a double bond in the allylic position run significantly faster in System 1 than calculated. This can now be attributed to the enhanced effects of resonance in allyl compounds, of which there is also clear chemical evidence. Thus, allyl halides are more reactive than alkyl halides, allyl ethers can be readily cleaved by hydrogenolysis and the allyl radical is considerably more stable than the propyl radical. These properties are considered to be due to the "triad" nature of resonance in the allyl radical (VII).

$$\overline{CH_2} - CH = \overset{+}{CH_2} \leftrightharpoons \overset{+}{CH_2} = CH - \overline{CH_2}$$

(VII)

The observed direction of the constitutive effect of the allyl group on R_M is seen to be the same as that of the *tert.*-butyl group. In accordance with the views already expressed, the "allyl" effect on R_M is considered as being due to the increase in hyperconjugation of the α-CH_2 group of the allyl group, because of the extension of conjugation by the allyl double bond. The effect of this is to loosen the hydrogen atoms of the allyl group even more than can be accounted for by the normal decrease in $\Delta R_M(H)$ for hydrogen atoms in the vicinity of the ring. This results in increased hydrogen bonding with the polar phase, and thus a decrease in R_M in System 1.

It is not surprising that the largest "allyl" effect is observed in *p*-benzylphenol, whose R_M value is 0.316 less than calculated—equivalent to more than one carbon atom. This is attributed to resonance in the benzyl group. (*Cf.* the marked reactivity of benzyl halides and the stability of the benzyl radical arising from the exceptional stabilization of structures involving the hyperconjugated CH_2 moiety.) The loosening of the two α-hydrogen atoms in *p*-benzylphenol is thus reinforced by the proximity of two benzene rings, which account for the unusual magnitude of the "allyl" effect in this compound. Confirmation of this concept of the "allyl" effect in the allylphenols is provided by comparing the R_M values of the allyl ethers. These, in contrast, show no anomalies and agree well with the calculated values. This is because, in ethers, the oxygen atom prevents interaction of the allyl group with the ring. (Indeed, *p*-benzyloxyphenol runs rather more *slowly* than required by theory—probably because it is not quite justifiable to use $\Delta R_M(O \text{ in } CH_2R)$, which is derived from aliphatic primary alkoxy groups, for the calculation of benzyloxy compounds.)

Turning now to the ether series, the chromatography of the alkoxyphenols reveals a remarkable variation in the value of $\Delta R_M(O)$, depending on the nature of the group attached to oxygen. We regard this effect also as electronic in origin and as

primarily due to a variation in the degree of polarization of the C–O bond. Although the steric effects of alkyl groups could operate by screening the oxygen atom, our results show that any such effects are overshadowed by the polar effects. A bulky group attached to oxygen would tend to make $\Delta R_M(O)$ more positive in System 1. But $\Delta R_M(O)$ in *p-tert.*-butoxyphenol is much more negative than in *p-n*-butoxyphenol, in spite of the considerable screening effect of the *tert.*-butyl group.

Hyperconjugation is no longer possible when alkyl groups are separated from the ring by an oxygen atom—as is demonstrated by several chemical studies. Thus, JONES[60] found pure inductive order in the rates of chlorination of alkyl phenyl ethers, compared to the BAKER-NATHAN order found in alkylbenzenes. The effect of different alkyl groups on $\Delta R_M(O)$ is, indeed, clearly related to their inductive effects. BERLINER AND BONDHUS[56] regard the inductive order in alkyl aromatic *ethers* as due to partial ionic bond formation, which in turn depends on the stability of the incipient alkyl carbonium ion (a resonance effect), and there is no doubt that the variation in $\Delta R_M(O)$, as shown in Table III, closely parallels the order of stability of the alkyl or aryl ion (or radical) involved. Thus, tertiary ethers are more readily cleaved by acids than primary ethers, while phenyl ethers are the most stable of all. The magnitude of the variation in $\Delta R_M(O)$ clearly rules out the possibility that molar volume effects are involved (in any case, the bond stretching in tertiary ethers would require a chromatographic effect in the opposite direction to that found, if molar volume were the determining factor). The chromatographic effect on $\Delta R_M(O)$ is almost certainly, then, due to the variation of electron density on oxygen due to resonance effects in the substituent group. Unlike the inductive effect on C–C polarization, this must profoundly affect the availability of the oxygen atom for interaction with solvent molecules; for example, by hydrogen bonding or formation of ether hydrates. (The anomalous $\Delta R_M(CH_2)$ values obtained by SMITH[61] and quoted by BUSH[47] for a homologous series of alkyl sulphides are, we believe, due to a similar variation in the value of $\Delta R_M(S)$. We would regard this as a further example of the way in which an undisclosed con-

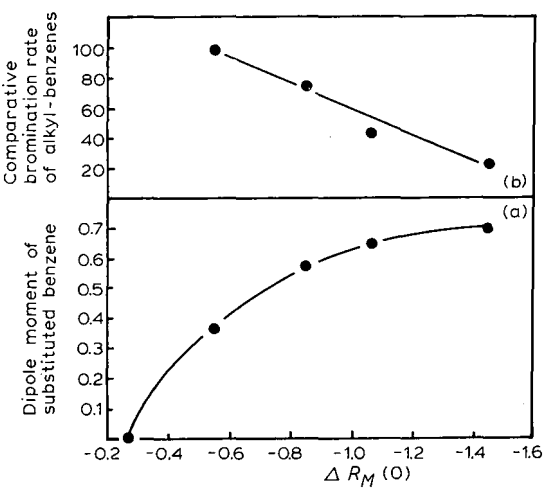


Fig. 3. Relationship between $\Delta R_M(O)$ in phenyl, primary, secondary, and tertiary phenyl ethers and (a) dipole moments of substituted benzenes and (b) comparative rates of bromination of alkylbenzenes.

stitutive effect can lead to results in apparent disagreement with MARTIN's equation.)

In order to illustrate the close relationship of the chromatographic parameters for oxygen with the permanent electronic polarizations of molecules, we have (as for the hydrogen parameters above) plotted the values of ΔR_M(O) against sets of physical and chemical data known to be attributable to such polarization effects. Curve (a) in Fig. 3 shows a plot of the atomic parameter ΔR_M(O) in phenyl, methyl, ethyl, isopropyl and *tert.*-butyl ethers against the dipole moments of a series of alkylated benzenes (*p*-diphenyl to *tert.*-butylbenzene)[62]. The smooth curve that is obtained provides evidence of the relationship between inductive order and chromatographic parameters. Curve (b) is a plot of ΔR_M(O) against the rates of bromination of alkylbenzenes (toluene to *tert.*-butylbenzene)[63]. Although (as might be expected since reaction rates are greatly susceptible to transition state effects) the points show rather more scatter, the relationship is similar to that demonstrated in Fig. 2 (b), the plot tending to be linear. If plots are made of ΔR_M(O) against the rates of chlorination of a series of branched ethers[62] and *p*-alkoxybenzoic acids[64] similar, almost linear, relationships are observed.

As already indicated in the experimental section, small differences in the resonance energy of similar molecules do not appear to affect R_M if they do not introduce a permanent polarization into the molecule. Thus, anthrols and phenanthrols have identical R_F values, although anthracene and phenanthrene differ in resonance energy by about 8 kcal.

SUMMARY

The chromatographic behaviour of seventy-seven phenols and closely related substances has been studied in a reversed phase system (ethyl oleate against 25 % aqueous ethanol) and the relationships between their R_M values and their structures elucidated and discussed. Constitutive effects in chromatography were studied by means of a new method, the use of atomic ΔR_M parameters. The methods of calculating these parameters for carbon, hydrogen and oxygen are described and illustrated. By this method it is possible to calculate the R_M value of any of these compounds from the R_M value of phenol itself. It is shown that the ΔR_M parameters for CH groups (arbitrarily expressed for convenience as atomic ΔR_M(H) parameters) vary depending on their proximity to the aromatic ring. Similarly, the atomic ΔR_M(O) parameters in ethers are profoundly influenced by the nature of the substituent vicinal to the oxygen atom. These effects are considered to be produced by permanent polarizations due to the resonance effects of alkyl groups in the molecules under consideration.

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THE APPLICATION OF GEL FILTRATION TO THE MEASUREMENT OF THE BINDING OF PHENOL RED BY HUMAN SERUM PROTEINS*

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INTRODUCTION

The development of gel filtration techniques, as described by PORATH AND FLODIN[1] and by GELOTTE[2], offers a convenient method for studying the binding of small molecules by proteins. WILCOX AND LISOWSKI[3] have used these techniques to investigate protein–metal complexes, and DEMOOR *et al.*[4] have carried out studies on the binding of corticoids by proteins, using gel filtration to separate the free and protein-bound corticoids.

The association of phenol red with proteins has been extensively examined by means of equilibrium dialysis and ultrafiltration. GROLLMAN[5] has demonstrated that the binding of phenol red by serum is due primarily to the albumin fraction. This albumin–phenol red association was shown to be pH dependent, with a maximum at about pH 4.5. No binding was demonstrable above pH 8 or 9. SMITH AND SMITH[6] found that the binding of phenol red by serum was proportional to the concentration of albumin. RODKEY[7] examined the binding of phenol red by sera of several species and studied the effect of hydrogen ion concentration on the association constants for phenol red and bovine serum albumin.

This communication is concerned with the application of gel filtration to measurements of the binding of phenol red by human serum and by human serum albumin, and suggests a method for routine serum albumin analysis. We have examined some of the factors which influence the association of phenol red with serum and serum albumin.

METHODS

Gel filtration experiments were carried out using the cross-linked polysaccharide Sephadex G-25** (medium particle size). The bed material was washed with distilled water. Fine particles were removed by repeated washing and decanting. The Sephadex, suspended in distilled water, was poured into columns 1.1 cm in diameter fitted with a coarse sintered glass disc. Unless otherwise stated, all columns were poured to a height of 12.5 cm.

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** Pharmacia, Uppsala, Sweden.

Human serum α-globulin (fraction IV) and crystalline human serum albumin were purchased from Pentex, Inc., Kanakee, Ill. Human serum γ-globulin was obtained from Squibb and Co. Serum proteins were dissolved in isotonic saline to a concentration of 4 g/100 ml. Phenol red (phenolsulfonephthalein, sodium salt) was obtained from Distillation Products, Rochester, N.Y. and was used without further purification. It was dissolved in distilled water to a concentration of $5 \cdot 10^{-3}$ M. Human serum was obtained by venipuncture and refrigerated, but not frozen, until needed.

Unless otherwise noted, all experiments were carried out in the following manner. One ml of serum was mixed with 2 ml of phenol red solution. The pH was adjusted to the desired point with dilute HCl or NaOH, with rapid agitation. After standing for 1 h at room temperature, an aliquot, usually 100 μl, was applied to the column and elution was started with distilled water. The effluent was collected in 10-drop fractions, using an automatic fraction collector equipped with a drop counting unit. To each fraction was added 1 drop of N NaOH and 3.0 ml of distilled water. The optical density of the solution was measured at 520 mμ, using a Bausch and Lomb Spectronic 20 Spectrophotometer.

Total protein and albumin were determined according to HAWK, OSER AND SUMMERSON[8], using the biuret reagent.

The bound phenol red was calculated as percent of total phenol red (100 × O.D. of bound phenol red/total O.D. of phenol red). All column separations were carried out in duplicate.

RESULTS

Fig. 1 shows the elution pattern of human serum albumin alone and of a mixture of human serum albumin and phenol red, pH 4.5. It may be seen that there are two phenol red peaks, one of which is superimposed on the protein peak and represents

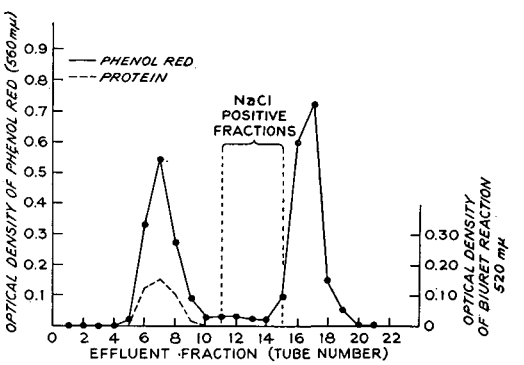

Fig. 1. Elution pattern of human serum albumin alone and of a mixture of human serum albumin and phenol red, at pH 4.5. Details of technique are given in text.

protein-bound phenol red. The second peak represents free, or unbound, phenol red. Some interaction between phenol red and the Sephadex is suggested by the fact that the free phenol red peak lags slightly behind the elution of added NaCl.

Sephadex appears to remove some protein-bound molecules, such as triiodothyronine, from protein, so that the apparent extent of binding is influenced by the

length of the column[9]. Fig. 2 shows the effect of varying the column length on the binding of phenol red by human serum. It is clear that phenol red binding is not influenced by column lengths between 7.5 and 12.5 cm and, therefore, the precise height of the bed material is not critical. A column length of 12.5 cm was chosen in order to obtain a satisfactory separation between the two phenol red peaks.

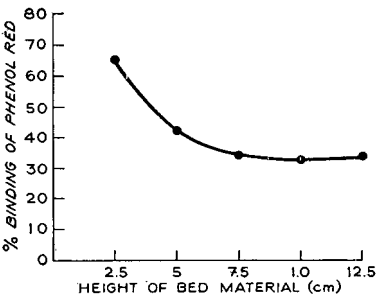

Fig. 2. Effect of Sephadex column length on the binding of phenol red by human serum.

Fig. 3 illustrates the influence of hydrogen ion concentration on the extent of binding of phenol red by human serum albumin. It is seen that there is a maximum binding at about pH 4.0–4.5 and a plateau between pH 6 and pH 7.5. The binding falls to zero at about pH 8.5.

The interaction of human serum γ-globulin (4 g/100 ml) with phenol red was studied over the range of pH 5–9. No binding could be demonstrated at any point. The interaction of human serum α-globulin (4 g/100 ml) with phenol red was measured at pH 4.5. Under these conditions approximately 0.5 % of the phenol red was bound by the protein, whereas about 25 % of the phenol red was protein-bound when a similar concentration of albumin was employed at pH 4.5.

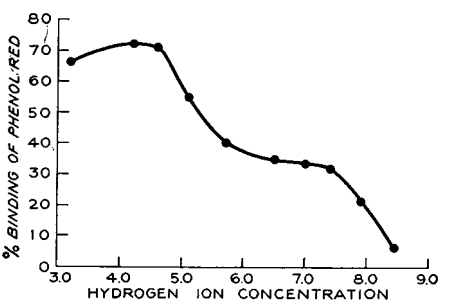

Fig. 3. Influence of hydrogen ion concentration on the binding of phenol red by human serum albumin.

The above results would indicate that, under the conditions described, virtually all of the phenol red bound by human serum is attached to albumin. The relationship between albumin concentration and phenol red binding is shown in Fig. 4. It may be seen that a direct proportionality exists between protein concentration and phenol red binding over the range of concentration of albumin of 4 mg/ml to 20 mg/ml. This is true both of crystalline human serum albumin and pooled human serum.

TABLE I

COMPARISON OF THE RESULTS OF SERUM ALBUMIN DETERMINATIONS BY SODIUM SULFATE FRACTIONATION AND BY PHENOL RED BINDING

Serum	*Albumin concentration (g/100 ml)*	
	Sodium sulfate	*Phenol red binding*
1	3.7	3.6
2	3.8	3.8
3	3.8	3.7
4	4.7	4.9
5	4.5	4.7
6	4.9	4.7
7	3.9	4.0
8	4.9	5.2
9	3.7	3.9
10	4.6	4.8

That this method is applicable to the measurement of albumin in human serum is shown by Table I. The albumin concentrations of several randomly selected human sera were determined by sodium sulfate fractionation and by phenol red binding. In most cases the two methods agree to 0.2 g/100 ml or better, a maximum difference of about 5 %.

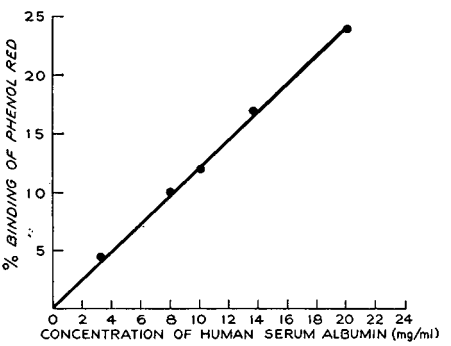

Fig. 4. Relationship between albumin concentration and the extent of binding of phenol red.

DISCUSSION

Application of gel filtration, using such materials as Sephadex, would seem to be well suited to the study of protein–small molecule binding. It is inherently simpler and less time consuming than is equilibrium dialysis, the most common technique used in the past. However, gel filtration cannot be applied to all systems, since the gel binds some small molecules sufficiently tightly so that it competes with protein for these compounds. However, where interaction with the bed is weak, the method can be used with good results.

It would appear that the albumin–phenol red system offers a good model for studying this application of gel filtration. Over reasonable column lengths the binding of phenol red by protein is constant and the weak interaction of phenol red with the bed material is sufficient to produce a good separation of bound and free forms of the dye.

Gel filtration studies have shown, as have ultrafiltration experiments, that phenol red is bound by human sera and that this binding is primarily due to the albumin in the serum. It would appear that gel filtration would offer a possible method for determining serum albumin levels and albumin/globulin ratios.

SUMMARY

The application of gel filtration to studies of the interaction of phenol red with serum proteins has been examined. Phenol red is bound primarily by serum albumin, although a comparatively minute amount is bound by serum α-globulins. Serum γ-globulins do not bind phenol red. The combination of phenol red with albumin is pH sensitive, with a maximum at pH 4.5. No binding was observed above pH 8.5. The extent of binding of phenol red by albumin within the concentration range tested, is proportional to the concentration of phenol red. The use of this technique for the determination of human serum albumin concentrations is demonstrated.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART V. THE HYDROLYSIS OF HEXAMETAPHOSPHIMIC ACID

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(Received June 5th, 1962)

STOKES[1] reported that tetrametaphosphimic acid is produced in the hydrolysis of hexametaphosphimic acid, but did not study the hydrolysis products further. Preliminary results of the hydrolysis of hexametaphosphimate are reported here, a more detailed study is being carried out and will be published later.

PREPARATION OF SODIUM HEXAMETAPHOSPHIMATE

Two grams of hexaphosphonitrilic chloride was dissolved in 10 ml of diethyl ether, and agitated with a solution of 5 g sodium hydroxide in 10 ml of water, for about two days, when the ether layer was free from phosphonitrilic halide.

The aqueous solution was separated off and the sodium hexametaphosphimate precipitated as a thick syrup by the addition of 5 ml of ethanol. The precipitate was washed by stirring with 1 ml samples of 60 % v/v aqueous ethanol, redissolved in the minimum of water, reprecipitated with ethanol and rewashed with 60 % v/v aqueous ethanol until free from sodium chloride. Stirring with renewed samples of ethanol, removed water from the sodium salt which was obtained as a white amorphous powder. The sodium salt was filtered off and dried *in vacuo* over sulphuric acid.

Found: P, 26.6; N, 11.4;

Calc. for $P_6N_6O_{12}H_6Na_6$: P, 27.4; N, 12.4.

The salt gave one spot in the three chromatographic solvents (Table I).

TABLE I

Solvent	R_x *value*
GASSNER's acid[3]	0.07
QUIMBY's neutral[4]	0.06
BIBERACHER's basic[5]	0.40

The retention volume of sodium hexametaphosphimate under the conditions reported in ref.[2] was a major peak at 320 ml, and a minor one at 250 ml. Since paper chromatography showed the absence of all other phosphate species, it was decided that the smaller peak must be due to a small amount of a different form of the hexa-

metaphosphimate ion. STOKES[1] postulated that it can exist in both ring and chain forms.

Potentiometric titration of hexametaphosphimate gave three inflection points, which (these are shown in Fig. 1) correspond to 4, 5, 6 replaceable hydrogens, with pK values of 2.80, 6.88 and 9.40 respectively. STOKES[1] prepared sodium salts of the hexamer containing 4.6, 6.0 and 6.4 atoms of sodium, and it appears that a number of

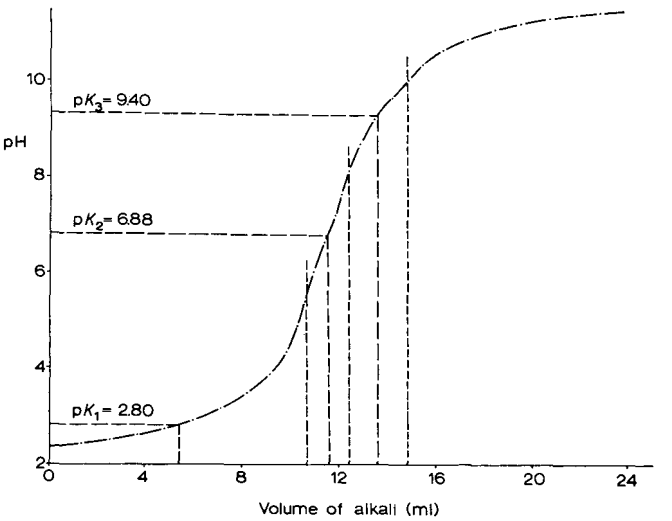


Fig. 1. Potentiometric titration of hexametaphosphimic acid.

salts exist in which the number of metal atoms is not a whole number. As with the pentamer[6], it appears that one, and possibly two units of the ring structure are in a different environment to the others. The appearance of two or three inflection points in the titration curves of the pentamer and hexamer respectively does not necessarily imply that they have chain structures.

PAPER CHROMATOGRAPHIC STUDY OF THE HYDROLYSIS OF SODIUM HEXAMETAPHOSPHIMATE AT 60° AND pH 3.6

Sodium hexametaphosphimate was dissolved in a sodium acetate–hydrochloric acid buffer pH 3.6, and maintained at 60°. Samples were removed at intervals and chromatographed in BIBERACHER's basic[5] and GASSNER's acid[3] solvents. The species detected by the BIBERACHER solvent, with their R_x values are given in Table II.

Samples chromatographed in GASSNER's acid[3] solvent showed large amounts of orthophosphate to be present, with pyrophosphate (R_x 0.76), and a trace of a phosphate species on the starting line.

The hydrolysis appeared to take a similar path to that of the pentamer and tetramer acids in the decomposition to trimeric ring imidophosphates, with the simultaneous production of orthophosphate. Only small quantities of trimetaphosphimate were formed in this hydrolysis, qualitatively much less than for the pentamer.

TABLE II

Time (h)	*Species present*
0	HexaMPm (0.40)
0.2	HexaMPm (0.40)
1	HexaMPm (0.40) + Ortho (1.0)
3	HexaMPm (0.40) + Ortho (1.0) + trace TMPm (1.20) + trace DITMP (1.45)
8	HexaMPm (0.40) + Ortho (1.0) + trace TMPm (1.20) + trace DITMP (1.45) + trace ITMP (1.80)
20	HexaMPm (0.40) + Ortho (1.0) + little TMPm (1.20) + DITMP (1.45) + ITMP (1.80)
48	Same as 20 h but ITMP increasing
100	Little HexaMPm (0.40) + Ortho (1.0) + little TMPm (1.20) + DITMP (1.45) + ITMP (1.80)
200	Ortho (1.0) + little TMPm (1.20) + DITMP (1.45) + large ITMP (1.80)
285	Large Ortho (1.0) + trace TMPm (1.20) + little DITMP (1.45) + large ITMP (1.80)

Abbreviations: HexaMPm = hexametaphosphimate; TMPm = trimetaphosphimate; DITMP = diimidotrimetaphosphate; ITMP = imidotrimetaphosphate; Ortho = orthophosphate.

The tetrametaphosphimate (TeMPm) reported by STOKES is difficult to identify, but the occurrence of a spot on the starting line in the acid solvent could be caused by the highly insoluble tetrametaphosphimic acid.

ION-EXCHANGE STUDY OF THE HYDROLYSIS AT 60° AND pH 3.6

Similar hydrolytic conditions to those used in the paper chromatographic study were employed, and samples were removed at intervals and subjected to ion-exchange separations. The results are shown in Fig. 2.

The elution patterns are rather complex in the fraction numbers 25 to 40. At 18 h, the peaks at fraction 25 and 32 are due to HexaMPm; at later intervals the peak at 25 tended to move forward, whilst that at 32 remained stationary. It is probable that the peak at 25 in these later fractions is probably pyrophosphate, since the HexaMPm has largely disappeared. A DITMP peak could be detected in each case at fraction 36, and although this decreased in size after 186 h, it gave a better resolved peak at that time, due to the corresponding decrease in HexaMPm at fraction 32. It was impossible to say whether TMPm or TeMPm was formed, but the complexity of the elution patterns in this region made their presence a distinct possibility. Trace TMP was found after 186 h.

The high concentration of orthophosphate formed was in agreement with the decomposition of HexaMPm to a trimeric ring plus three orthophosphate groups or their equivalent in chain phosphates. Great accuracy is not possible because of the difficulties of estimating the concentration of DITMP formed.

MECHANISM OF THE REACTION

The hydrolysis of hexametaphosphimate is thought to occur by two mechanisms, one giving DITMP and orthophosphate by a ruptured ring mechanism[2,6,7], and the

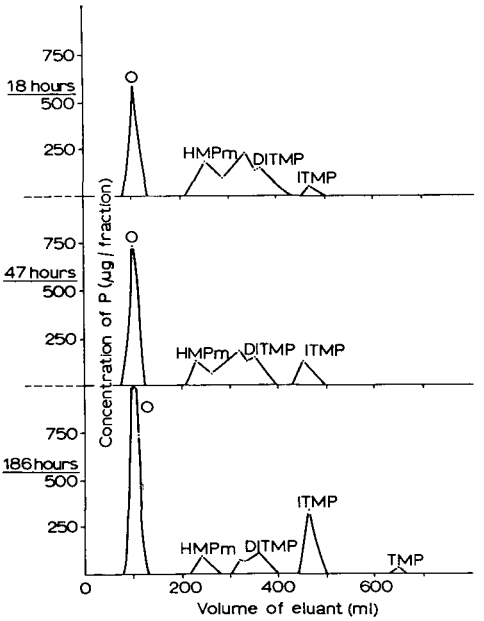
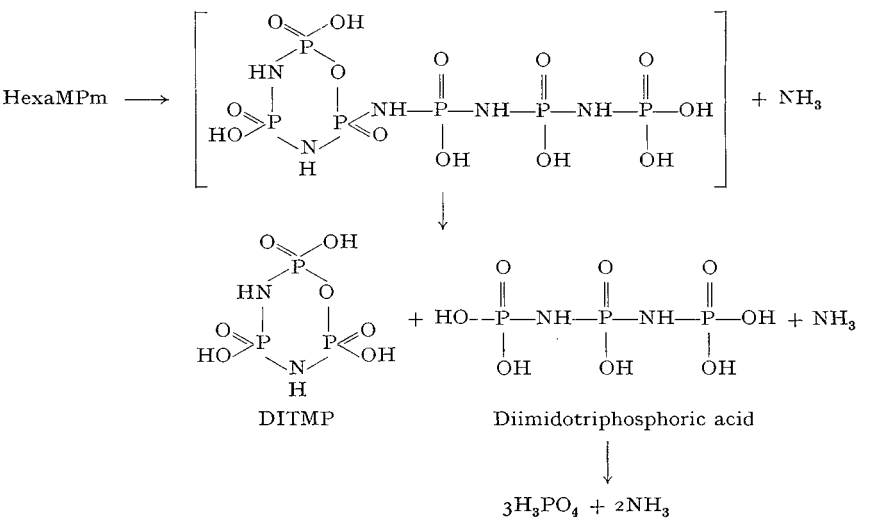


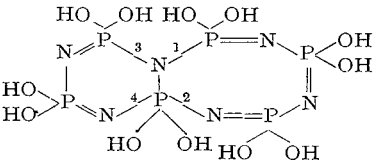
Fig. 2. Elution patterns for the hydrolysis of hexametaphosphimate at pH 3.6 and 60°.

other giving TMPm or TeMPm and orthophosphate involving branching across the ring.

The first of these is essentially the same as that described for the tetramer[7] and pentamer[6] in which the ring breaks, and on reformation does so in such a way that a six-membered ring is formed, and a chain containing three phosphorus atoms remains, which degrades rapidly to orthophosphate or pyrophosphate.

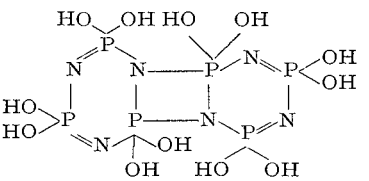


The intermediate postulated as occurring in the second mechanism has the following structure:

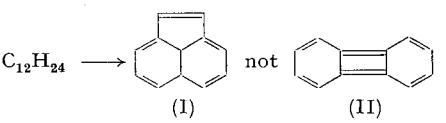


which according to the two P–N bonds which are broken would yield TMPm or TeMPm. Thus 1 and 2 would give TMPm, whilst 3 and 4 would give TeMPm.

It was thought that the hydrolysis of HexaMPm might yield two molecules of TMPm if branching could occur in the ring in two places as shown below, but the low yield of TMPm does not suggest this.



But the organic compound analogous to this structure is very rarely formed, thus cyclododecane condenses to structure I, and not the diphenylene type structure II[8].



(I) (II)

As with the pentamer, there can be little doubt that the first stage of the hydrolysis of hexametaphosphimate is a breakdown to a trimeric ring imidophosphate with the elimination of three molecules of orthophosphate. Again, no definite evidence for the formation of tetrametaphosphimate was obtained.

SUMMARY

A preliminary study of the hydrolysis of sodium hexametaphosphimate in weakly acid solution is described. The products of the reaction are trimeric ring imidophosphates, orthophosphate and ammonia. A reaction mechanism is proposed to explain the formation of these products.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART VI. THE HYDROLYSIS OF SODIUM HEPTAMETAPHOSPHIMATE AND OCTAMETAPHOSPHIMATE

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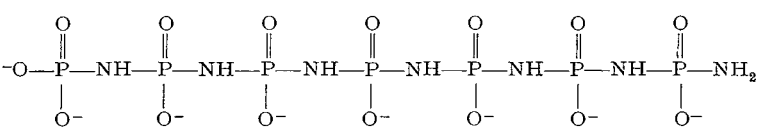
STOKES reported that the metaphosphimic acid series ended at the hexamer, and that the heptamer and octamer members existed in a chain form,



although he only isolated the heptamer acid.

STOKES[1] hydrolysed sodium heptametaphosphimate with acetic acid, and found that tetrametaphosphimic acid (characterised by observation of its crystalline form) was formed. STOKES did not isolate the octameric phosphonitrilic chloride or the acid, but found that on hydrolysing the oil (presumably higher cyclic polymers) left after removal of the heptameric phosphonitrilic chloride, a mixture of higher acids was obtained. These acids on further acid hydrolysis, decomposed into tetrametaphosphimic acid, diimidotrimetaphosphate and triimidotetraphosphate.

The possibility of such chain metaphosphimic acids existing in acid solution, is very doubtful since they would be expected to degrade directly to orthophosphate, as do imidodiphosphate or diimidotriphosphate. We believe that these higher acids exist as ring acids, but lack of sufficient material prevented many characterisation tests from being performed. Work is in progress to prepare more of these materials but the yields are extremely low. Analysis of the infra-red spectra of the metaphosphimic acids from the trimer to the octamer show certain interesting trends, and details will be published elsewhere[2]. Paper chromatographic results indicate that the heptameric and octameric acids hydrolyse by paths very similar to the lower acids, and in no way suggest that they have different types of structure.

PREPARATION OF SODIUM HEPTAMETAPHOSPHIMATE AND SODIUM OCTAMETAPHOSPHIMATE

Both these acids were prepared by exactly similar preparations as those described for sodium pentameta-[3] and hexametaphosphimates[4].

Sodium heptametaphosphimate

Found: P, 27.0; N, 11.5.
Calc. for $P_7N_7O_{14}Na_7H_7$: P, 27.8; N, 12.6.

Sodium octametaphosphimate

Found: P, 27.5; N, 11.8.
Calc. for $P_8N_8O_{18}Na_8H_8$: P, 28.2; N, 12.7.

A PAPER CHROMATOGRAPHIC STUDY OF THE HYDROLYSIS OF SODIUM HEPTAMETAPHOSPHIMATE

Sodium heptametaphosphimate was dissolved in a sodium acetate–hydrochloric acid buffer pH 3.6, and was heated at 60°. Samples were removed at intervals and eluted in BIBERACHER's basic solvent[5]. The species detected, with their R_x values, are given in Table I.

TABLE I

Time (h)	*Species (R_x values)*
0	HeptaMPm (0.55) only
0.2	HeptaMPm (0.55) + Ortho (1.0)
2	HeptaMPm (0.55) + Ortho (1.0) + trace DITMP (1.45)
6	HeptaMPm (0.55) + Ortho (1.0) + little DITMP (1.45)
24	HeptaMPm (0.55) + Ortho (1.0) + little TMPm (1.20) + DITMP (1.45) + trace ITMP (1.80)
55	HeptaMPm (0.55) + Ortho (1.0) + little TMPm (1.20) + large DITMP (1.45) + ITMP (1.80)
97	HeptaMPm (0.55) + Ortho (1.0) + little TMPm (1.20) + large DITMP (1.45) + ITMP (1.80)

Abbreviations: HeptaMPm = heptametaphosphimate; TMPm = trimetaphosphimate; DITMP = diimidotrimetaphosphate; ITMP = imidotrimetaphosphate; Ortho = orthophosphate.

The hydrolysis was quite rapid compared with that of the lower metaphosphimic acids (trimer excluded) and the formation of orthophosphate after only 0.2 h was particularly noticeable. The orthophosphate increased with time and was present in considerable concentration after 55 h. DITMP was also present in large quantities at 24 h and 55 h, but TMPm was never present in large amounts. As DITMP was detected before TMPm, and this indicates that these are formed by two different mechanisms rather than TMPm being formed as a first step, and then DITMP being formed from the TMPm.

Considerable tailing occurred below the orthophosphate spot, probably due to some chain phosphates, but a spot near the starting line was probably TeMPm, and it was present in similar concentrations to the TMPm.

A PAPER CHROMATOGRAPHIC STUDY OF THE HYDROLYSIS OF SODIUM OCTAMETAPHOSPHIMATE

Sodium octametaphosphimate was dissolved in a sodium acetate–hydrochloric acid buffer pH 3.6, and was heated at 60°. Samples were removed at intervals and eluted

in BIBERACHER's basic solvent[5]. The species detected, with their R_x values, are given in Table II.

TABLE II

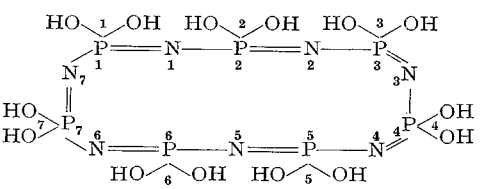
Time (h)	*Species (R_x values)*
0	OctaMPm (0.50)
0.2	OctaMPm (0.50) + Ortho (1.0)
2	OctaMPm (0.50) + Ortho (1.0) + trace DITMP (1.45)
6	OctaMPm (0.50) + Ortho (1.0) + DITMP (1.45)
24	OctaMPm (0.50) + Ortho (1.0) + trace TMPm (1.20) + DITMP (1.45) + trace ITMP (1.80)
55	OctaMPm (0.50) + Ortho (1.0) + trace TMPm (1.20) + DITMP (1.45) + ITMP (1.80)
97	OctaMPm (0.50) + Ortho (1.0) + trace TMPm (1.20) + large DITMP (1.45) + little ITMP (1.80)

The rate of hydrolysis was comparable to the rate of hydrolysis of the heptameric acid. Orthophosphate was formed almost immediately and after 55 h was present in large quantities. TMPm was again present in only small concentration, and was detected only after DITMP was detected, indicating that TMPm and DITMP are formed by different mechanisms.

Tailing occurred below the orthophosphate spot, and was partially due to OctaMPm, and to chain phosphates, but a small amount of TeMPm was also present.

THE MECHANISM OF HYDROLYSIS

Although HeptaMPm and OctaMPm were only studied qualitatively by paper chromatography, it was shown that the first step of the hydrolysis was a breakdown to a trimeric imidophosphate with production of vast amounts of orthophosphate[3,4]. Small amounts of TMPm were formed in each case, probably by a mechanism involving bonding across the ring. DITMP was formed in greater concentration, probably through the mechanism involving ring rupture, as for lower metaphosphimates. DITMP was detected before TMPm in the hydrolysis of the higher acid indicating that they were formed by two different mechanisms. The presence of TeMPm is explained by the mechanism involving bonding across the ring similar to that for the preparation of TMPm.



Bonding between P(2) and N(5) gives TeMPm, whilst bonding between P(3) and N(5) will give TMPm. Work is in progress to elucidate the hydrolysis of the higher metaphosphimic acids, but yields of the higher cyclic polymers especially the heptamer and octamer, are very low.

SUMMARY

A preliminary study of the hydrolysis of sodium hepta- and octametaphosphimates in weakly acid solution is described. From paper chromatographic studies, the products of the reaction appear to be trimeric ring imidophosphates, orthophosphate and ammonia. A reaction mechanism is proposed to explain the formation of these products.

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CHROMATOGRAPHY ON PAPER IMPREGNATED WITH ION-EXCHANGE RESINS

VII. ION EXCHANGE WITH ORGANIC SOLVENTS

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INTRODUCTION

In a previous paper[1] in this series we have shown that the mechanism of adsorption on ion-exchange resins can be studied by chromatography with resin loaded papers, and in particular the comparison of several resins is rendered very simple by chromatographing on several papers simultaneously with the same solvent. The present communication utilises this simple technique for the investigation of several problems. Firstly no work with organic solvents on the inorganic ion exchangers (except ref.[2]) such as zirconium phosphate has been reported as far as we know. Secondly the claim was made[3] that evidence for an anionic chloro-complex of thorium was obtained by adsorption studies on anion exchangers, although neither solvent extraction nor partition chromatography have given evidence for such a complex. Preliminary results on this question have already been reported in the preceding communication[1] and have indicated that the mechanism of the retention of thorium on anion exchangers seems to be rather a kind of partition chromatography where the resin supports the stationary phase. This work was extended and confirmed with numerous solvents. Finally we believe that with the comparative method used we can present a better picture of the processes involved in the adsorption of inorganic substances on ion exchangers from organic solvents, than has been obtained so far from equilibrium and column experiments.

Ion exchange with organic solvents has already been the topic of numerous publications over the last fifteen years[4]. More detailed studies of analytical interest were recently made by FRITZ AND PIETRZYK[5] who examined the anion exchange behaviour of numerous metal ions in a number of solvents containing hydrochloric acid and by KORKISCH *et al.*[6] who studied the behaviour of thorium and uranium in numerous solvents containing various mineral acids at various concentrations.

EXPERIMENTAL AND RESULTS

In all experiments the following four papers were developed in the same solvent at a temperature of 20 $\pm$ 1° in tightly closed small volume jars (26 cm $\times$ 15 cm diam.) by the ascending method:

(1) Amberlite SA-2 paper containing 45 % of Amberlite IR-120 (a sulphonic resin).

(2) Amberlite SB-2 paper containing 45 % of Amberlite IRA-400 (a quaternary ammonium resin).

(3) Zirconium phosphate paper prepared according to ALBERTI AND GRASSINI[7] by precipitating zirconium oxychloride inside the filter paper with phosphoric acid.

(4) Whatman No. 1 cellulose paper.

The solvents examined all contain 1 *N* or 2 *N* acids since intermediate R_F values are obtained on zirconium phosphate paper in this region of acidity. The following organic solvents (of chemically pure grade) were studied: methanol, ethanol, isopropanol and acetone. The main interest of this investigation was centred around the behaviour of thorium and uranium. So as to observe also the behaviour of typical transition and non-transition elements, copper and lanthanum were chromatographed with thorium and uranium. A wider range of ions was studied in acetone mixtures and ethanol since these seemed to yield the most promising results.

The solutions of the ions to be studied were usually made by dissolving the salt (chloride, nitrate or sulphate respectively) in 1 *N* or 2 *N* acid. Thorium nitrate was evaporated 3 times with conc. HCl and then dissolved in 2 *N* HCl as recommended by KORKISCH AND TERA[3] to prevent hydrolysis. Nitrate and sulphate solutions of thorium were also made by evaporating with the concentrated acid and subsequent dilution in the cold.

Uranium and copper could be detected on all papers with potassium ferrocyanide.

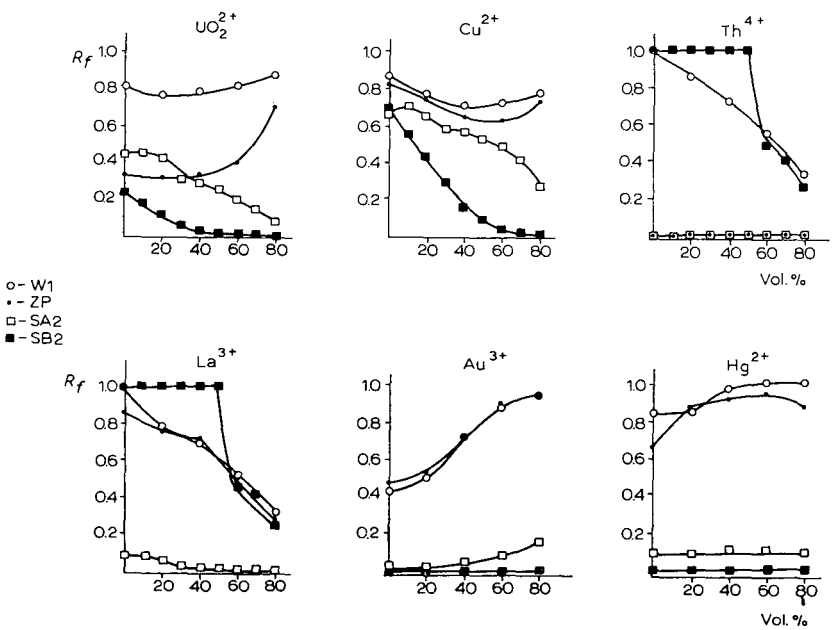

Fig. 1. R_F values of some metal ions in methanol–2 *N* HCl mixtures. Ordinates: R_F values; abscissae: vol. % of methanol in mixtures of methanol–water–HCl all being 2 *N* with respect to HCl. ○ = W1: Whatman No. 1 paper; ● = Z.P.: zirconium phosphate paper; □ = SA-2: Amberlite SA-2 paper (strong acid sulphonic type resin *ca.* 45 %); ■ = SB-2: Amberlite SB-2 paper (strong base quaternary ammonium type resin *ca.* 45 %).

Thorium and lanthanum were detected with thoron and in absence of zirconium phosphate also with alcoholic ammoniacal 8-hydroxyquinoline.

Anion exchange papers usually gave rather elongated spots for Th(IV) and La(III) for intermediate R_F values. However, only the values of the centres of the spots were recorded in the results.

Figs. 1–7 show the variation of the R_F value when the water in 1 *N* or 2 *N* acids is gradually replaced with organic solvents from 0 % to 80 %.

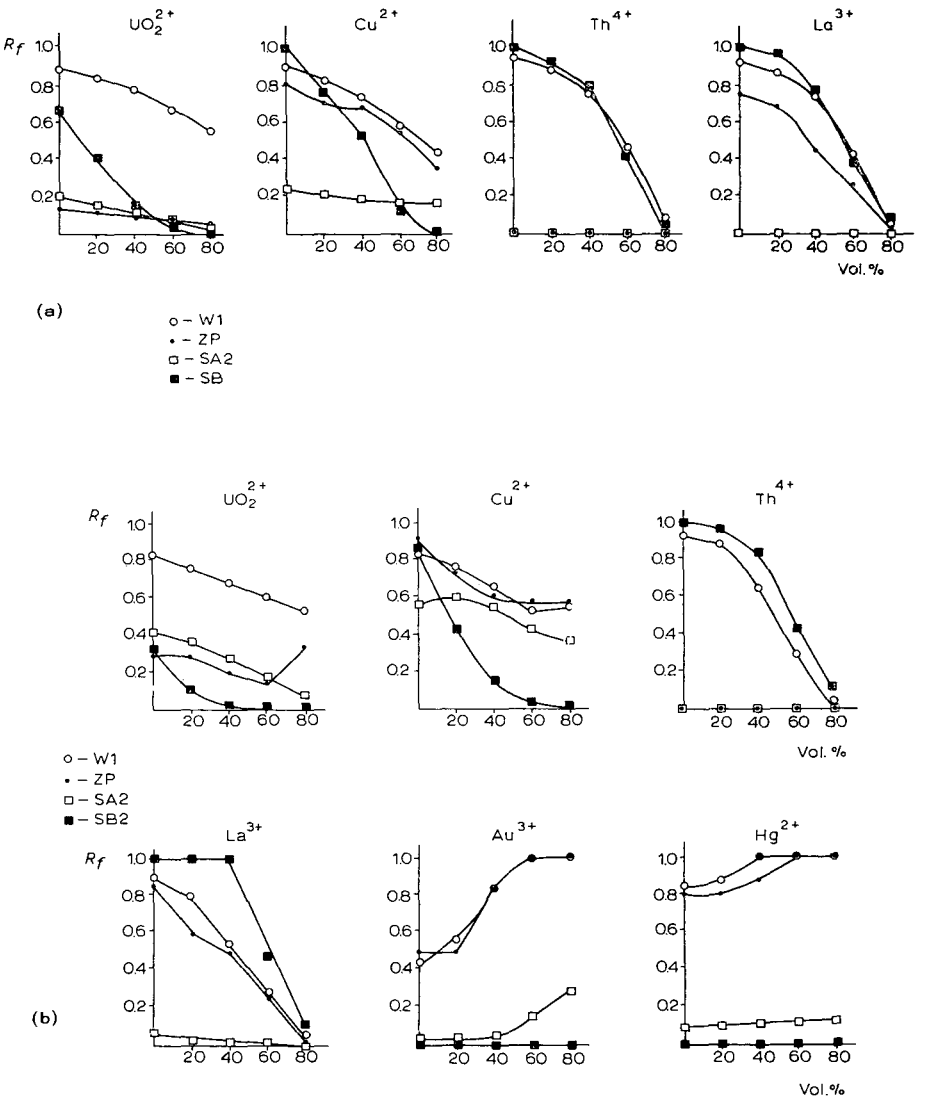

Fig. 2. R_F values of some metal ions in (a) ethanol–1 *N* HCl and (b) ethanol–2 *N* HCl mixtures. Symbols etc. as in Fig. 1.

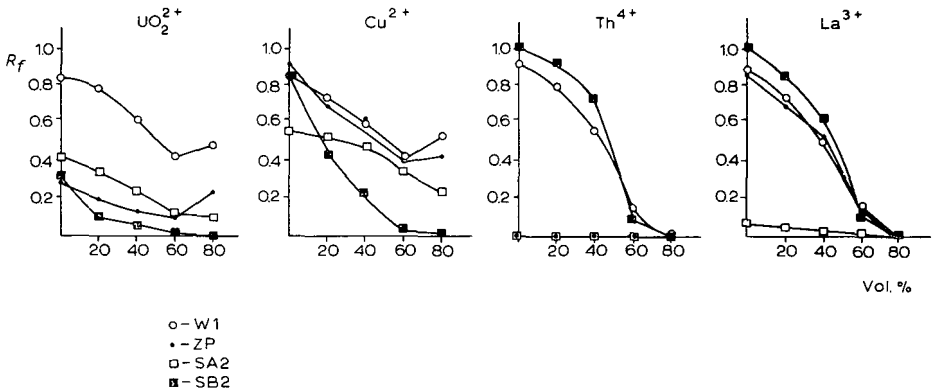

Fig. 3. R_F values of some metal ions in isopropanol–2 N HCl mixtures. Symbols etc. as in Fig. 1.

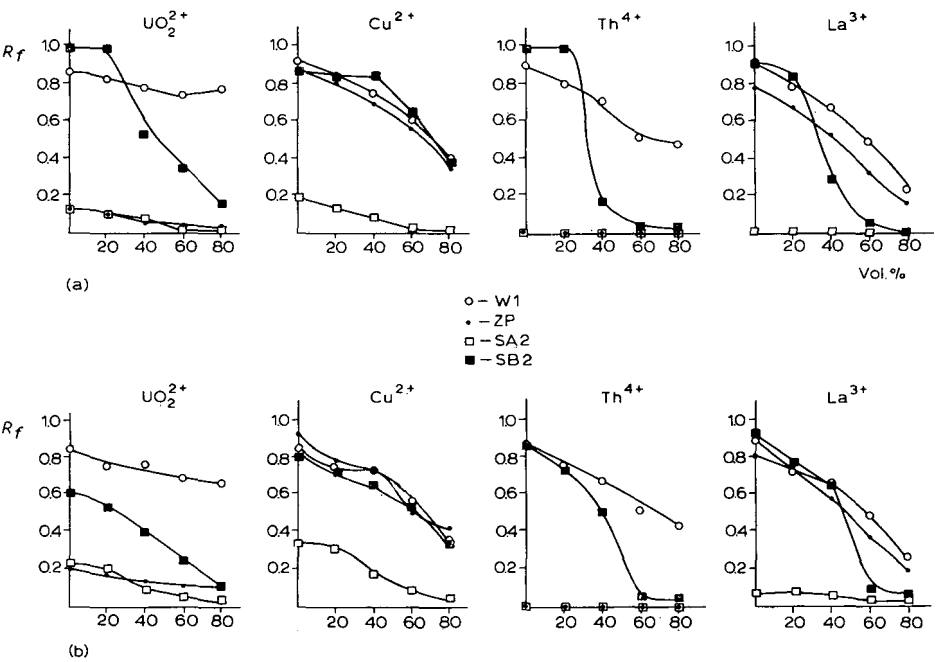

Fig. 4. R_F values of some metal ions in (a) ethanol–1 N HNO_3 and (b) ethanol–2 N HNO_3. Symbols etc. as in Fig. 1.

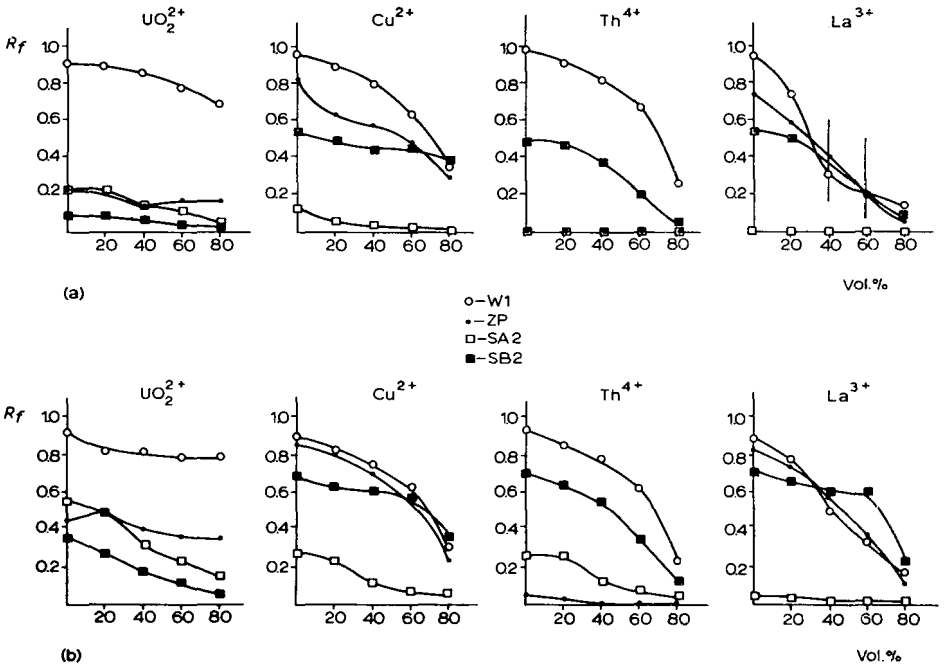

Fig. 5. R_F values of some metal ions in (a) ethanol–1 N H_2SO_4 and (b) ethanol–2 N H_2SO_4. Symbols etc. as in Fig. 1.

DISCUSSION

Lanthanum

La(III) in HCl is strongly adsorbed on the sulphonic resin paper from all concentrations of methanol, ethanol, isopropanol and acetone. The small decrease of the R_F values may be attributed to a small "partition effect" superimposed on the cation exchange adsorption. From HCl the R_F values on cellulose paper (Whatman No. 1), anion exchange paper and zirconium phosphate paper are almost identical and decrease regularly with the increase in the concentration of the organic solvent. It must therefore be concluded that anion exchange plays a negligible role in the retention of La(III) and that the adsorption on zirconium phosphate is also very small in 1–2 N acid.

From nitric acid there is some evidence for adsorption on anion exchangers in higher concentrations of alcohols and acetone (Figs. 4a, 4b, 7a). This may be due to physical adsorption on the resin network by a neutral complex or to actual anion exchange.

In sulphuric acid there are small differences between anion resin papers and cellulose papers. However, they seem to be negligible as are also those for the zirconium phosphate paper. Thus with the exception of nitric acid there seems to be no anion exchange of La(III) from organic solvents and little retention by zirconium phosphate, the main phenomena being cation exchange with sulphonic groups and partition.

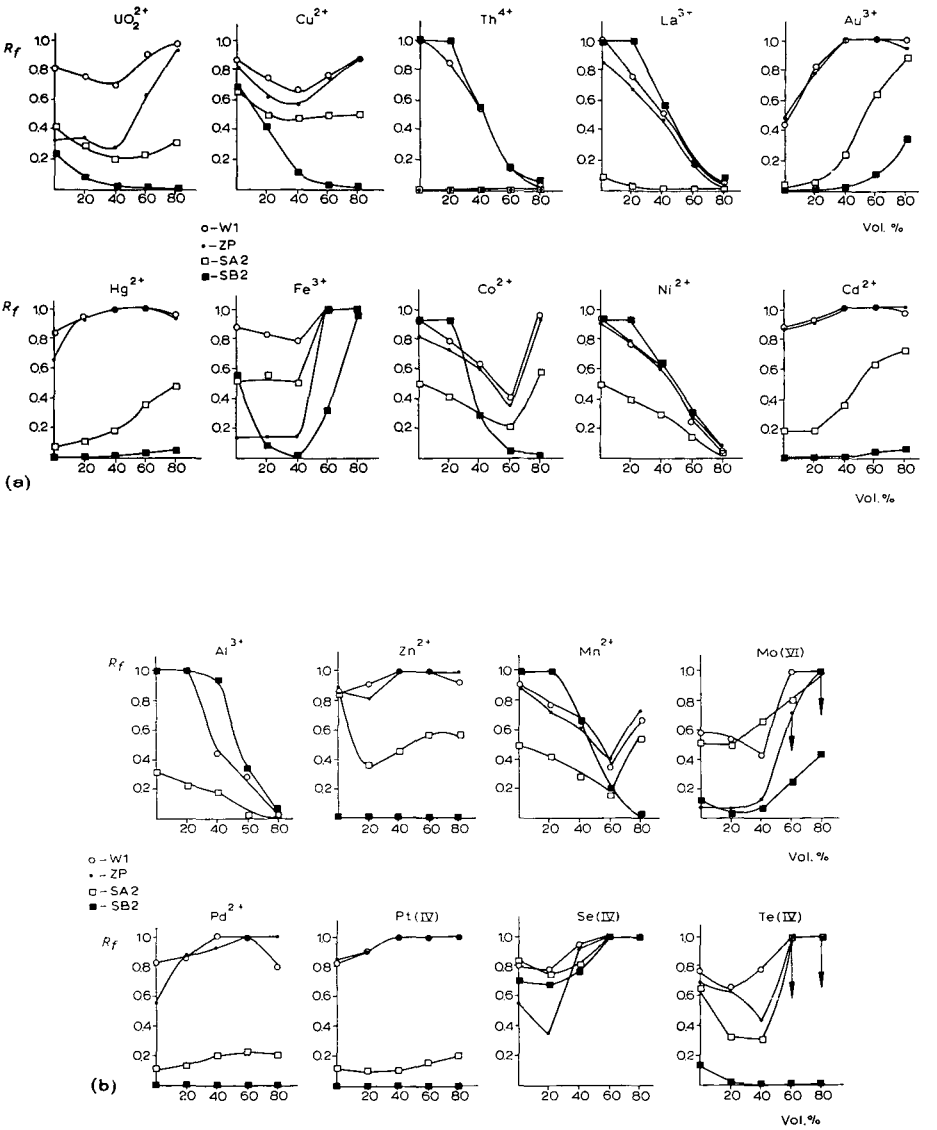

Fig. 6. R_F values of a number of metal ions in acetone–2 N HCl. Symbols etc. as in Fig. 1.

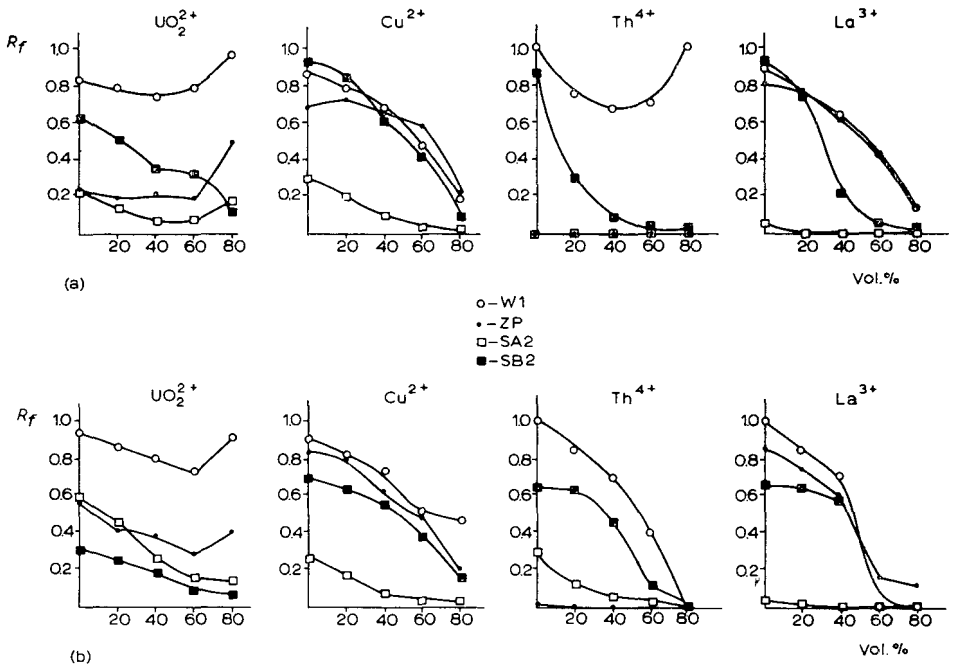

Fig. 7. R_F values of some metal ions in (a) acetone–2 N HNO_3 and (b) acetone–2 N H_2SO_4. Symbols etc. as in Fig. 1.

Thorium

Thorium(IV) is strongly adsorbed on zirconium phosphate and sulphonic resin papers from all acids and all solvents. From hydrochloric acid the adsorption on anion exchange paper and on cellulose paper is identical except in low concentrations of organic solvents where cellulose adsorbs more than the resin paper. From nitric acid the tendency to adsorption on anion exchangers is very marked and the R_F value curves of the cellulose paper and the anion exchange paper diverge considerably above 40 % of organic solvent.

From sulphuric acid there is an increased adsorption on the anion exchange paper already in the aqueous solution. Both cation and anion exchange papers have lower R_F values with an increase in the organic solvent concentration which suggests a partition effect in addition to the ion exchange effect.

From these results it is evident that anion exchange does not contribute to the adsorption of Th(IV) on the various papers from HCl, while it does from HNO_3 and H_2SO_4.

In sulphuric acid (Figs. 5b, 7b) another interesting effect may be observed. Th(IV) is more strongly adsorbed on zirconium phosphate than on the sulphonic resin. In 2 N H_2SO_4 a sulphonic resin must be much more ionised than the phosphoric groups of zirconium phosphate. It thus seems that the retention of Th(IV) on the zirconium phosphate is due to complex formation between Th(IV) and the phosphoric acid groups rather than to an electrostatic attraction.

Uranium (VI)

U(VI) is only slightly adsorbed from 2 *N* HCl–methanol on cellulose paper, but quite strongly on both cation and anion resin paper. There seems to be an increased adsorption which does not depend on a partition effect. Since there is an increase on both resin papers we would like to suggest that a neutral species which adsorbs on the organic network of the resin is responsible for this adsorption. This neutral species is formed as the water of hydration is removed from the uranyl ion.

A similar effect is shown in ethanol with 1 *N* or 2 *N* HCl and isopropanol although there is also a slight partition effect as well.

Zirconium phosphate adsorbs U(VI) from aqueous solution but less from organic solvents; in fact acetone–2 *N* HCl may be used to desorb it completely while this is not so easy with high concentrations of aqueous or alcoholic HCl (see Fig. 8). This effect does not seem to have been recorded previously and may have importance in

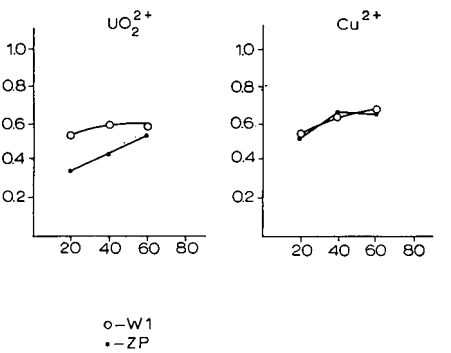

Fig. 8. R_F values of UO_2^{2+} and Cu^{2+} ions on Whatman No. 1 (open circles) and zirconium phosphate (black dots) with mixtures of ethanol–conc. HCl as solvents. Ordinates: R_F values; abscissae: vol. % of conc. HCl in ethanol.

the treatment of uranium solutions. The only organic solvent mentioned for such a desorption in the literature is tributyl phosphate[2].

From nitric acid (–ethanol or acetone) there is little retention on cellulose paper, a gradual increase of adsorption on anion resin paper and a much greater adsorption on cation resin and zirconium phosphate papers. This seems to be in agreement with solvent extraction studies which suggest an equilibrium of cationic and neutral species. The increase of adsorption on the anionic paper seems to be due to the existence of a neutral species which, however, is not adsorbed on the much more polar cellulose. The fact that the adsorption on zirconium phosphate decreases slightly with increasing acetone concentration seems to confirm this. From sulphuric acid U(VI) is only little retained by cellulose paper and quite strongly on all three exchangers. The existence of anionic sulphato complexes is well-known, and hence the adsorption on the anion resin may be accounted for.

Copper

There is little adsorption on cellulose or zirconium phosphate from alcoholic or acetone –HCl mixtures. The adsorption on the anion resin increases considerably with increase in the organic solvent concentration, more neutral and anionic chloro-com-

plexes being formed, as has been observed in numerous other systems previously. There is also an adsorption on the cationic resin which varies little with the solvent concentration. It is stronger from 1 *N* than from 2 *N* HCl and seems to be due to the cationic chloro-complexes as well as the neutral ones. Thus as the amount of cations decreases with increasing solvent concentration the physical adsorption of neutral complexes increases accordingly.

In nitric acid the curves for anionic resin, cellulose and zirconium phosphate coincide, thus retention on these three seems to be solely due to partition. On cationic resin paper there is strong adsorption which increases in higher solvent concentration due to an additional partition effect. In sulphuric acid the picture is essentially as in nitric acid with a possible slight anion exchange effect which seems to be independent of the organic solvent.

The behaviour of anionic chloro complexes and some anions

Figures 1, 2b, 6 show also the adsorption of chloroauric acid and chloromercuric acid from acetone, methanol and ethanol containing 2 *N* HCl. As has been shown previously[8] adsorption on the cation resin paper and cellulose paper must be considered as physical adsorption akin to inversed phase chromatography. The effect of organic solvents is studied here for the first time and shows clearly that there is a competition between the tendency to adsorb on one non-polar surface and the tendency to dissolve in a non-polar solvent. The adsorption on the anion exchanger clearly shows that the adsorption is due to anion exchange plus physical adsorption and is always greater than on the cationic resin. No adsorption on zirconium phosphate can be observed from any of the solutions examined.

Fig. 9 shows the adsorption of chloride, bromide, iodide and thiocyanate from ethanol–0.5 *N* $LiNO_3$ on anion resin paper and cellulose paper. As was previously

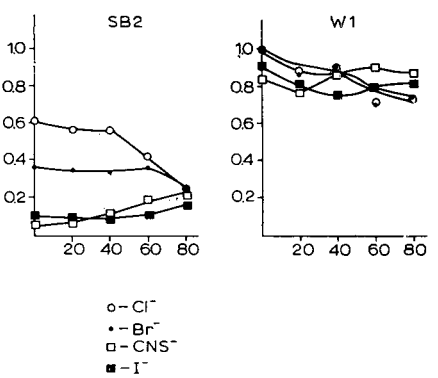

Fig. 9. R_F values of chloride, bromide, iodide and thiocyanate with ethanol–0.5 *N* $LiNO_3$ as solvent. Ordinates: R_F values; abscissae: vol. % ethanol in ethanol–water–$LiNO_3$ mixtures which are 0.5 *N* with respect to $LiNO_3$. ○ = Cl^-; ● = Br^-,; □ = CNS^-; ■ = I^-. SB-2 = Amberlite SB-2 paper; W 1 = Whatman No. 1 paper.

shown[9] with equilibrium studies, thiocyanate desorbs with increasing ethanol concentration and chloride adsorbs more strongly. This effect seems to be mainly due to the

change in the hydration of the ions on the one hand and their increased solubility in the organic phase on the other.

CONCLUSIONS

The retention of ions on ion-exchange papers from solutions of acids in organic solvents can be due to a variety of mechanisms:

1. Ion exchange, that is, actual electrostatic attraction between ionised groups of the ion exchanger and ionised species in solution.

2. Complex formation with groups such as sulphonic or phosphoric acid groups of the ion exchanger.

3. Partition between the water retained on a polar support, which may be cellulose or also a resin equipped with polar groups.

4. Adsorption on the organic network (either paper or resin) of species which extract readily into organic solvents.

5. Complex formation due to the presence of the organic solvent which changes the water concentration in complexing equilibria as well as changes of hydration of non-complexed ions.

The following effects were observed:

(i) U(VI) can be desorbed from zirconium phosphate with acetone while it is only possible to desorb it with aqueous acids in high concentrations. Fast desorption of U(VI) from organic resins with organic solvents proved equally impossible.

(ii) Chloroauric acid and similar strongly adsorbed halo-complexes may be desorbed from organic resins with organic solvents, the effect depending on the nature of the solvent.

(iii) No evidence for an anionic Th(IV) chloro-complex could be obtained, although complexing with nitric acid and sulphuric acid may be observed readily.

ACKNOWLEDGEMENTS

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SUMMARY

The adsorption of a number of metal ions from organic solvents (such as methanol, ethanol, isopropanol, acetone) mixed with acids (such as HCl, HNO_3 or H_2SO_4) was studied on cellulose paper, zirconium phosphate impregnated paper, sulphonic resin paper and quaternary ammonium resin paper. As the mechanisms responsible for the adsorption the following were suggested: ion exchange, complex formation with the groups of the exchangers, partition with the water held on the paper, adsorption on the organic network of the paper or resin and change of the complex equilibria due to the presence of the organic solvent.

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PAPER ELECTROPHORESIS IN THE STUDY OF THE CHEMICAL EFFECTS PRODUCED DURING β DECAY OF ^{132}Te TO ^{132}I

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^{132}Te (77.7 h)–^{132}I (2.30 h) is an ideal system for the study of the chemical consequences of β decay[1]. It has already been investigated by several authors[1,2] by solvent extraction and precipitation experiments broadly involving the following steps: (1) addition of macroquantities of iodine in its several oxidation states to the solution of ^{132}Te in 0.1 *N* HNO_3 and allowing the mixtures to stand for 16 h for the growth of ^{132}I, (2) separation and purification of the carriers and (3) determination of the radioactivity of each fraction. The presence of strong oxidising agents, IO_3^- and IO_4^-, as carriers during the growth of iodine is undesirable. Secondly, even in the most recent method recommended for milking ^{132}I from ^{132}Te, a contamination of 0.1 μg of stable tellurium per milliliter of the product solution and $5 \cdot 10^{-3}$ % of radioactive tellurium has been reported[3]. The presence of tellurium is not desirable when iodine is meant for clinical purposes. In view of these problems we were led to search for an alternative method for the separation of the different oxidation states of iodine from one another without use of carriers and also from those of tellurium. The present paper reports the feasibility of paper electrophoresis for this purpose. This technique has already been applied to the study of the products of the Szilard-Chalmers reaction in various compounds[4–7].

EXPERIMENTAL

^{131}I and ^{132}Te solutions used in these studies were supplied by C.E.A. (Saclay, France).

For low voltage electrophoresis the usual glass plate technique described by LEDERER AND WARD[8] was employed. High-voltage electrophoresis was carried out by the method and with the apparatus described by GROSS[9]. Arches No. 302 paper strips were used for the separations.

The position of the ions on the inactive electrophoregrams was located by the help of suitable reagents. Radioelectrophoregrams were scanned by the Frieseke-Hoepfner FH 452 automatic scanner.

RESULTS AND DISCUSSION

Electrophoretic migration on paper of tellurite, tellurate, iodide, iodate, and periodate has been studied by LEDERER[10] and GRASSINI AND LEDERER[11]. The results of these authors as well as those of JACH *et al.*[6] on iodide, iodate and periodate indicate

that the periodate ion gives more than one spot with a comet extending up to the distance moved by iodate under the same conditions. This behaviour of periodate thus hampers the complete separation of iodate and periodate and will complicate the study of the system which interests us with two more ions, namely, tellurite and tellurate. We therefore thought it of interest to investigate first of all the cause of this behaviour of periodate ions and subsequently to develop a method to avoid its many spots and the comet.

(1) *Paper electrophoresis of periodate ion*

Periodate ion is an energetic oxidising agent in an acid medium. Its oxidising action becomes, however, mild and selective in an alkaline medium[12]. The results of our preliminary experiments with low-voltage electrophoresis showed that even in *N* NaOH IO_4^- gives two spots, one at the point of application and the other at the distance traversed by iodate under the same conditions, with a comet in between. This showed that during electrophoresis the periodate ion is partially reduced to iodate ion. In order to verify whether this reduction resulted from the cellulose of the paper as has already been suggested[12], we carried out the electrophoresis of periodate ion, in *N* NaOH, on Whatman GF/A glass-paper strips and on plaster of Paris plates. Electrophoregrams similar to those obtained on cellulose paper were again obtained. The possibility of the cellulose being responsible for the reduction of periodate during electrophoresis was thus eliminated. The other factors that could reduce IO_4^- were the heat generated during electrophoresis and the long duration of the experiment. Periodate ions are known to decompose under the action of heat and by aging in solution[12]. When we eliminated these two factors by using the high-voltage electrophoresis apparatus of GROSS where arrangements for cooling the paper strip had been made and the time of run of the experiment was reduced to about one-fifth of that necessary for low-voltage electrophoresis, the whole of periodate, applied as $^{131}IO_4^-$ or as inactive periodate, remained at the point of application (Fig. 1). Similar electrophoregrams were obtained when the concentration of the electrolyte was varied in the range 0.01–1.0 *N* NaOH.

When sodium chloride solution was used as electrolyte for high-voltage electro-

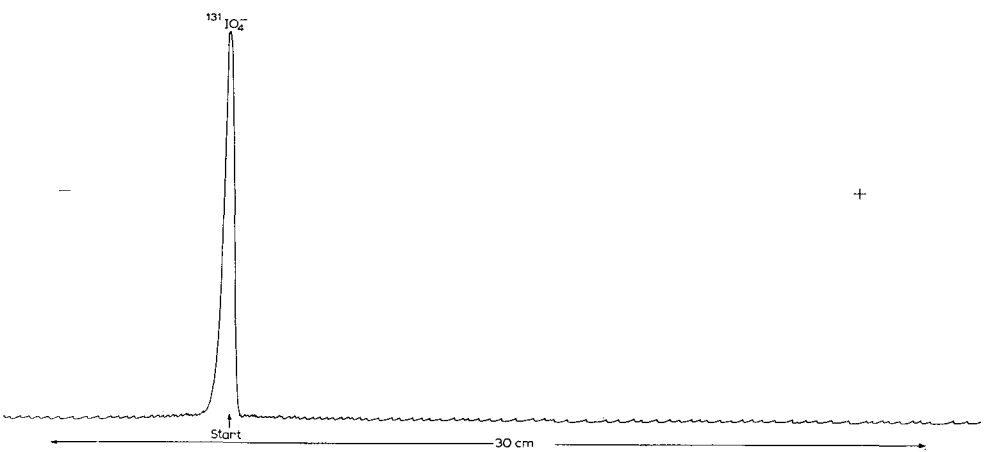

Fig. 1. High-voltage electrophoregram (1250 V, 15 min; *N* NaOH) of $^{131}IO_4^-$ on 4 cm wide paper.

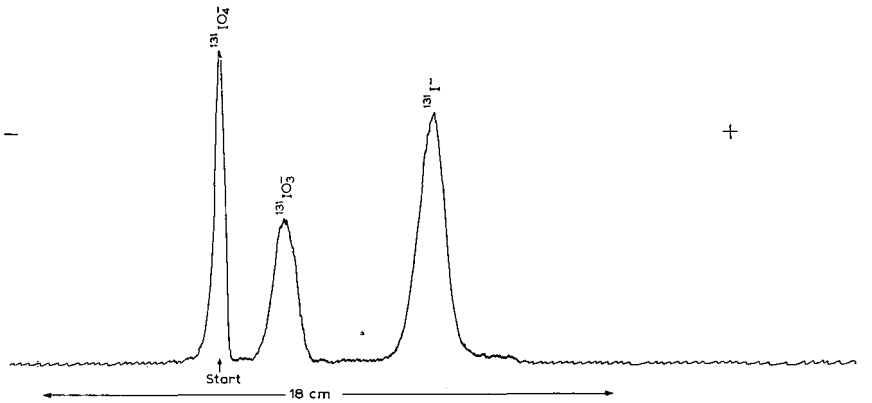

Fig. 2. High-voltage electrophoregram (1250 V, 15 min; N NaOH) of $^{131}I^-$, $^{131}IO_3^-$ and $^{131}IO_4^-$ on 4 cm wide paper.

phoresis, periodate ion remained at the starting point for concentrations lower than 0.5 N. In higher concentrations, and specially in 1.0 N NaCl, electrophoresis showed a faint anionic comet with its origin at the point of application where the major portion of the IO_4^- lay.

(2) *Separation of iodide, iodate and periodate*

Figs. 2 and 3 show the typical electrophoregrams obtained for $^{131}I^-$, $^{131}IO_3^-$ and $^{131}IO_4^-$ applied to the same strip. These ions could be separated very well by using sodium hydroxide as electrolyte in the range 0.01 to 1.0 N. In lower concentrations of the electrolyte the bands were somewhat wider but they were still far apart.

(3) *Separation of iodide, iodate and periodate from tellurite and tellurate*

The results of GRASSINI AND LEDERER[11] indicate that in 0.1 N NaOH, tellurate and periodate, and tellurite and iodate have electrophoretic mobilities of the same order. We found that increase in concentration of the electrolyte does not improve the

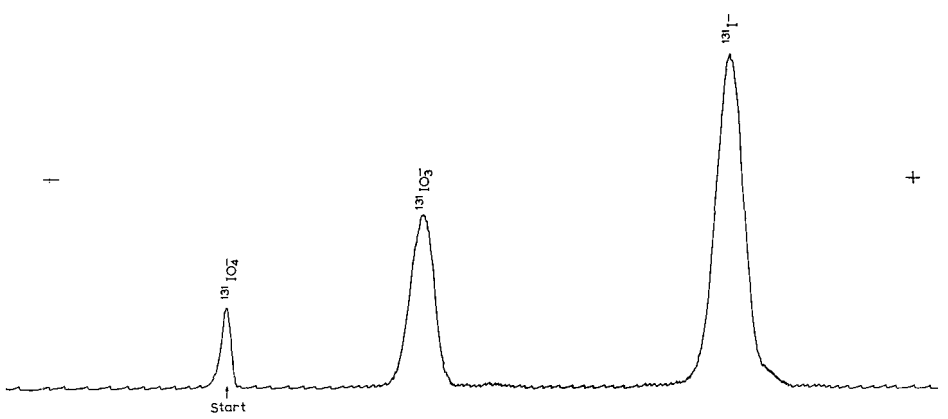

Fig. 3. High-voltage electrephoregram (800 V, 30 min; N NaOH) of $^{131}I^-$, $^{131}IO_3^-$ and $^{131}IO_4^-$ on 4 cm wide glass paper.

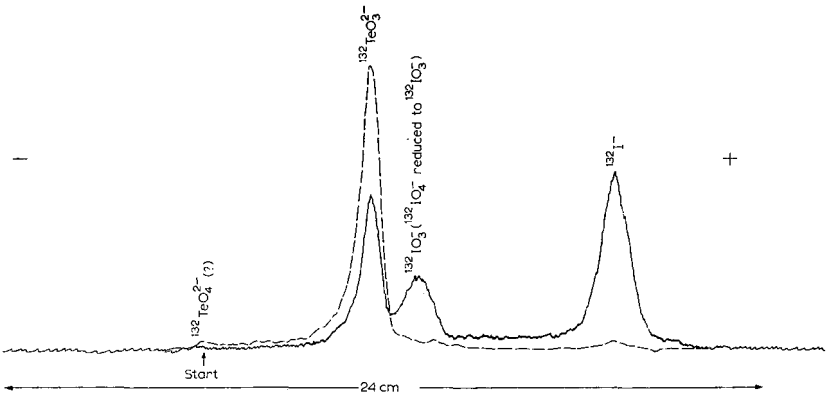

Fig. 4. Low-voltage electrophoregram (300 V, 1 h; $N/2$ NaCl) of alkaline solution of $^{132}TeO_3^{2-}$ in radioactive equilibrium with ^{132}I, on 2.7 cm wide paper. ——— after electrophoresis; – – – – – after 24 h.

separation of these two groups of ions. Electrophoresis in ammonium carbonate solution also does not show promise of their separation[10]. We attempted a separation by using sodium and potassium chloride solutions. Figs. 4 and 5 show the separations obtained with low- and high-voltage electrophoresis respectively. In low-voltage electrophoresis, periodate ion is reduced and moves as iodate while tellurate remains at the point of application. Therefore low-voltage electrophoresis can be used to separate tellurite, tellurate, (periodate + iodate), and iodide. In high-voltage electrophoresis, as we have already mentioned, periodate remains at the point of application. High-voltage electrophoresis in sodium or potassium chloride ($< 0.5\ N$) can therefore give the separation of tellurite, iodide, iodate and periodate in solution. It does not permit the separation of tellurate from periodate and is therefore not suitable for the study of the chemical effects of β decay when the starting substance is tellurate.

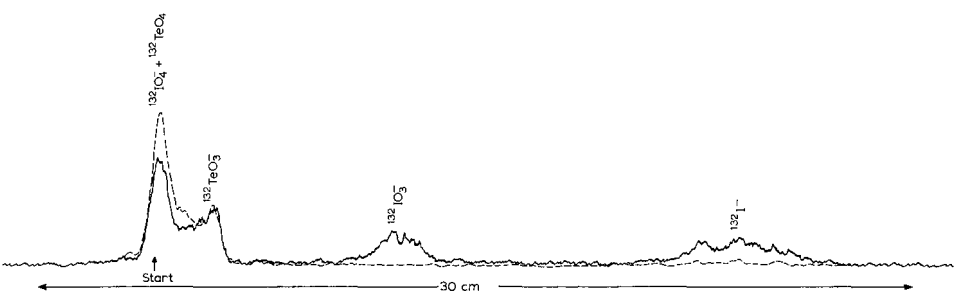

Fig. 5. High-voltage electrophoregram (3000 V, 15 min; $N/2$ NaCl) of alkaline solution of ($^{132}TeO_3^{2-}$ + $^{132}TeO_4^{2-}$) in radioactive equilibrium with ^{132}I, on 2.7 cm wide paper. ——— after electrophoresis; – – – – – after 24 h.

ACKNOWLEDGEMENTS

We express our sincere thanks to Dr. F. DOBICI, Miss G. GRASSINI and Dr. M. LEDERER, who suggested the experiment with plaster of Paris plates and initiated us in the method of their preparation.

SUMMARY

The low-voltage and high-voltage paper electrophoretic behaviour of iodide, iodate, periodate, tellurite and tellurate ions has been studied in sodium hydroxide and sodium chloride solutions used as electrolyte. Iodide, iodate and periodate can be very well separated in 0.01–1.0 N NaOH or in sodium chloride solutions of concentration less than 0.5 N. Simultaneous separation of iodide, iodate, periodate, tellurite and tellurate could not be realised. One could, however, obtain the following separations: tellurate, tellurite, (iodate + periodate), and iodide in NaCl by low-voltage electrophoresis, periodate, tellurite, iodate and iodide by high-voltage electrophoresis in sodium chloride (< 0.5 N).

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THE QUANTITATIVE SEPARATION OF PERIODATE AND IODATE BY THIN-LAYER ELECTROPHORESIS ON STRIPS OF PLASTER OF PARIS

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Electrophoretic separation of inorganic anions on paper has found applications in analytical as well as radiochemical problems and, while paper electrophoresis has been employed[1-5] for the separation of periodate and iodate, this method has not proved satisfactory. Two or more spots have been observed on some papers[2,4] and Whatman No. 3MM has been suggested to be superior to other papers[5]. Since periodate is a strong oxidising agent in an acid as well as an alkaline medium and since it also forms complexes with polyhydroxy compounds (such as cellulose) which are believed to be the first stage of the reduction reaction, we considered cellulose *a priori* unsatisfactory for quantitative and trace scale separations of periodate and iodate. The reaction of complex formation is a suspected cause of the multiple spots observed and the reduction reaction a cause of the reduction comet which has been observed in several separations[6].

In this paper we shall describe the investigation of inorganic supports to replace cellulose in paper electrophoresis. Preliminary work with Whatman glass fibre paper gave indifferent results, yielding with periodate, amongst others, a cationic spot which oxidised acid potassium iodide. Glass fibre paper was therefore not further studied. Plaster of Paris in thin layers on glass plates was then tried, as it had been found satisfactory for several chromatographic separations[7]. It was noted that periodate gave one single spot at the start when chromatographed with 0.05*M* ammonium carbonate and that iodate moved as a diffuse trail close to the liquid front. Sodium hydroxide in various concentrations (1 *N*, 0.1 *N* and 0.01 *N*) was also tried but was found to attack the plaster of Paris strips. During electrophoresis on plaster of Paris, iodate moves as a compact spot and is readily separated from periodate, which forms one single spot at the point of origin.

In the experimental part below we were able to show that it is possible to separate macro amounts quantitatively on plaster of Paris and that trace amounts, produced by neutron irradiation of potassium periodate (Szilard-Chalmers reaction), gave considerably increased yield by comparison with electrophoretic separations on paper run parallel to the separation on plaster of Paris.

EXPERIMENTAL

(*a*) *Preparation of thin layers of plaster of Paris*

It is important to control the purity of the plaster of Paris used. Some samples gave a

blue colour with starch–iodide and had to be rejected. Commercial plaster of Paris (supplied by Hopkins and Williams) gave the best results of the various products tried.

About 25 g were stirred with 30 ml of distilled water and after about 3 min poured into a mould made of glass plates as shown in Fig. 1; another glass plate was then

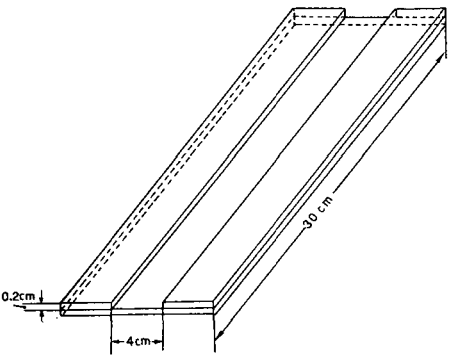

Fig. 1. Arrangement of glass slides to form the mould for the preparation of the thin layers.

placed over this and the whole left about 48 h in an oven at 60°. Thin glass plates (about 1 mm thick) are recommended for radioactive substances to avoid self-shielding.

(b) Electrophoretic separation

The Jouan apparatus for paper electrophoresis was used to support the thin layers. Contact between the electrode vessels and the horizontally placed layer was made with two pieces of Whatman No. 1 paper. As electrolyte, 0.05*M* ammonium carbonate was employed throughout and was sprayed onto the thin layer to saturation. For quantitative work thin layers without glass support were found to have sufficient rigidity if supported by two glass rods at the sides. The volume of solution to be analysed was placed on the strip moistened with the electrolyte by means of an Agla syringe. Samples of 0.05 ml were usually applied and these were placed in the previously pencil-marked centre.

Potentials of 300 to 400 V for 1.5 to 2 h (with 5 to 10 milliamps) gave complete separation of iodate from periodate. Radioactivity measurements along the thin layers were carried out with the counter described by LINSKENS[11].

(c) Quantitative analysis

The portions of the thin layer containing the periodate and the iodate were placed in glass-stoppered Erlenmeyer flasks, broken up and titrated with *N*/100 thiosulphate, after addition of excess acid and potassium iodide, to a starch end-point. Since we did not trust the stability of aqueous solutions of periodate and iodate, aliquots of the solution to be placed on the thin layer were also analysed each day by titrating the periodate with *N*/100 arsenite in alkaline solution and the sum of periodate and iodate with thiosulphate in acid solution on a separate sample[10]. Table I gives the

results obtained before and after electrophoretic separation. Only quantities of the order of 1 mg can be analysed by this method owing to the low solubility of potassium periodate in aqueous solutions.

TABLE I

QUANTITATIVE ANALYSIS OF MACRO AMOUNTS OF PERIODATE AND IODATE

Periodate (mg) present	*Periodate (mg) found*	*Iodate (mg) present*	*Iodate (mg) found*
0.30	0.30	1.10	1.14
0.86	0.86	1.58	1.56
0.36	0.36	0.27	0.27
1.06	1.08	2.48	2.54
0.47	0.47	0.69	0.65
0.19	0.19	1.36	1.36
1.06	1.12	2.48	2.40
0.47	0.48	0.67	0.67
0.21	0.21	1.26	1.24
0.12	0.12	0.23	0.24
0.36	0.36	0.25	0.25
0.30	0.30	1.10	1.13

(d) Separation of radioactive periodate and iodate

Samples of potassium periodate were irradiated for 1 h in a Triga swimming pool reactor in a flux of about 10^{12} neutrons/cm^2/sec. Annealing was then carried out on some samples for 15 min at 240°. The results obtained by electrophoresis on plaster of Paris indicate that about 80 % of radioactive periodate and 20 % of iodate are so formed and this agrees well with the findings of ATEN *et al.*[8]. A typical separation is shown in Fig. 2. A parallel separation carried out on cellulose paper is shown in Fig. 3 and shows that the periodate peak is considerably lower, presumably due to reduction.

Separations on plaster of Paris and paper of irradiated samples which were not annealed are shown in Figs. 4 and 5 and again the amount of periodate found on paper is considerably lower than that on plaster of Paris. These experiments were repeated several times and parallel separations on paper were carried out. The relative percentages of periodate and iodate are shown in Tables II and III.

TABLE II

Experiment	*% Activity due to KIO_4 irradiated without annealing. Electrophoresis on:*	
	Plaster of Paris strip	*Whatman 3MM paper*
1	12	3
2	10	1.8
3	12	2
4	9	2.7
5	8.7	2.8
6	10	2.9
7	13	2

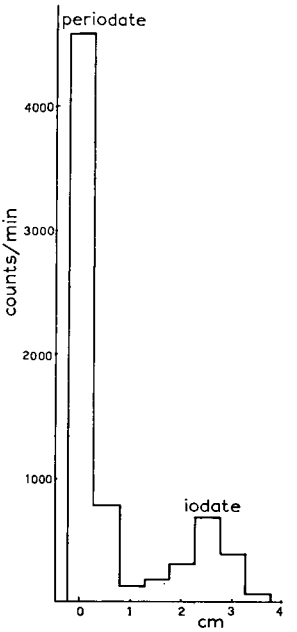

Fig. 2. Separation of radioactive $K^{128}IO_4$ and $K^{128}IO_3$ as formed by irradiation and subsequent annealing. Separation on a thin layer of plaster of Paris with 400 V for 1.5 h with 0.05 *M* ammonium carbonate as electrolyte.

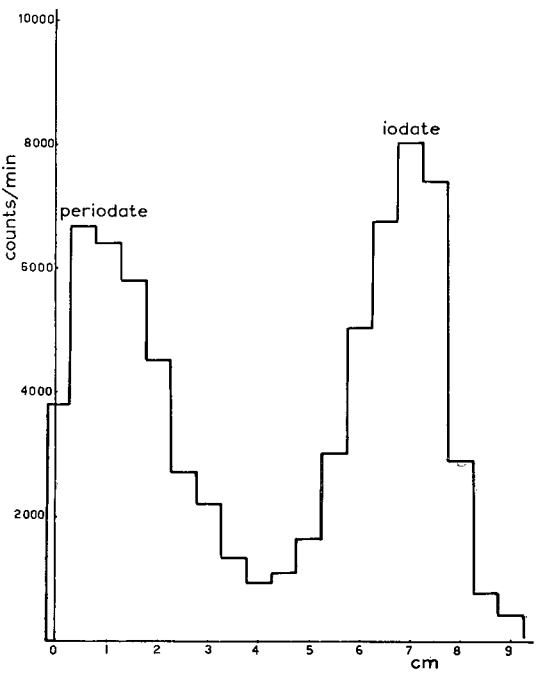

Fig. 3. As in Fig. 2 using Whatman No. 3MM paper as support for the separation instead of plaster of Paris.

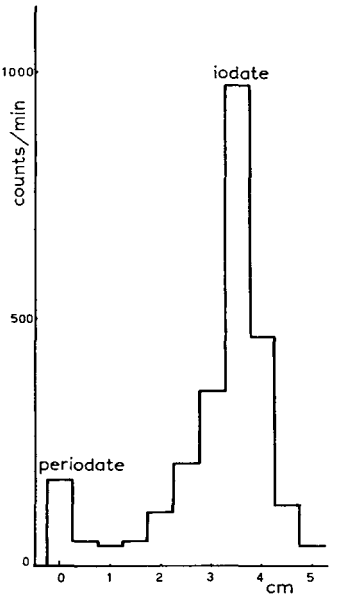

Fig. 4. As in Fig. 2 without annealing. Separation on plaster of Paris with 400 V for 2 h.

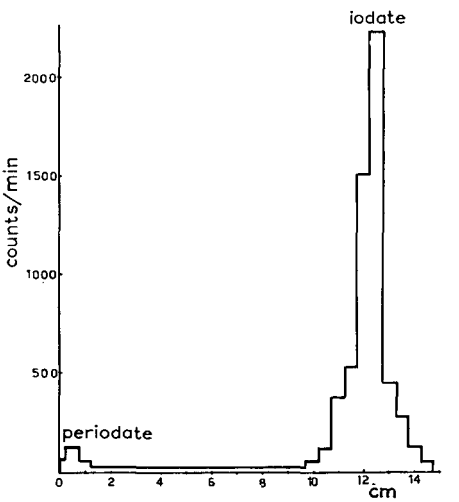

Fig. 5. As in Fig. 4 on Whatman No. 3MM paper instead of plaster of Paris.

The values for samples without annealing agree with the findings of CLEARY *et al.*[9]. It would thus seem that, under the usual conditions for paper electrophoresis, considerable amounts of trace periodate are converted to iodate.

TABLE III

Experiment	*% Activity due to KIO_4 irradiated and annealed at 240° for 15 min. Electrophoresis on:*	
	Plaster of Paris strip	*Whatman 3MM paper*
1	85	48
2	79	37
3	81	44
4	80	45
5	85	39

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The authors wish to thank Prof. V. CAGLIOTI AND Dr. M. LEDERER for their valuable suggestions.

SUMMARY

The electrophoretic separation of periodate and iodate was carried out on thin layers of plaster of Paris with 0.05*M* ammonium carbonate as electrolyte with 300–400 V for 1.5 to 2 h. Quantitative separations of milligram amounts and separation of trace amounts are reported.

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Short Communications

Paper chromatographic behaviour of low molecular weight hexosamine-containing compounds from human urine

Approximately the same amounts of bound hexosamine are excreted in the urine either in the form of non-dialysable mucoproteins and/or polysaccharides, or in the form of low molecular weight compounds[1-3]. The great attention of scientists focussed on urine colloids in the past years has led us to investigate these low molecular weight compounds which may be in close relationship with mucoproteins.

The presence of these compounds in urine was mentioned by several authors, but only BOAS[1] made an attempt to investigate them in detail. In his preliminary report he could distinguish at least three distinct fractions, separated chromatographically on cellulose columns from lyophilized dialysate of urine.

The concentration of dialysable hexosamine in urine is about 50 μg/ml[1,3] and, therefore, low molecular hexosamine-containing compounds have to be concentrated before chromatographic analysis on paper. Our purification procedure is as follows. Pooled normal male urine was precipitated with 6 volumes of ethanol and filtered. The filtrate was concentrated *in vacuo* to one tenth of its volume and the greater part of the salts removed on ion-exchange columns (Zerolit 225-H^+, X 4.5, 50–100 mesh, 5 × 62 cm, and Zerolit FF-$HCOO^-$, 16–50 mesh, 5 × 31 cm). The water eluate from the latter column contained substances not adsorbable on ion exchangers under our experimental conditions. It was again concentrated *in vacuo* and then fractionated by chromatography on a charcoal–Celite column[4] (the charcoal was purchased from Messrs. Spolek pro chemickou a hutní výrobu, ČSSR; before use it was mixed in the ratio of 1:1, w/w, with Celite 535, Light & Co.). The column was step-wise eluted with water and water–ethanol mixtures (up to 30 % ethanol, v/v) and, finally, with aqueous acetone (30 %). The ten fractions thus obtained were examined by chromatography on paper. At least fourteen Elson-Morgan[5] positive spots yielding the purple colour, characteristic of N-acetylamino sugars, were obtained, as well as two other spots, which gave a red coloration. The R_F values for the most intense spots are listed in Table I. The substances designated as IIb and IVb were present in relatively greater amounts. The former was eluted with water, the latter with ethanol (10 %).

The substances described above contain, with one exception, neither glucuronic acid, nor sialic acid. The fraction X, eluted with aqueous acetone, contained 5 % sialic acid, as estimated by Bial's method[6]. The substance X is ninhydrin-positive, while the others are ninhydrin-negative and consequently, in character most similar to the hexosamine-containing oligosaccharides found in human colostrum by KUHN *et al.*[7]. They are also accompanied by several other neutral sugars. They might be present in fractions of urine ultrafiltrate separated on Sephadex[8]. The total yield of these substances accounts for 10–25 % of the dialysable hexosamine, so that it may be

presumed that other types of low molecular weight hexosamine-containing substances such as glycopeptides, are also excreted in urine, as shown by recent observations[9,10].

The details of our purification procedure will be published elsewhere. Further experiments are in progress to elucidate the composition and structure of the substances described in this communication.

TABLE I

R_F VALUES AND COLOUR REACTIONS OF LOW MOLECULAR WEIGHT HEXOSAMINE-CONTAINING SUBSTANCES

Solvent systems: S_1 = pyridine–ethyl acetate–water, 1:2:2 (v/v/v)[7], circular development; S_2 = *n*-butyl alcohol–acetic acid–water, 4:1:5[5], descending; S_3 = *n*-butyl alcohol–pyridine–water, 6:4:3[5], circular. No. 1 Whatman paper; solutions containing 1 mg of each fraction were spotted on the paper. For comparison, the R_F values of glucosamine (GN) and N-acetylglucosamine (AcGN) are given.

Substance	*Solvent systems*			*Colour reactions*[a]		
	S_1	S_2	S_3	*Elson-Morgan*[b]	*Aniline phthalate*	*Aniline-diphenylamine*
IIa_2		0.14	0.18	red[c]		no reaction
IIb	0.28	0.27	0.58	purple[c]	brown	yellow-green
IIc	0.42	0.33	0.65	blue-gray		
IVa	0.10	0.04	0.10	gray	brown-red	
IVb	0.30	0.10	0.40	purple[c]	brown-red	blue-green
VIa	0.03	0.00	0.10	purple	brown	
VIIa	0.02	0.00	0.05	gray	no reaction	no reaction
VIIb	0.60	0.22	0.55	gray	no reaction	no reaction
X			0.12	purple		
GN	0.15	0.09	0.30	red		
AcGN	0.30	0.30	0.50	purple[c]		

[a] The reactions were performed according to HAIS AND MACEK[5], where they are designated D47, D34, and D35.

[b] In solvent systems S_1 and S_2 the colours are not quite clear and have some gray or brown coloration.

[c] Reacts in the cold especially on chromatograms from S_3.

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The separation of metals on ion-exchange modified cellulose paper using complexing agents

Whatman ion-exchange modified cellulose paper has been used previously for the chromatographic separation of metals by means of simple eluting agents. It was observed in some later chromatographic experiments that the addition of complexing agents gave evidence of a relationship between the R_F value of the metal ion complex and the gross stability constant of that complex. This subject has now been studied in greater detail.

Experimental

Whatman P. 20 cellulose phosphate, cation-exchange paper and DE.20 diethylaminoethylcellulose, anion-exchange paper was cut into strips of size 20 × 2 cm and small holes punched at each end of the strip. The apparatus was very simple and consisted of test tubes 20 × 3.8 cm, each with two indentations in the upper lip. Bent glass rods supported the strip and weighted the bottom. The test solution (0.02 ml) containing metal(s) equivalent to 1 mg of each per ml was transferred to the strip along a line about 3 cm from one end, by means of a micropipette. The exact position of this band was recorded by means of a thin pencil line. The eluting solution (50 ml) was added to the open tube and the strip hung, so that the lower end containing the test spot was just immersed. Elution by upward displacement was continued until the solvent front was about 5 cm from the upper end of the strip, *i.e.* in about 1 h. The strip was then removed, the position of the elution front marked, and the position of metal or metals detected with the appropriate reagent. R_F values were determined by normal methods and stability constants for comparison were obtained from the literature[1].

Separations on cellulose phosphate. Each 50 ml of the eluting solution consisted of

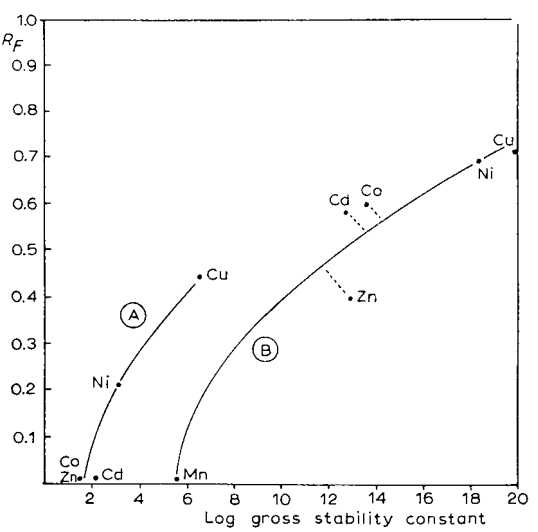

Fig. 1. Elutions with M ammonium nitrate containing (A) pyridine; (B) ethylenediamine.

M ammonium nitrate and contained 1.5 ml of the complexing agent. In the first series, simple divalent metals were tested in conjunction with two amines. Results in Fig. 1 show an increase in R_F value with that of the recorded stability constant. Similarly, if any metal was tested in the presence of complexing agents of similar type then the same relationship was shown to apply (see Fig. 2). Similar tests on

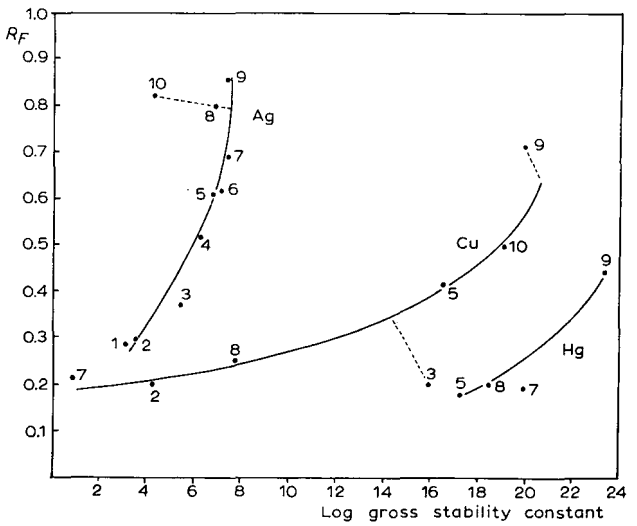

Fig. 2. Elutions with *M* ammonium nitrate containing (1) trimethylamine; (2) triethanolamine; (3) diethanolamine; (4) diethylamine; (5) monoethanolamine; (6) ethylamine; (7) *n*-butylamine; (8) methylamine; (9) ethylenediamine; (10) pyridine; (11) N-methyl-ethylenediamine.

weaker cation-exchange papers, *e.g.* CM and CT, were not satisfactory as the metals moved together.

Separations on DE paper. Complexing agents were not added as separate components to the eluting solution; instead ammonium salts of acids with known complexing powers were used, *e.g.* ammonium chloride and ammonium thiocyanate solutions. Some results shown in Fig. 3 show a reversed relationship compared to that obtained on cation-exchange papers.

The results obtained bear comparison with many standard techniques used with ion-exchange resins in the presence of complexing agents. The use of ion-exchange paper provides a convenient method for the semi-quantitative study of related complexes of similar metals, though the anomalous behaviour under certain conditions must be taken into account.

As an example of this, certain metals are known to be very strongly absorbed by cellulose phosphate[2] probably because of covalent complex formation. If the stability of this bond is considerably higher than that between the metal and the complexing agent present in the eluting solution, then little or no movement will take place.

The experimental techniques described may be used to effect many convenient and rapid chromatographic separations of metals on ion-exchange cellulose paper. For example, various groups of transition metals may be separated into narrow bands using ammonium nitrate solution and amines. A separation of certain noble

metals on DE paper using 2 *N* HCl has been published[3] and the order of separation is again consistent with the principle. It has been possible to predict the separation

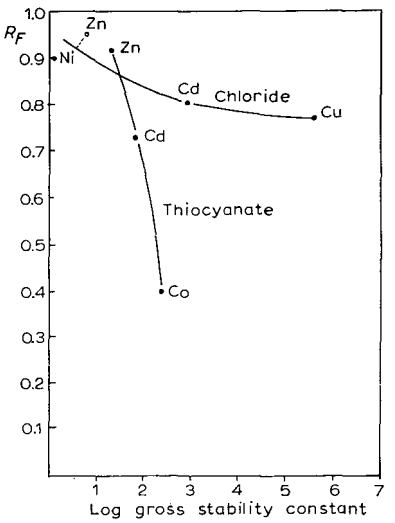

Fig. 3. Elutions with ammonium chloride and thiocyanate (nitrates of metal salts applied to paper).

of certain groups of metals on ion-exchange paper from data published on stability constants of complexes.

W. & R. Balston, Ltd., N. F. KEMBER
Maidstone, Kent (Great Britain) A. FARMER

[1] *Stability Constants of Metal-ion Complexes*. Special Publications 6 and 7 of the Chemical Society, London, 1957 and 1958.
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J. Chromatog., 10 (1963) 106–108

Papierchromatographie von ätherischen Ölen

Die klassischen Arbeiten, Bestandteile ätherischer Öle durch Dünnschichtchromatographie[1–4] und Gaschromatographie[5,6] aufzutrennen, sind allgemein bekannt und haben in den vergangenen Jahren eine weite Verbreitung gefunden. Nachteilig ist bei der Dünnschichtchromatographie, dass eine quantitative Bestimmung der aufgetrennten Substanzen etwa durch Densitometrie nur äusserst schwer durchführbar ist. Sie wurde bisher nur einmal von HEFENDEHL[7] am Beispiel des Menthofurans im

Pfefferminzöl versucht. Die Gaschromatographie als zweite Methode ist wegen ihrer Aufwendigkeit praktisch nur in der Industrie oder grossen Laboratorien durchführbar.

Insofern besässe die Papierchromatographie, namentlich zur quantitativen Bestimmung aufgetrennter Bestandteile grosse praktische Vorteile. Auch hier liegen schon zahlreiche Arbeiten, allerdings mit anderen Stoffen vor. Bis auf ein von CASTIGLIONI[8] beschriebenes Verfahren, das Geraniol und Linalool direkt auf Papier zur Auftrennung bringt, gehen die meisten bisher veröffentlichten Verfahren den Umweg über die Auftrennung entsprechender Derivate, beispielsweise indem Ketone und Aldehyde als 2,4-Dinitrophenylhydrazone zur Chromatographie kommen. In dieser Weise haben SCHULTE UND STORP[9-11] Aldehyde und Ketone ohne Elution vom Papierchromatogramm direkt bestimmt. Diese Methode setzt voraus, dass die Umsetzung der Carbonylverbindungen mit dem Reagens 100%ig erfolgt. Andererseits lassen sich aber damit nicht alle Bestandteile eines ätherischen Öls erfassen, so dass diese Methode nur eine Teillösung darstellt.

Anknüpfend an frühere Arbeiten auf dem Phosphatidgebiet[12,13] haben wir ein mit Paraformaldehyd imprägniertes Papier* entwickelt, das sich zur "Direktpapierchromatographie" von ätherischen Ölen und zur densitometrischen Bestimmung ihrer Bestandteile eignet. Als Lösungsmittel haben sich die Systeme *n*-Hexan–*n*-Heptan–Eisessig (15:15:2 V.T.) und Cyclohexan–Äthylacetat (97:3 V.T.) sowie Trichloräthylen bewährt. Hiermit werden bei der aufsteigenden Chromatographie über eine Laufstrecke von 15 cm und bei einer Laufzeit von 45 Min. (für Hexan–Heptan–Eisessig)

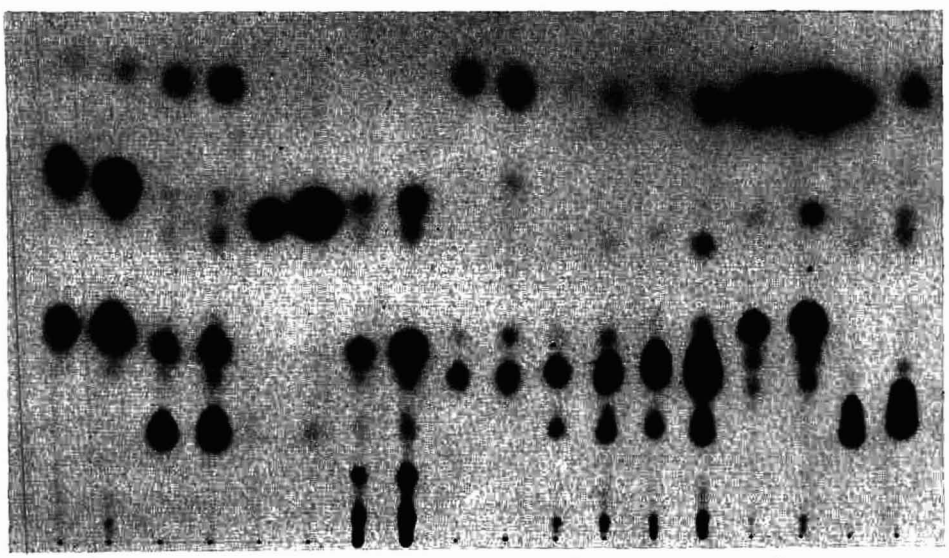

Fig. 1. Aufsteigend angefertigtes Chromatogramm auf mit Paraformaldehyd imprägniertem Ederol-Papier. Fliessmittel: *n*-Hexan–*n*-Heptan–Eisessig (15:15:2). Anfärbung: Osmiumtetroxyd, danach Antimon-(V)-chlorid, zuletzt 2,4-Dinitrophenylhydrazin. 1 = Ol. Lavandul.; 2 = Ol. Serpylli; 3 = Ol. Anisi; 4 = Ol. Aurant. flor.; 5 = Ol. Chamomill.; 6 = Ol. Meliss.; 7 = Ol. Citronell.; 8 = Ol. Majoran.; 9 = Ol. Geran. afric.

* Das Papier wird nach unseren Angaben von der Firma J. C. Binzer, Hatzfeld/Eder (Deutschland) unter der Bezeichnung Ederol No. 208/P hergestellt.

in den meisten Fällen ausreichende Trennungen erreicht (siehe Fig. 1). Damit liegt die Laufzeit in derselben Grössenordnung wie bei der Dünnschichtchromatographie. Bei schwierigeren Trennproblemen ist die absteigende Technik vorzuziehen. Für Serienanalysen kann auch die Rundfiltermethode herangezogen werden. Ätherische Öle werden 1:10 und die Testsubstanzen 1:30 mit Chloroform oder *n*-Heptan verdünnt und davon aus feinen Kapillaren 0.001–0.005 ml im Abstand von 2 cm auf die Startlinie des Chromatogramms aufgetragen. Eine Kammersättigung wird durch Einhängen von Filtrierpapieren, die mit dem Fliessmittel getränkt sind, erreicht.

Zum Sichtbarmachen der Flecke verwendet man Osmiumtetroxyd (0.25 g/5 l-Kammer), Antimon-(III)-chlorid (10 %ig in Chloroform, besprühen und trocknen bei 80–85°), Antimon-(V)-chlorid (besprühen mit 10 %iger Lösung in Tetrachlorkohlenstoff), 2,4-Dinitrophenylhydrazin (besprühen mit gesättigter Lösung in 2 *N*-Salzsäure), modifiziertes Ehrlichs-Reagens und Anisaldehyd–Schwefelsäure–Methanol 1.5:1.5:97 (besprühen und trocknen bei 40–50°), wobei die aufeinanderfolgende Anwendung mehrerer Anfärbreagenzien wie z.B. Osmiumtetroxyd, Antimon-(V)-chlorid und 2,4-Dinitrophenylhydrazin bereits eine gewisse chemische Differenzierung ermöglicht. Die Empfindlichkeit dieser Reagenzien liegt für die ätherischen Ölbestandteile bis auf einzelne Ausnahmen zwischen 2–0.2 μg. Für die Anfärbung zum Zwecke der densitometrischen Bestimmung ungesättigter Verbindungen ist Osmiumtetroxyd sehr gut geeignet.

Einzelheiten dieser neuen Methode werden demnächst veröffentlicht.

Den Firmen Frey und Lau, Hamburg-Bahrenfeld, Dragoco sowie Haarmann und Reimer, Holzminden, danken wir für die freundliche Überlassung von Proben ätherischer Öle bzw. reiner Substanzen, der Firma J. C. Binzer, Hatzfeld/Eder, für Papiermuster.

Unser Dank gilt ferner der Deutschen Forschungsgemeinschaft und dem Fond der Chemie für Forschungsbeihilfen.

Institut für Pharmazeutische Arzneimittellehre der Universität, München (Deutschland)

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1 E. STAHL, *Pharmazie*, 11 (1956) 633.
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Eingegangen den 27. Juli 1962

* Aus der Dissertation P. MUSCHOLL, München (in Vorbereitung).

An improved method for the gas chromatographic separation of estrogens*

The role of estrogens in human metabolism has emphasized the need for determining micro-quantities of these compounds. Methods previously described employing column partition chromatography[1], paper chromatography[2], a combination of countercurrent distribution and paper chromatography[3] and acetylated compounds in gas chromatography[4] have been either time consuming or insensitive. In a previous report from this laboratory[5] the three estrogens: estrone, 17β-estradiol and estriol were successfully separated but the results presented in this report represent a superior separation of estrone and 17β-estradiol and a shorter retention time for estriol.

Experimental

The gas chromatographic separation of a synthetic mixture of estrone, 17β-estradiol and estriol was accomplished utilizing a Barber-Colman model 10 gas chromatograph equipped with a ^{90}Sr ionization detector.

A 6 ft. long, "U" shaped, pyrex glass column with an internal diameter of 5 mm

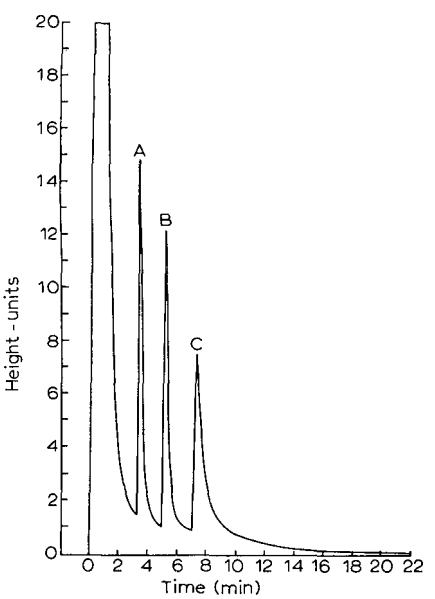

Fig. 1. Gas chromatographic separation of (A) 1.5 μg 17 β-estradiol, (B) 1.5 μg estrone and (C) 5.0 μg estriol. Conditions: Column, 6 ft. 5 mm I.D. pyrex glass column; 3% QF-1 on 100/120 Gas Chrom-P; 243°, 60 ml/min flow rate. Detector, 272°. Flash heater, 303°.

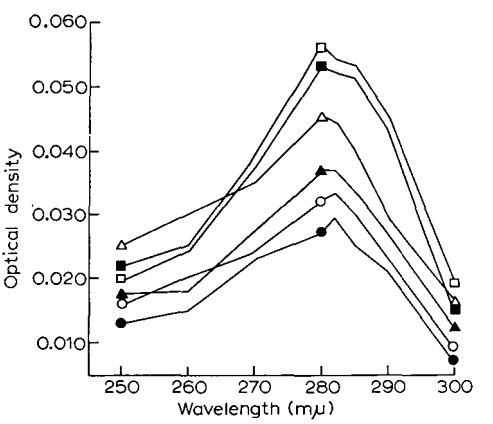

Fig. 2. Ultraviolet absorption spectrum for estrogens prior to and following passage through column. (△) 3 μg estrone before passing through column; (▲) 3 μg estrone after passing through column; (○) 3 μg 17β-estradiol before passing through column; (●) 3 μg 17β-estradiol after passing through column; (□) 5 μg estriol before passing through column; (■) 5 μg estriol after passing through column.

* This investigation was supported in part by a research grant from The Heart Association of Southeastern Pennsylvania.

was packed with a stationary phase of 3% QF-1 (fluorosilicone) on 100/120 Gas Chrom-P (Applied Science Laboratories, Inc., State College, Pa.). The temperature of the column was maintained at 243°, the detector at 272° and the "flash heater" at 303°. Argon was employed as the carrier gas and a flow rate of 60 ml/min was maintained. The column was previously conditioned, while vented to the air, at 253° and at the operating pressure of the carrier gas for 24 h.

The samples were introduced to the column with a 10 μl microsyringe employing a spectroquality reagent grade of dioxane as the solvent.

Results and discussion

Two criteria were used to determine and prove the thermal stability of the estrogens in the gas chromatographic separation technique described. The first was an examination of the chromatogram over an extended length of time. Had any decomposition occurred, new peaks, skewing or broad peaks would have been observed. At no time were any of these conditions observed under the operating conditions described. The chromatogram of the three compounds is shown in Fig. 1. The second criterion was to determine the ultraviolet absorption spectra after passing the compounds through the column and comparing them to reference spectra. In all cases the absorption maxima were identical prior to and following passage through the column as may be observed in Fig. 2. This indicated thermal stability of the compounds.

The retention times of the three compounds are shown in Fig. 1.

Previous evidence for the gas chromatographic separation of estrogens has been presented by WOTIZ AND MARTIN[4] and VANDENHEUVEL *et al.*[6] but the problem of thermal instability was encountered in both reports and as a result separations were difficult. WOTIZ AND MARTIN[4] found it necessary to acetylate the estrogens prior to introducing them to the columns. The symmetry of the peaks, as observed in our results as seen in Fig. 1, indicated the thermal stability of the compounds under the conditions described.

The methods employed in this study are being applied to biological material and will be presented in a future report.

Acknowledgement

Estrone was generously supplied by Eli Lilly and Company, Indianapolis, Indiana.

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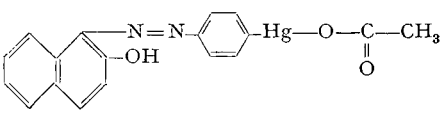
Notes

Anfärbung von Sulfhydryl-Verbindungen nach papierelektrophoretischer Trennung

Verbindungen mit -SH-Gruppen lassen sich papierelektrophoretisch in 16–17 Stunden bei 220 V auf mit Acetatpuffer (pH 6.0, μ = 0.1) getränkten Streifen (Schleicher & Schüll 2043 b Mgl, ausgewaschen) trennen. Für die anschliessende Nachweis-Reaktion ist es von Bedeutung, dass die Trocknung entweder im Stickstoff-Strom oder bei niedriger Temperatur (Gefriertrocknung) erfolgt. Trocknung bei Zimmertemperatur und in der Atmosphäre führt zur praktisch vollständigen Oxydation aller SH-Verbindungen:

In Erweiterung der von BENNET UND YPHANTIS[1] vorgeschlagenen Methode wurde 1-(4-Acetoxymercuriphenylazo)-naphthol-2



zum Nachweis auf dem Papier angewandt. Es handelt sich dabei um eine geänderte Vorschrift von FLESCH UND KUN[2], die 1-(4-Chlormercuriphenylazo)-naphthol-2 benutzten. Für den Nachweis wird der getrocknete Streifen mit einer Lösung von 3 mg 1-(4-Acetoxymercuriphenylazo)-naphthol-2* in 100 ml Amylacetat mit Stickstoff aus der Bombe besprüht. Nach 3–5 Minuten werden die Streifen in Amylacetat kurz ausgewaschen.

TABELLE I

Verbindung	*Reaktion*	*Nachweisgrenze in g/cm²*	*Elektrophoretische Beweglichkeit bei 4 °C in cm/h*
Äthylmerkaptan	+	10^{-6}	
Cystein	+	$7 \cdot 10^{-8}$	+0.57
Cystin	—		
GSH (Glutathion)	+	10^{-7}	+0.40
GSSG	—		+0.50
Methionin	—		
Thiouracil	+	$2 \cdot 10^{-5}$	
Thioharnstoff	+	10^{-5}	
Albumin	+	10^{-5}	
Globulin	+	10^{-5}	
Co-Carboxylase	—		

* Für die Herstellung des Reagens danke ich Herrn Drs. J. H. F. BAAK (Organisch-chemisches Laboratorium der Universität, Nijmegen).

Alle Sulfhydryl-Verbindungen geben orange-rote Flecken (Tabelle I). Die Nachweisgrenze liegt zwischen $2 \cdot 10^{-5}$ und $7 \cdot 10^{-7}$ g/cm^2. Die Farbreaktion auf dem Papier kann verstärkt werden, wenn man den besprühten und ausgewaschenen Streifen in Chlorwasserstoff-Dampf bringt.

Die Farbreaktion bleibt mehr als 24 Stunden beständig.

Botanisches Laboratorium der Universität, J. A. M. SCHRAUWEN
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[1] H. S. BENNET AND D. A. YPHANTIS, *J. Am. Chem. Soc.*, 70 (1948) 3522.
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J. Chromatog., 10 (1963) 113–114

Gas chromatographic analysis of aqueous alcohols

II. Quantitative analysis of aqueous butanol solutions containing non-volatile salts

In a previous communication[1] a procedure was reported for the qualitative gas chromatographic analysis of aqueous alcohols. The method makes use of a mixed stationary phase of sorbitol–di-(2-ethylhexyl) sebacate, the sorbitol acting as a retardant for water. Because of the very bad tailing of the water peak it was not possible to perform quantitative analyses by the peak area normalisation method. BREALEY *et al.*[2] overcame this difficulty in the analysis of aqueous solutions by the use of an internal standard. This technique has now been successfully applied to the analysis of aqueous *n*-butanol solutions, *n*-propanol serving as the internal standard. It has been found in this work that more accurate results were obtained when the concentration of the internal standard in the sample approached that of the component to be determined.

Peak areas for both *n*-butanol and *n*-propanol increased linearly with sample size up to a volume of 10 μl of the pure components. For larger samples a decrease in detector (katharometer) response was noted.

The retention time of water was found to be a function of the absolute quantity injected; the larger the quantity, the shorter the retention time. For aqueous solutions of *n*-butanol, the largest sample that could be injected without obscuring the butanol peak by that of water was 100 μl. This gave rise to a detection limit (katharometer) for *n*-butanol in water of 0.01%.

The results obtained by this method are compared in Table I with those of a colorimetric procedure based on oxidation with dichromate[3], as well as with the results obtained by difference from the determination of the water content by the Karl Fisher method. The effect of the internal standard concentration on the accuracy of the results is also indicated in this table.

Peak areas were measured by triangulation and a calibration factor of 1.08 was

TABLE I

COMPARISON OF ANALYTICAL RESULTS OBTAINED BY GAS CHROMATOGRAPHIC AND CHEMICAL METHODS

Sample number[a]	*Internal standard added* %	*Sample size* μl	*Weight % n-butanol*			
			Known composition	*Gas chromatography*	*Dichromate oxidation*	*By difference from water content*[b]
1	1.92	30		0.19	0.19	
2	2.10	30		1.48	1.54	
3	2.10	30		0.45	0.47	
4	93.1	10	88.98	88.3		88.9
5	68.5	5	94.49	94.7	94.5	94.4
	1.67	5		93.0		
6	72.5	5	86.50	86.7	87.4	87.0
	3.81	5		85.5		

[a] These samples did not contain salts.
[b] Water determined by Karl Fisher method.

used to normalise the *n*-butanol peak area with that of *n*-propanol. The operating conditions have been reported[1].

The chromic acid oxidation method is sensitive to interference by halide ions, so that for aqueous butanol solutions containing halide salts, it is necessary to separate the alcohol from the sample by distillation prior to analysis. As most of the solutions analysed in these laboratories contained relatively high concentrations of calcium chloride, the gas chromatographic method, therefore, proved especially suitable. However, to avoid contaminating and blocking the column with non-volatile salts it was necessary to ensure that only the vapours reached the column. Most commercial instruments are fitted with injection blocks which are intended to produce rapid volatilization of the sample. Although such injection blocks serve as traps for non-volatiles, few of these instruments are designed for ease of dismantling and cleaning. BREALEY *et al.*[2] designed a special, easily dismantled injection block for the analysis of pharmaceutical preparations containing non-volatiles, for use with capillary pipettes.

Fig. 1 shows a similar injection block designed for use with syringes carrying a two-inch needle. The basic principle is the same as that of BREALEY *et al.* but the design has been simplified. The injection block, which attaches directly to that of the commercial instrument, consists essentially of an electrically heated copper block in which a small removable brass cup, filled with glass wool, is seated. To clean the block, the four securing bolts (not shown in the diagram) are opened, the head removed, and the glass wool replaced. The 100 W heating element, the details of which are not shown in the diagram, produces a maximum temperature of 300°. Temperature control was achieved by means of a rheostat.

As water is not measured directly by this method the extent of the hydration of the calcium chloride residue left in the injection block would not affect the analytical result. However, a partially hydrated residue could undergo slow dehydration by the carrier gas stream, a condition which could be expected to give rise to an unstable base line. Although the calcium chloride–calcium chloride monohydrate equilibrium temperature is reported to be 260°, an injection block temperature of 220° was found sufficient to give a stable base line.

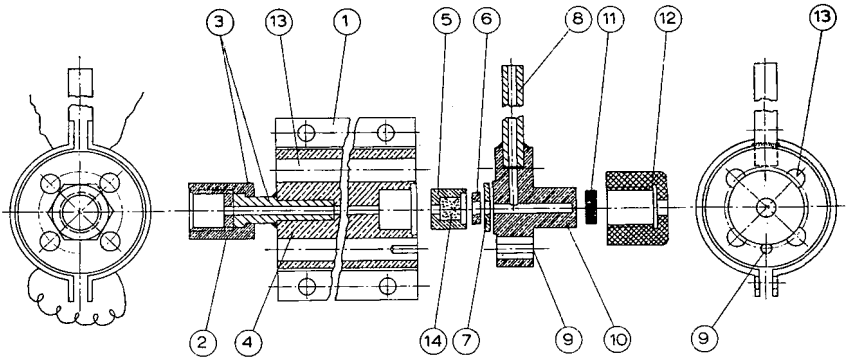

Fig. 1. Injection block designed for use with syringes carrying a two-inch needle.

1. Electrical heating jacket retainer
2. Annealed copper gasket
3. Connection to instrument injection port
4. Copper block
5. Brass cup for glass wool
6. Brass retainer for glass wool
7. Annealed copper gasket
8. Carrier gas inlet
9. Thermocouple well
10. Removable head
11. Silicone rubber septum
12. Transite cap (septum retainer)
13. Holes for securing bolts
14. Glass wool

The trapping efficiency for non-volatiles of this injection block was examined after several days of operation. The original injection block of the instrument was rinsed with distilled water—no chlorides could be detected in these washings.

We are indebted to Messrs. BREALEY, ELVIDGE AND PROCTOR for furnishing us with further information on their injection block, to Messrs. OFER AND GLASBERG of the Instrumentation Department for constructing and assisting in the design of the injection block, to the Analytical Department for the chemical analyses, and to the Israel Mining Industries for permission to publish this work.

Organic Chemistry Department, Israel Mining Industries Laboratories, Haifa (Israel)

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[1] M. ROGOZINSKI, L. M. SHORR AND A. WARSHAWSKY, *J. Chromatog.*, 8 (1962) 429.
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Received June 4th, 1962

News

INTERNATIONAL SYMPOSIUM ON ADVANCES IN GAS CHROMATOGRAPHY

January 21st–24th, 1963

This symposium will be held at the Sheraton-Lincoln Hotel, Houston, Texas and is organised by Dr. A. ZLATKIS, Department of Chemistry, University of Houston, Houston, Texas (to whom inquiries may be addressed).

Papers will be read by J. E. LOVELOCK, O. F. FOLMER, B. J. GUDZINOWICZ, D. H. DESTY, C. S. G. PHILLIPS, R. P. W. SCOTT, H. R. FELTON, E. BAYER, J. A. DORSEY, D. D. DEFORD, M. J. E. GOLAY, J. H. PURNELL, J. C. GIDDINGS, H. W. JOHNSON, JR., J. H. KNOX, R. D. SCHWARTZ, I. HALASZ, A. T. JAMES, A. KARMEN, E. C. HORNING, A. I. M. KEULEMANS, S. R. LIPSKY, H. J. DAWSON and I. DORETZKY.

XITH ANNUAL COLLOQUIUM, ST. JANS HOSPITAAL, BRUGES
"PROTIDES OF THE BIOLOGICAL FLUIDS"

May 2nd–5th, 1963

For application and further information about this Colloquium, please apply to: The Laboratory of St. Jans Hospitaal, Bruges, Belgium.

NEW JOURNALS

The number of publications in the field of gas chromatography has increased to such an extent that, apart from the *Journal of Chromatography* (which will continue its usual policy of publishing papers on all aspects of chromatography including gas chromatography), there will appear as from January 1963:

Journal of Gas Chromatography, published by Preston Technical Abstracts Co., Evanston, Ill. It is intended that each monthly issue will contain a minimum of six contributed articles and several short contributed notes. There will also be a book and patent review section. Meetings, symposia and courses on gas chromatography will be covered as well as the activities of associations concerned with gas chromatography.

Radiochimica Acta. This new journal is being edited by A. H. W. ATEN, JR., H. J. BORN, E. GLUECKAUF, P. REGNAUT, F. STRASSMANN and R. WOLFGANG and published by Akademische Verlagsgesellschaft, Frankfurt a. M.–Academic Press, New York–London.

The first issue has already appeared and contains an introduction by OTTO HAHN and twelve original papers.

NEW BOOKS

Gas Chromatography, by JOHN H. KNOX, Methuen and Co., Ltd., London, in the series Methuen's Monographs on Chemical Subjects, 1962, viii + 126 pages, price 15s.

Gas Chromatography, a symposium held under the auspices of the Analysis Instrumentation Division of the Instrument Society of America, June 1961, edited by N. BRENNER, J. E. CALLEN AND M. D. WEISS, Academic Press Inc., New York and London, 1962, xv + 719 pages, price $ 22.00.

Gas Chromatography: Principles, Techniques and Applications, by A. B. LITTLEWOOD, Academic Press Inc., New York and London, 1962, 507 pages, price 107 s 6d.

Gas–Liquid Chromatography, by S. DAL NOGARE AND R. S. JUVET, Interscience, New York, 1962, xviii + 450 pages.

Biochemical Applications of Gas Chromatography, by H. P. BURCHFIELD AND E. E. STORRS, Academic Press, New York and London, 1962, 680 pages, price $ 22.00.

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CONTAMINATIONS EN CHROMATOGRAPHIE GAZ–LIQUIDE D'ESTERS D'ACIDES GRAS RADIOACTIFS SUR PHASE STATIONNAIRE POLYESTER

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L'étude du métabolisme des lipides par marquage isotopique au ^{14}C nécessite la mesure de la radioactivité spécifique des acides gras ou de leurs dérivés. Diverses techniques ont été décrites concernant la mesure de la radioactivité des esters méthyliques d'acides gras fractionnés par chromatographie gaz–liquide. Des deux types de méthodes, d'une part mesure continue automatique de l'activité des vapeurs émergentes, cumulative[1] ou différentielle[2], d'autre part mesure discontinue de l'activité de fractions collectées individuellement [3–5], seule cette dernière s'applique au cas d'acides gras très faiblement radioactifs.

Les esters méthyliques d'acides gras séparés sur phase polyester, même quand ils sont parfaitement séparés, sont légèrement contaminés par suite de la nature même du polyester[6]. Les phénomènes de transestérification qui se produisent au cours de l'analyse entre les esters d'acides gras et le polyester font que chaque ester est contaminé par une fraction des esters émergeant avant lui. Cette contamination est en général négligeable, mais, si le contaminant est un ester très radioactif, elle peut conduire à des erreurs d'interprétation concernant l'ester contaminé (par exemple, dans une expérience d'incorporation d'acétate ^{14}C dans les acides gras d'un animal, linoléate contaminé par du palmitate très marqué). Il est donc important, si l'on n'a pas effectué l'isolement et la purification des fractions par d'autres moyens analytiques, de connaître l'ordre de grandeur de ces contaminations.

Le présent travail décrit une méthode simple de collecte et de mesure de radioactivité ^{14}C d'esters méthyliques d'acides gras fractionnés par chromatographie gaz–liquide sur polyester, la détermination des contaminations d'une fraction par une autre fraction et le principe de leur correction.

CONDITIONS EXPÉRIMENTALES

Le fractionnement est effectué sur phase polyéthylène glycol adipate, préparé avec la quantité minimum du catalyseur acide *p*-toluène-sulfonique. Les colonnes (diamètre 4 mm, longueur 1.10 m, 1 g de polyester/3 g de support Chromosorb W 80–100 mesh) sont conditionnées pendant 3 jours à la température d'analyse, 180°, afin de

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réduire l'émission continue de polyesters volatils. Le détecteur est la balance à densité de gaz de MARTIN. Les quantités totales d'esters d'acides gras fractionnés varient de 0.5 à 2.5 mg.

A l'émergence de la colonne, les vapeurs sont collectées par condensation sur cristaux d'anthracène qualité scintillation selon la méthode de KARMEN *et al.*[7] (Fig. 1).

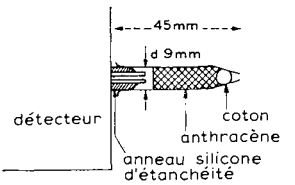

Fig. 1. Collecteur anthracène.

Dans les conditions d'analyse, débit du gaz porteur azote de 40 ml/min, la collecte est presque totale (mieux que 90 %) pour le méthyl-palmitate et les esters à chaîne plus longue, avec des cristaux d'anthracène purs, non enduits de graisse apiezon ou silicone. Les esters condensés sur la partie antérieure du tube sont entraînés par quelques gouttes de toluène qualité scintillation. On complète ensuite le remplissage du tube par l'anthracène. L'intervalle de temps entre l'instant de détection des vapeurs et celui de leur émergence étant négligeable, le repérage sur le chromatogramme des fractions collectées est pratiquement concomitant de leur émergence.

Les mesures de radioactivité ^{14}C sont effectuées en scintillation solide dans le spectromètre Packard "Tricarb" modèle 314 EX. Le tube est logé dans le flacon en verre de 20 ml d'usage courant. Les conditions de mesure ont été déterminées pour donner à la fois un rendement acceptable pour le ^{14}C et un très bas bruit de fond, condition absolue de mesure convenable des très faibles radioactivités:

tension aux bornes des phototubes 980 V,

chenal spectral de mesure défini par la fenêtre 10–50,

amplification A' 10 gain 100.

Dans ces conditions, le rendement de mesure ^{14}C est de 31 % et le bruit de fond d'environ 4.5 impulsions/min seulement.

EXAMEN DES CONTAMINATIONS

L'observation des contaminations est rendue possible par le marquage isotopique des contaminants. Nous avons examiné le bruit de fond de collecte et étudié la contamination par du méthyl-palmitate 1-^{14}C, injecté seul ou ajouté à un mélange d'esters méthyliques d'acides gras non radioactifs.

(1) *Bruit de fond de collecte*

Après analyse d'esters et autres composés radioactifs, une colonne polyester émet continuellement des vapeurs radioactives constituant le bruit de fond de collecte. L'excès du bruit de fond de collecte (mesure directe) sur le blanc (dispositif collecteur et flacon de mesure) est proportionnel à la durée de la collecte. À titre indicatif, cette contamination, variable avec le passé de la colonne, est de l'ordre de 1 à 2 imp/min

pour une durée de collecte égale aux 2/10 du temps d'émergence du méthyl-palmitate, de l'ordre de 30 min dans nos analyses.

(2) *Traînées du pic contaminant*

On peut suspecter comme causes de contamination les traînées, antérieure et postérieure, d'un pic, traînées invisibles sur la ligne de base. Pour apprécier ce facteur, nous avons injecté du méthyl-palmitate 1-^{14}C et mesuré l'activité ^{14}C présente dans les collectes effectuées à divers niveaux avant et après le pic. Nous schématisons dans la Fig. 2 les niveaux moyens et les intervalles de collecte et nous rapportons les

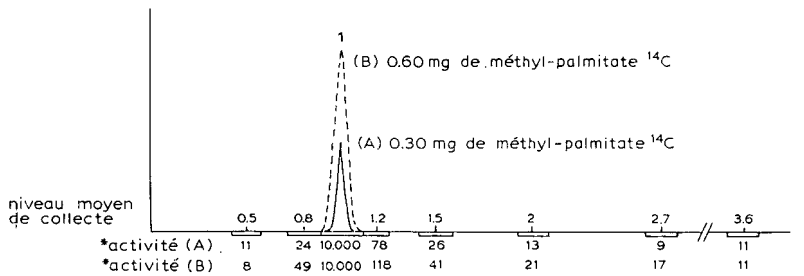

Fig. 2. Contamination des collectes après injection de méthyl-palmitate ^{14}C. Unité de temps: temps d'émergence du méthyl-palmitate. Durée de chaque collecte: 0.2.
* Activité = activité collectée—bruit de fond de collecte antérieur à l'injection.

activités de contamination observées à ces niveaux. L'unité de temps est le temps d'émergence du méthyl-palmitate et l'activité collectée dans le pic contaminant est ramenée à 10,000 imp/min. On effectue deux expériences pour, respectivement, 0.30 et 0.60 mg de méthyl-palmitate injecté.

Dans les conditions normales d'expérience, la contamination par traînées de pic est généralement négligeable, sauf évidemment pour les pics contigus au pic contaminant. Bien que les pics relatifs aux esters méthyliques d'acides gras, même dissymétriques, vérifient certaines des propriétés d'une distribution normale[8], les activités observées dans les traînées du pic contaminant sont très supérieures aux activités escomptées d'après une distribution normale. La dissymétrie de la contamination, plus élevée après qu'avant le pic, est opposée à la dissymétrie du pic, de descente plus rapide que la montée. Des phénomènes de transestérification, rétention et émission ultérieure des esters d'acides gras, sont responsables d'une contamination dissymétrique de ce type. Les acides gras transestérifiés par échange avec l'acide adipique du polyester sont renouvelés par des acides gras ultérieurs transestérifiés à leur tour. Les polyesters volatils émis continuellement par la phase stationnaire participent aussi à ces transestérifications, conduisant ainsi au bruit de fond de collecte.

En plus des phénomènes de transestérification, on doit invoquer d'autres mécanismes de rétention, dans la phase stationnaire ou sur le support, suivis de réémission. D'après les observations de MEINERTZ ET DOLE[9] concernant des analyses sur colonne apiezon et sur colonne polyester "rapide" — réduisant les risques de transestérification — il nous paraît que la contamination par transestérification est la plus à craindre. Enfin, outre ces divers processus, encore mal connus, la contamination

peut résulter de l'émission, très lente et non détectée sur le chromatogramme, de composés radioactifs provenant d'analyses antérieures.

(3) Contamination d'une analyse

Les phénomènes de transestérification étant continus tout au long de l'analyse, on s'attend à des transestérifications d'autant plus appréciables que les dimensions à la fois des pics contaminants et des pics contaminés sont plus importantes, les pics les plus contaminés étant les plus proches des pics contaminants. Dans le but de vérifier cette hypothèse, nous avons injecté trois fois successivement du méthyl-palmitate inactif à la suite de l'expérience désignée par B dans la Fig. 2. Dans la Fig. 3, sont

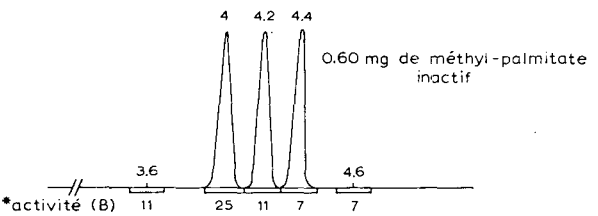

Fig. 3. Contamination des collectes. Suite de l'experience (B) de la Fig. 2 (mêmes légendes).

données les valeurs des activités collectées. Le premier pic entraîne effectivement une fraction plus importante du contaminant palmitate et le bruit de fond de collecte reprend ensuite sa lente décroissance.

Pour apprécier l'ordre de grandeur des contaminations résultantes dans une analyse complète (acides gras de phosphoglycérides), nous avons ajouté du méthyl-palmitate ^{14}C à un ensemble d'esters inactifs, collecté diverses fractions au cours de l'analyse, et mesuré l'activité de ces fractions, pour deux dimensions d'analyse. Dans la Fig. 4 sont schématisées les conditions de l'expérience et les activités de contamination observées au niveau de différents esters.

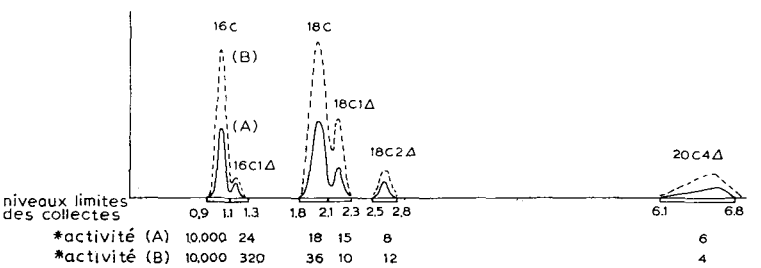

Fig. 4. Contamination des collectes, par le méthyl-palmitate ^{14}C, dans une analyse d'esters méthyliques d'acides gras de phosphoglycérides (mêmes légendes que Fig. 2). (A): analyse de 1 mg d'ester environ. (B): analyse de 2 mg.

Compte tenu des durées de collecte, les contaminations sont du même ordre de grandeur que précédemment (Fig. 2) à partir du stéarate. La forte contamination du palmitoléate provient surtout, évidemment, d'une mauvaise séparation du palmitate.

DISCUSSION ET CONCLUSIONS PRATIQUES

Dans une analyse complète, les esters sont contaminés par une fraction de tous les esters émergeant avant eux. Les résultats obtenus ci-dessus permettent de calculer l'ordre de grandeur des contaminations relatives à chacun d'eux.

Le contrôle du bruit de fond de collecte au cours de l'analyse, si possible en encadrant les divers esters, permet d'apprécier les contaminations. Dans la pratique, on contrôle les corrections de contaminations provoquées par les esters les plus radioactifs. Le calcul des contaminations selon le principe établi ci-dessus n'est qu'approximatif mais permet de décider si les activités attribuées à un ester donné sont valables ou non. Dans ces conditions de rectification, l'activité observée au niveau du linoléate après administration d'acétate ^{14}C chez l'animal est de l'ordre de grandeur d'une activité par contamination.

Il est clair que, lorsque les rectifications ainsi calculées sont importantes devant les activités mesurées, il faut, pour obtenir des résultats valables, effectuer de nouvelles analyses selon des méthodes différentes.

Dans le cas où les activités spécifiques des esters d'acides gras saturés sont très différentes de celles des esters d'acides insaturés, on séparera selon des méthodes conventionnelles soit les acides saturés des acides insaturés, soit les esters d'acides saturés des esters d'acides insaturés[10,11]. Après ce fractionnement préalable, on effectuera sur deux colonnes distinctes l'analyse et la collecte des esters méthyliques des acides gras saturés d'une part, des acides insaturés d'autre part. Selon la nature des problèmes étudiés et selon les résultats ainsi obtenus, on pourra, au moins dans certains cas, se dispenser de recourir aux méthodes classiques de fractionnement qui nécessitent des quantités appréciables de produits.

Enfin, étant donné que, d'une part les séparations sont meilleures, d'autre part les phénomènes de transestérification et de rétention par le support sont d'autant moins notables que la température est moins élevée, on aura intérêt à effectuer les analyses sur polyester à une température modérée, de l'ordre de 180° pour le polyéthylène glycol adipate.

Le méthyl-palmitate 1-^{14}C utilisé dans ce travail a été mis à notre disposition par le Commissariat à l'Energie Atomique, Département de Biologie, selon le contrat No. 2761.

RÉSUMÉ

Les fractions collectées au cours d'une analyse d'esters méthyliques d'acides gras par chromatographie gaz–liquide sur phase stationnaire polyester sont contaminées par suite de phénomènes de transestérification. L'ordre de grandeur des contaminations par du méthyl-palmitate ^{14}C est déterminé dans diverses conditions d'analyse et l'on discute certaines conclusions pratiques.

SUMMARY

The methyl esters of fatty acids collected during analysis by gas–liquid chromatography on a polyester stationary phase are contaminated owing to trans-esterification processes. The contamination by ^{14}C methyl palmitate is essayed under different conditions of analysis. Practical conclusions are discussed.

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IMPROVED LIQUID ELUTION COLUMN SYSTEM

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INTRODUCTION

Liquid column chromatography (LCC) is one of the methods of choice for the preparative separation of fragile biological materials and compounds of very high molecular weight. The method may never equal the remarkable performance of the rival gas–liquid chromatography (GLC) in respect to speed and packing stability, but would seem to be highly susceptible to improvement in other points of technique. In some cases the fault can be traced to some inadequacy in equipment design brought about by the use of glass. Yet, except in special applications requiring an all-glass apparatus, the use of glass can be limited to the column itself. Here visibility is helpful in guiding packing operations and in techniques where the movement of bands needs to be followed. Therefore, any non-contaminating material is justified which will help by introducing desirable features in other parts.

Sample loading, particularly in partition chromatography, is a delicate operation requiring skill and experience. While the purpose of this procedure is to minimize the volume of the starting band and to promote its even distribution, none of the several techniques proposed hitherto is infallibly successful in achieving this result or compares in speed, simplicity and reproducibility with the injection technique used in GLC. A successful adaptation of the latter to LCC as described below involves mechanical features only accessible through the use of metal.

Similarly, a removable column bottom fitting was designed to eliminate post-column dead volume effects as described by VANDENHEUVEL[1], and other potential drawbacks of conventional systems. Use of easily replaceable filter paper disks in the packing support system, has made available a range of porosities and a uniformity in pore size not found in commonly used sintered glass, and has eliminated cleaning problems. Removability of the bottom fitting, which has also become a feature of commercial equipment, is helpful in techniques calling for the stepwise extrusion of the packing, and in general, in column unloading and cleaning.

The above, and other features described below, have required designs resulting in a system where complete tightness at glass to metal junctions is safely obtained with a little compression of standard "O" rings. This may be achieved through the use of precision bore glass tubing for the column.

VANDENHEUVEL AND HAYES[2], BAULD AND GREENWAY[3], BUSH[4], and others, have insisted upon selecting for the column glass tubing of uniform bore, free of defects, in order to promote packing uniformity.

Precision bore tubing columns answer this description ideally and furthermore they permit the safe application of useful packing techniques having potential drawbacks in ordinary columns. Thus, when a filter paper disk is placed over the column packing to protect the top layer during sample loading operations, uneven contact along the disk periphery may promote formation of preferential flowpaths. Skewness in elution bands may also result from defects in the top layer structure when pressure is being applied with the packing piston, and the latter happens to deviate from the vertical even slightly. Both drawbacks are eliminated by the use of perfectly fitting packing tools in precision bore tubing columns as described by VANDENHEUVEL AND VATCHER[5].

Temperature control of the column has long been established as an important factor contributing to the reproducibility of LCC results, particularly in partition chromatography. Jacketed columns are therefore used extensively. On the other hand, columns of various diameters and lengths are routinely used in many laboratories to cope with a variety of problems. The authors found it very convenient to group in a simple thermostated tank columns of various diameters. Fastened through "O" ring unions, the columns are effectively protected and remain completely visible at all times. Fittings for 1, 1/2 and 3/8 in. columns are described in the present article along with a thermostated tank system used by the authors. By reducing appreciably the time usually spent in erecting, disconnecting and cleaning the equivalent number of single column units, by eliminating the related paraphernalia, the breakage, and much of the bench space that would otherwise be occupied, compact and versatile systems of this sort improve the management of LCC problems.

DESCRIPTION OF EQUIPMENT

*Multicolumn tank system**

Glass columns A, A... (Fig. 1) are fitted in tank B. Windows C, forming the front and back of the tank are made of 1/4 in. thick Lucite fastened to the tank frame by 1 in.-spaced, 1/16 in. diam. flat-head metal screws. Water tightness is obtained by a 1/16 in. thick, 1/4 in. wide Neoprene gasket cemented to the tank frame and lightly coated on the Lucite side with silicone grease. The frame itself is built by silver-soldering two vertical plates E to the top and bottom plates of the tank. Side plates E extend below the tank to base plate F. The tank frame and base plate are made of 1/4 in. thick plate. A series of fittings G and H** are lead-soldered to the bottom and top plate respectively, permitting quick fastening of columns. These are ready for use when fitted with bottom fitting J.

Thermostated water, supplied from a bath not shown, is circulated through the tank (inlet K, outlet L). Alternately, the tank itself is fitted with a heating element and temperature regulator, and the water recirculated through an outside pump.

The rate of flow of the solvent being fed through M (elevated reservoir, or pump) is read on flow meter N and adjusted by needle valve O. The solvent is led through

* Unless stated otherwise, all metal parts described under this heading are made of brass.
** Details of parts G, H, J, R and S are given below.

the tank by stainless steel coil P. The supply of constant temperature solvent emerging at Q can be connected to any of the columns in use. It is shown connected to stainless steel injection device R and column piston S.

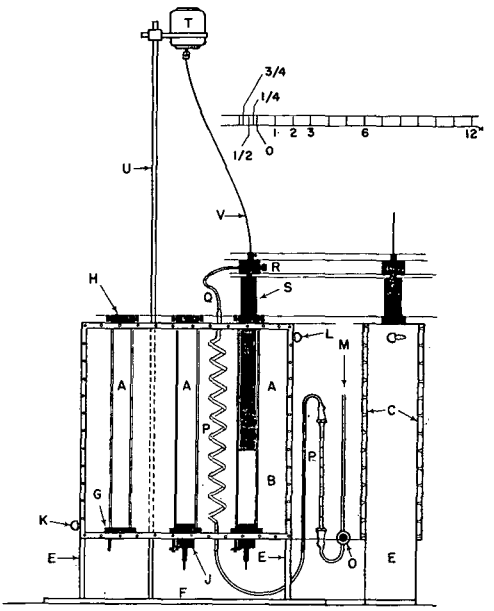

Fig. 1. Multicolumn tank system.

Stirrer motor T, supported by rod U is connected to R by a length of piano wire or a flexible shaft V.

In our present set up, 6 columns (two of each of the three diameter sizes described below) are fitted in a tank less than 11 in. wide. A total effective column length of 5.5 ft. is obtained thereby in a tank light and compact enough to be easily transported. The columns, the only glass parts in this equipment, are well protected from shock by the fastening system used and by the tank itself.

Columns are fastened in the tank through the arrangement shown in Fig. 2 where fittings G and H corresponding to 1 in. columns A, A... are described. Tightness is ensured by compression of "O" rings 3 and 6 obtained by screwing threaded collars 1 and 4 on corresponding collars soldered to bottom plate BP and top plate TP respectively. Neoprene gaskets 2 and 5 protect the column ends and prevent vertical motion. Only the diameters of parts just described need be adapted to fit columns of other sizes.

*Column fittings**

Bottom fittings J for the 1 (I, II, III), 1/2 (IV), and 3/8 in. (V) columns are shown in Fig. 3. All include three main parts, *i.e.*, threaded cap 7, central threaded spindle 8, and tightening sleeve 9. In the 1 in. column only, perforated cone 10 is included in

* All metal parts described under this heading are made of type 316 stainless steel.

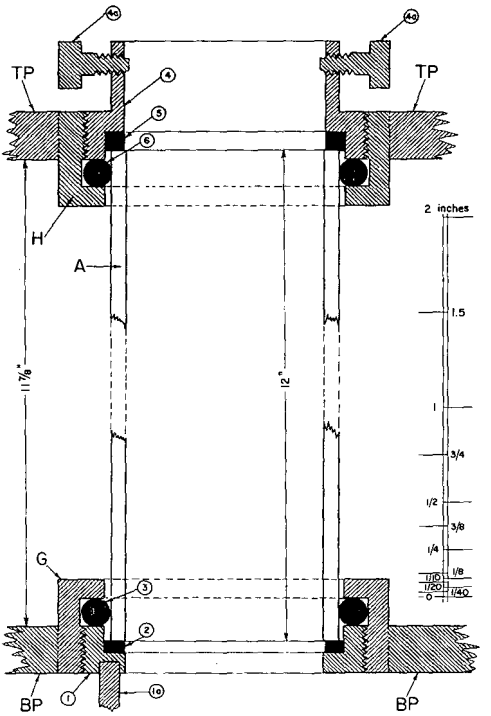

Fig. 2. Axial section showing tank fittings G and H for 1 in. column.

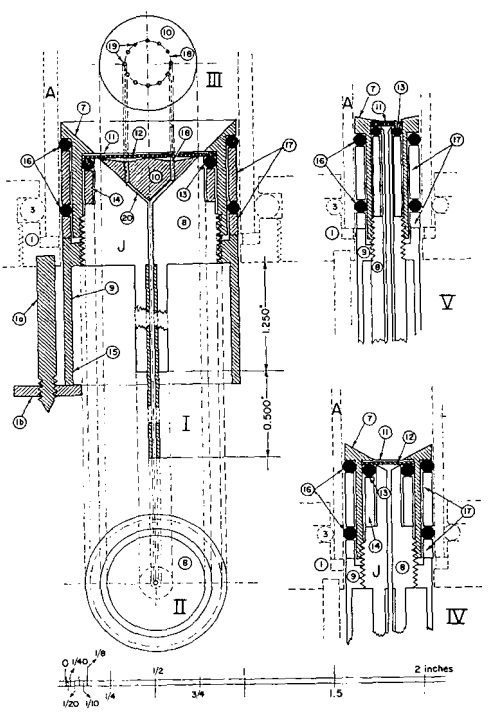

Fig. 3. Axial sections of bottom fittings for the 1 (I), 1/2 (IV) and 3/8 in. (V) columns. Bottom view of 1 in. fitting shown in (II); top view of perforated cone 10 shown in (III).

order to fill the space below the filtering plate. In all columns, the latter is composed of filter paper disk 11 supported by metal gauze disk 12. The disks are squeezed firmly by "O" ring 13 which ensures complete tightness. The "O" ring is compressed by pressure transmitted by spacer ring 14 when parts 7 and 8 are screwed together. Tightening of the bottom fitting in a glass column is obtained simply by rotating 15 while holding 8 by the central outlet tube. It is then locked in position by screwing 1b on 1a.

Inside edges at both ends of the glass column must be polished by grinding to avoid damage to "O" rings.

In the 1 in. column, liquid flowing through 11 and 12 collects in groove 18, flows from holes 19 into space 20 around the recessed cone apex, and emerges in the central outlet tube.

Column piston S (Fig. 4) comprises piston head 21 which is tightened by compressing "O" rings 22. This is obtained by rotating tightening sleeve 23 while holding injection head R immobile. Pressure is thereby transmitted to the "O" rings by

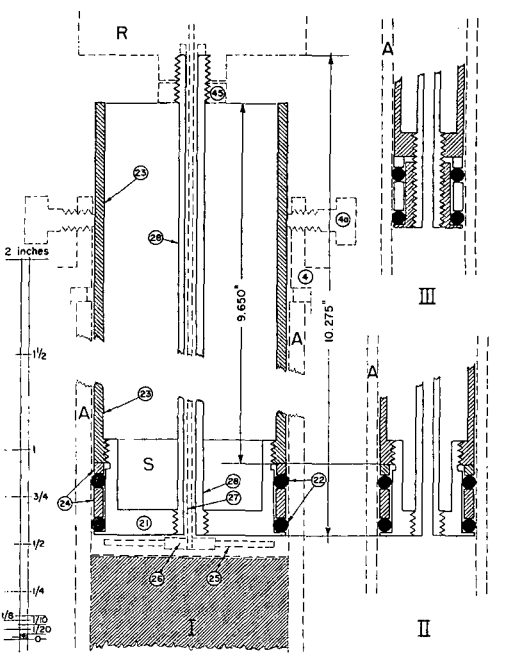

Fig. 4. Axial sections of column pistons for 1 (I), 1/2 (II) and 3/8 in. (III) columns.

spacer rings 24. Screws 4a are then used to lock the column head in position. This position should be such as to leave just enough space between packing and piston head to permit rotation of stirrer arms 25. Six such arms are fastened to hub 26. Stirrer shaft 27 passes through tube 28 connecting column piston S to injection device R. Parts 28 and 21 are cemented together by heat-cured epoxy resin* laid within their threaded junction.

* Epon adhesive VIII, Shell Corporation.

Injection device R shown in Fig. 5 can be used with all columns down to 3/8 in. diameter. It consists of two main threaded metal parts 29 (bottom) and 30 (top). Tightness at the junction of tube 28 and part 29 is ensured by Teflon washer 31. A larger Teflon insert 32 is located between 29 and 30. Leakage is prevented by gland 33 ("O" ring and spacer ring) and by "O" ring 34. Both "O" rings are compressed when 29 and 30 are screwed together. Insert 32 then forms the ceiling of injection

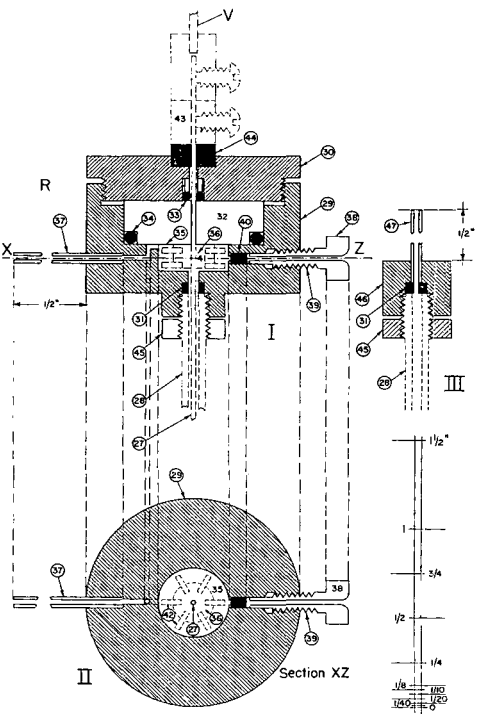

Fig. 5. Axial section of injection device (I) and adapter (III). Transverse section XZ (II).

chamber 35. The latter contains stirrer 36 fastened to stirrer shaft 27. Solvent proceeds to the injection chamber through inlet 37 while the sample is injected through port 38. The 22 to 24 gauge needle of the syringe containing the sample is inserted through screw 39 and pierces septum 40*. The effective length of the 3/4 in. syringe needle is adjusted by a guard so that when the latter butts against 38, the needle will almost reach hub 41 of the stirrer as it emerges between the two rows of stirrer arms 42. Slowly incoming solvent will mix thoroughly with the injected sample. The resulting mixture will be carried down quickly and completely to the mixing chamber atop the column packing since the combined free volume of chamber 35 and tube 28 is only 0.15 ml.

Stirrer shaft 27 is connected to motor drive shaft V by the collar and screw arrangement shown. Adjustment of the pressure of hub 41 on Teflon insert 32 is obtained through collar and screw 43, while metal to metal friction is avoided by

* 1/16 in. thick fluorocarbon rubber plug preceded by 0.005–0.010 in. thick Teflon disk.

Nylon washer 44. Connecting tube 28 is locked to part 29 by hexagonal nut 45. Fig. 5 (III) shows the connector replacing injection head R for intercolumn connection.

Table I shows the sizes and materials for all "O" rings used in the fitting for 1, 1/2 and 3/8 in. columns.

TABLE I

INSIDE DIAMETER, I.D., CROSS-SECTIONAL DIAMETER, W, AND MATERIALS OF "O" RINGS FOR COLUMN FITTINGS OF VARIOUS SIZES

Related part	*"O" ring**	*Material***	*Actual dimensions**** *(in.) of standard "O" rings*					
			1 in. column		*1/2 in. column*		*3/8 in. column*	
			I.D.	*W*	*I.D.*	*W*	*I.D.*	*W*
G, H (Fig. 2)	3 and 6	A	1.109	0.139	0.612	0.103	0.487	0.103
J (Fig. 3)	13	B	0.614	0.070	0.145	0.070	0.056	0.060
J (Fig. 3) and S (Fig. 4)	16 and 22	Back A Front B	0.864	0.070	0.364	0.070	0.239	0.070
R (Fig. 5)	33	A	I.D.	0.029				
			W	0.040				
	34	B	I.D.	0.614				
			W	0.070				

* Number refers to number in figure quoted.

** A = Silicone, fluorosilicone or fluorocarbon rubbers; B = Teflon for general application; in most applications, fluorocarbon rubber.

*** ± tolerances (consult Catalog 5700, Parker Seal Co., Culver City, Calif.).

COLUMN OPERATION

Packing and loading

The middle column shown in Fig. 1 is fitted with bottom fitting J and ready to be packed. As a preliminary step, all air is expelled from the bottom fitting by connecting the solvent supply to the bottom outlet and allowing solvent to rise just above the filter paper while a slight vacuum (rubber bulb) is being applied from the top of the column. The column outlet is then closed and the packing slurry poured into the column.

Any packing mixture and any packing method described in the literature can be used. The packing procedure described by VANDENHEUVEL AND VATCHER[5] is particularly suitable to the present system since it takes advantage of precision bore columns by using precision packing tools.

Packing operations should be concluded by inserting a precisely cut, slightly oversized, thick filter paper disk which is pressed against the levelled packing with the packing piston. The necessary pressure is reproducibly obtained by placing the same weight on a plate fastened at the end of the packing piston shaft for the same length of time.

The column head is introduced after cautiously removing the packing piston and adding some solvent. Excess solvent will eventually enter the piston head and be discharged in a beaker held at the solvent inlet. Repeated suction applied to this inlet while the piston is being lowered will remove all air bubbles from the system.

The piston is finally locked in the final position already described. The solvent inlet is then connected to the solvent supply and solvent allowed to run from the column outlet into a beaker. Whatever conditioning is required by the LCC method used is carried out at this stage. In any case, solvent is allowed to percolate until the eluate is perfectly clear. The column outlet is then connected to whatever collecting or monitoring device is required.

Loading of the sample is carried out after arresting the flow of solvent and starting the stirrer motor (300–400 r.p.m.). While a liquid mixture can be injected as such, a solution or fine suspension of the liquid or solid sample in the minimum amount of solvent is generally used. A finely divided solid or liquid dispersion may result from mixing of the sample within the injection chamber. Solvent must be admitted very slowly at a rate and for a time predetermined experimentally. 2–3 ml in excess of the amount needed to obtain a clear solution in the mixing chamber above the packing is usually sufficient. The flow rate is then adjusted to the desired operational level and the stirrer motor switched off.

Column combinations

The smallest column length used with the present equipment is 2.5 in. for any of the column diameters. This can reach a maximum of 11.5 in. with one column and 23 in. with two. In the latter case the bottom outlet of the first column is connected to the inlet of the second with small gauge plastic tubing*, the second column being fitted with the adapter shown in Fig. 5.

The interesting effects described by HAGDHAL[6] can be obtained by interconnecting columns of decreasing diameter. These have the advantage of visibility over the metal-clad column system proposed by this author.

When a monitoring instrument is used with solvent of constant composition, some of the solvent supply is diverted to the standard cell as described by VANDENHEUVEL AND SIPOS[7]. With linear gradient elution the same procedure is often sufficient. In non-linear situations, however, complete compensation for solvent variations can only be achieved with solvent percolated through a twin column, the solvent supply being equally divided between the two columns.

Any unused column fitted with J (Fig. 1) will function as a mixing device for continuous gradient solvent production. The required volume of starting solvent (*cf.* LEBRETON[8]) is placed in the column and the column head is lowered to the top of the liquid**. Continuous mixing of diluting solvent flowing from the bottom produces the required graded solvent emerging from tube 37 (Fig. 5).

DISCUSSION

The present equipment resulted from the modification of an early model where 1/4 in. wall precision bore tubing was used. This type of tubing, a current production item in 1956*** is only available on special order today. Although the substitution of inexpensive 2 mm wall precision bore tubing necessitated a more elaborate design, the resulting equipment proved equally effective and simple to use.

* Gauge 18 Teflon spaghetti, Fluorocarbon Products Inc., Camden 1, N. J.
** The normal stirrer is replaced by a simple wire loop reaching the bottom of the column.
*** Precision Glass Products Company, Philadelphia 38, Pa.

In both column bottom fitting (Fig. 3) and column piston (Fig. 4) use is made of two external "O" rings. Soft fluorocarbon or silicone rubber is used for the backing "O" ring in order to obtain enough lateral expansion to ensure tightness under low compression. Made of tougher, non-contaminating Teflon, the "O" ring on the solvent side will expand only enough to prevent diffusion from either side.

Use of Teflon insert 32 (Fig. 5) avoids metal to metal friction of moving parts while stirrer 36 prevents segregation and immobilization of still undissolved sample particles. Both the rate of flow during the loading period and the dead volume constituted by the chamber above the packing should be kept as small as possible to ensure minimum initial band volume. Although the proposed loading procedure should be effectively equivalent to that usually practiced, the results are far less dependent on operator skill and experience in being determined by two easily reproducible conditions, *viz.*, the flow rate of solvent and the length of time assigned for this flow.

The example shown in Fig. 6 was chosen to permit comparison with GLC data obtained with the same mixture of methyl esters. It will be noted that a small negative peak appears at point F. This is an artifact resulting from a slight change in

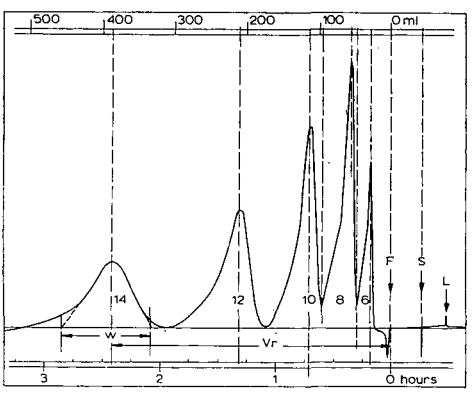

Fig. 6. Separation of methyl esters of the even numbered saturated fatty acids from C_6 to C_{14} by partition chromatography. Sample, 70 μl injected at L. From L to S, sample loading at reduced flowrate. Constant flowrate (2.62 ml/min) from S to end. Solvent front at F. In 1 in. column, 80 g siliconized firebrick holding 19 ml iso-octane. Developing solvent, 82% (v/v) ethanol–H_2O. Chromatogram obtained with recording differential refractometer described by VANDENHEUVEL AND SIPOS[7].

solvent composition following exchange with the injected sample. Point F corresponds to the solvent front and the small negative "ghost" peak is an enlarged reflection of the initial band. Indeed it is found that a plot of the logarithms of true emergence volumes V_r (counted from F to the peak maxima positions) against carbon numbers is rectilinear. The KEULEMANS[9] expression: N.T.P. = 16 $(V_r/w)^2$, yields 150 as the number of theoretical plates corresponding to n-C_{14} for this 8 in. column, or 225 theoretical plates per foot. This is comparable to 230 theoretical plates per foot (n-C_{14}) found by HAWKE *et al.*[10] with a 6 mm polyethylene glycol succinate column in the GLC separation of the same esters. Separations obtained with other column packings, some resulting in even better resolution and reduced tailing, will be described in a future article.

CONCLUSIONS

LCC equipment has not reached the degree of development and effectiveness found in its GLC counterpart. This is partly due to prejudice regarding design and materials from which the rival method appears to be free. Undoubtedly, successful work will still be carried out in simple LCC apparatus. It is even safer to predict, however, that without the limitations imposed by conventional equipment, LCC will make faster progress. The proposed sample injection device and column fittings unite several novel features with others already described in a combination eliminating many pitfalls in packing, loading and general procedure. The compact multicolumn arrangement, on the other hand, should improve sturdiness, management and versatility. Primarily designed for the comparative study of packing materials, this equipment is well suited to the standardization of conditions and the routine application of many forms of LCC, above all, partition chromatography. The high column resolution demonstrated by the example in Fig. 6 should encourage a search for further improvements.

SUMMARY

A compact, sturdy and versatile thermostated multicolumn system is described. The design of the stainless steel fittings for the 1, 1/2, and 3/8 in. precision bore glass columns permits facile adaptation of the system to most liquid column chromatography problems while virtually eliminating many pitfalls involved in packing and sample loading.

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A QUALITATIVE AND QUANTITATIVE STUDY OF THE SEPARATION OF UROPORPHYRIN OCTAMETHYL ESTERS I AND III BY DIOXAN CHROMATOGRAPHY

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Several methods are available for identification of uroporphyrin isomers; these include (a) dioxan chromatography (FALK AND BENSON[1]), (b) decarboxylation (EDMONDSON AND SCHWARTZ[2]) followed by lutidine chromatography of the coproporphyrin thus produced (ERIKSEN[3], RIMINGTON AND MILES[4]) or chromatography of the coproporphyrin esters (CHU, GREEN AND CHU[5]), (c) separation on Hyflo columns (CHU AND CHU[6]), (d) determination of melting point and mixed melting point (NICHOLAS AND RIMINGTON[7]), (e) infra-red spectrophotometry (FALK AND WILLIS[8]), (f) X-ray diffraction pattern (KENNARD AND RIMINGTON[9]). Some conflict between results obtained by dioxan chromatography and those obtained by other methods has been encountered both in this laboratory and by other authors (WATSON AND BERG[10], BOGORAD AND MARKS[11]). Some light has been thrown on the problem by the work of BOGORAD AND MARKS[11] who demonstrated, by means of labelled compounds, that when mixtures of uroporphyrin I and III esters were separated by dioxan chromatography the resulting spots each contained both isomers. Since the dioxan method is the least laborious there was a great need for its thorough investigation with a view to improving its reliability and sensitivity.

In the present investigation an attempt has been made to find optimal conditions for separating quantitatively mixtures of the isomers containing known amounts of uroporphyrin I varying between 0 and 100 % of the total ester. Estimation of the amount of ester in the I and the III positions respectively of the developed chromatogram was carried out by elution and spectrophotometry. It seems that quantitative separation of mixtures containing 50 % or less of the I isomer depends closely on the total amount of ester applied and subsequently eluted from the paper. In its final form, the technique may also be used for the separation of mixtures of unknown isomeric composition and for their quantitative evaluation.

MATERIALS

Solvents

The kerosene used was colourless commercial heating paraffin dried over calcium chloride and filtered. Chloroform, B.P., was washed three times with water, dried over calcium chloride and filtered. (It was found essential that washed chloroform, especially that used for elution, should not be used after five days storage.) Ethanolic

chloroform was prepared by adding ethanol to a concentration of 1 % (v/v) to chloroform treated as above. 1,4-Dioxan was purified by a method similar to that of EIGENBERGER[12] but with incorporation of a step designed to remove peroxides. Crude dioxan (1 l) was refluxed for 7 h (without the application of an air stream) in the presence of 120 ml of 4 % (w/v) hydrochloric acid. Peroxides were removed by shaking the mixture with excess of solid sodium sulphite: some solid potassium hydroxide was then added and the heavy white precipitate of inorganic salts removed by decantation and filtration. Shaking with potassium hydroxide pellets was then repeated until there was no further formation of an aqueous layer. The solvent was then dried over sodium wire and distilled as described by EIGENBERGER. Although free from peroxides, the dioxan at this stage still contained some aldehydic material which had not been removed by the original refluxing. This impurity was removed by fractional freezing, collecting the fraction which froze at 11.5°. The dioxan (b.p. 101–103°; m.p. 11.5°) was stored in dark bottles at 4°, at which temperature peroxide formation was reduced to a minimum.

Standard reference substances

Uroporphyrin I octamethyl ester, m.p. 293°, (uro I ester) was fraction Ai shown in Fig. 1 of the paper of RIMINGTON AND MILES[4]; further data concerning it are given by RIMINGTON AND SVEINSSON[13]. Uroporphyrin III octamethyl ester, m.p. 255–258°, (uro III ester) was the synthetic ester of TARLTON, MACDONALD AND BALTAZZI[14]. Coproporphyrin I tetramethyl ester, m.p. 253–256°, (copro I ester) was isolated from calf meconium. Coproporphyrin III tetramethyl ester, m.p. 155/170°, (copro III ester) was obtained from *Corynebacterium diphtheriae* by the method of GRAY AND HOLT[15]. Protoporphyrin IX dimethyl ester, m.p. 230°, was prepared from haemin by the method of GRINSTEIN[16]. Biosynthetic specimens were obtained by incubation of haemolysates of human erythrocytes with porphobilinogen, isolation of the uroporphyrin fraction and its esterification.

METHODS

Chromatography

Chromatography of the esters followed essentially the method of FALK AND BENSON[1]. Solutions of standard uro I and uro III esters were prepared at a concentration of 1 μg/10 μl in washed chloroform. This was achieved by weighing slightly more than the desired amount, dissolving, measuring the extinction at 405–406 mμ of a suitably diluted aliquot, using $E^{1\%}_{1\,cm} = 2300$ for uro III (RIMINGTON[17]) and $E^{1\%}_{1\,cm} = 2264$ for uro I (RIMINGTON[18]) and adjustment of volume. Mixtures of uro I and uro III esters were then prepared in varying ratios from these solutions, and stored at 4° when not in use. The concentrations of the chloroform solutions of the uro esters obtained from enzymic incubation were estimated by eqn. (1) below. Such solutions were then taken to dryness and the esters redissolved in sufficient chloroform to give 1 μg/10 μl.

Spots were applied by means of a micro-pipette, graduated in 5 μl, along a baseline 2 cm from one edge of a 21 cm square of Whatman No. 1 paper such that they would run with the grain of the paper.

Development of the chromatograms was carried out by the ascending method

at 22–26°. (Within this temperature range no variation in chromatographic behaviour occurred). Glass cylinders, one for each run (25.0 cm internal height; 13.2 cm internal diameter) were used. An inverted Petri dish was placed in position at the bottom of the tank and the solvent used to saturate the atmosphere was placed in the bottom of the tank, the lid greased carefully with yellow soft paraffin and placed in position. 15–30 min later the solvents for the developing mixture were pipetted into a second Petri dish, mixed well and the Petri dish placed on the inverted dish. The chromatogram, rolled in a cylinder, was then inserted and the lid replaced quickly and firmly. After development and drying, the spots were detected by their fluorescence in ultraviolet light. The tanks were cleaned, greased and equilibrated for each chromatogram.

First run: Atmosphere, 10 ml ethanolic chloroform; developing mixture, 4 ml kerosene + 6 ml ethanolic chloroform. Development was continued until the top of the spots as seen in ordinary light had moved 4 cm from the base-line (indicated by a mark made previously at the edge of the paper). Time 15–20 min. After drying the paper and marking lightly in pencil the positions of the spots (the dotted line in Figs. 1, 5 and 6) the base of the paper was cut off 0.5 cm below the bottom of the spots. No isomer separation takes place during this run, which is designed merely to separate the esters from impurities which are left on the base-line.

Second run: Atmosphere, 7 ml dioxan; developing mixture, 4.0 ml kerosene + 1.0 ml dioxan (effect of variation in volume of dioxan was tested, see RESULTS, but unless otherwise indicated a volume of 1.0 ml was used). Development was continued until the solvent had reached the top of the paper (1.5 h). The paper was dried thoroughly (30 min in a warm place) and the spots marked very lightly in pencil.

For calculation of R_F values, the base-line was taken as the centre of the spot after the first run.

Elution of chromatograms

Fluorescent spots were marked lightly with pencil, cut out from the paper and each eluted in 3 ml of freshly washed chloroform by standing in stoppered tubes for 1 h with occasional shaking. The extinction of the chloroform solutions was measured in a Unicam model SP. 500 photoelectric spectrophotometer, at a 1 cm light path. The complete process, from application of material to spectrophotometric reading, was carried out within 4 h. The ester content of the eluates was calculated from the following expression (for derivation, see APPENDIX):

$$\mu g \text{ uro ester} = [2D_{405-6} - (D_{389} + D_{430})] \times 2.721 \times V \qquad (1)$$

where V = volume of solution;

D = extinction at wave-length indicated by the subscript.

Decarboxylation

5–20 μg of the uro esters obtained by enzymic incubation were decarboxylated by the method of EDMONDSON AND SCHWARTZ[2]. The decarboxylation tubes were sealed at a pressure equal to 1.5 mm Hg after holding them at that pressure for 10 min. Decarboxylation of this quantity of uro ester at this pressure yielded 100% recovery of the decarboxylation product, coproporphyrin. If the tubes were sealed in air or while attached to the water pump, however, very large or complete destruction

of the porphyrin ensued, an effect also noted by EDMONDSON AND SCHWARTZ[2]. The free coproporphyrin thus produced was prepared for lutidine chromatography by one of the following means: (a) pH was adjusted to 4 with saturated sodium acetate and the coproporphyrin transferred quantitatively to ether; the ether was washed three times with water and the aqueous washings extracted with fresh ether. The ethereal solutions were combined, washed with a very small quantity of water and evaporated to dryness. (b) As (a) followed by esterification and subsequent hydrolysis by the method of FALK, DRESEL, BENSON AND KNIGHT[19] using methanol–H_2SO_4 (95:5, v/v) as the esterification mixture.

Samples prepared by either (a) or (b) were dissolved in 2 *N* NH_4OH for lutidine chromatography. When duplicate samples were taken and one prepared by method (a) the other by (b) they were found to give the same result on lutidine chromatography and, therefore, method (a) was made the standard method of preparation because of its relative simplicity.

Lutidine chromatography

Isomer identification of the free coproporphyrin obtained by decarboxylation of the uro esters was carried out essentially by the method of ERIKSEN[3].

RESULTS

Variation in ratio of kerosene to dioxan

A series of runs was made to determine the optimal ratio of kerosene to dioxan required to separate quantitatively 1 μg of a 50:50 mixture of uro I and III esters. The results set out in Table I demonstrate clearly that decrease in the concentration of dioxan caused less of the I isomer to be carried up with the III isomer. It was apparent that the optimal ratio required to separate 1 μg of a 50:50 mixture was of the order of 4.0:1.0.

TABLE I

EFFECT OF VARIATION IN RATIO OF KEROSENE TO DIOXAN ON SEPARATION OF 1 μg OF A 50:50 MIXTURE OF URO I AND III ESTERS

Kerosene: dioxan	*Ester eluted*		
	I position (μg)	*III position (μg)*	*Apparent ratio I:III*
4.0:1.30	0.27	0.49	36:64
4.0:1.25	0.24	0.50	32:68
4.0:1.20	0.28	0.52	35:65
4.0:1.15	0.32	0.47	41:59
4.0:1.10	0.26	0.43	38:62
4.0:1.05	0.52	0.47	53:47
4.0:1.00	0.51	0.45	53:47

Variation in quantity of ester mixture applied

The separation was studied of a range of quantities of a 50:50 mixture of uro I and III esters by means of kerosene–dioxan mixtures of ratio 4.0:1.00 and 4.0:1.05 respectively. On visual examination, spots could be seen in the I and III positions in all

cases, but it was obvious that along the series the uro I spot increased in intensity and size much more than did the III spot, (Fig. 1). The results of elution from two chromatograms, set out in Table II, demonstrate that the quantity of ester eluted from the I position became an increasing proportion of the total ester applied as the amount

TABLE II

EFFECT ON SEPARATION OF VARIATION IN AMOUNT OF 50:50 MIXTURE OF URO I AND III ESTERS APPLIED

Kerosene: dioxan	*Ester applied (μg)*	*Ester eluted*		
		I position (μg)	*III position (μg)*	*Apparent ratio I:III*
4.0:1.05	0.30	0.02	0.18	10:90
	0.50	0.17	0.28	38:62
	0.75	0.27	0.39	41:59
	1.00	0.52	0.47	52:48
	1.50	0.95	0.70	58:42
	2.00	1.34	0.72	65:35
4.0:1.00	0.30	0.03	0.17	15:85
	0.50	0.16	0.28	36:64
	0.75	0.36	0.41	47:53
	1.00	0.51	0.45	53:47
	1.50	0.92	0.61	60:40
	2.00	1.37	0.71	66:34

of the latter increased; the theoretical 50 % separation was obtainable only at one point. The two solvent ratios used gave essentially similar results. In subsequent work a kerosene–dioxan ratio of 4.0:1.0 was employed.

Recovery of esters by elution from chromatograms

From a comprehensive survey of our data, elution from chromatograms was found to vary between the extreme limits of 50 % and 100 %, most usually approximating

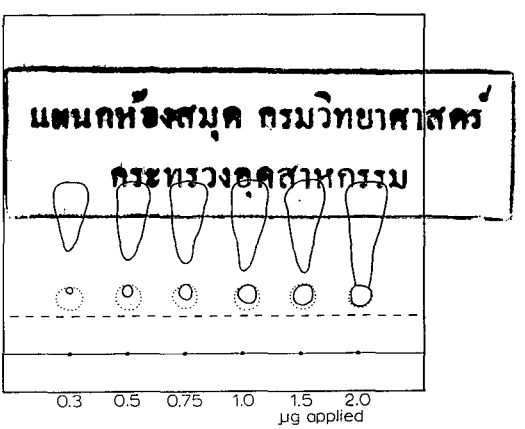

Fig. 1. Paper chromatogram obtained by applying a series of amounts of a 50:50 mixture of uroporphyrin I and III esters (kerosene–dioxan = 4.0:1.0). Dotted lines mark positions of spots after the first run and the solid lines their positions after final development. The bottom of the paper was cut off along the horizontal dashed line after the first run.

70%. The reason for this variability remains unknown; it does not depend upon greater stability or preferential elution of one or other isomer since a similar range of variability was found at all isomer ratios.

It was noted that better agreement with expected values was obtained in any

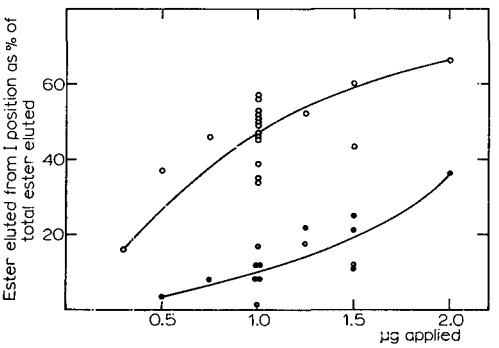

Fig. 2. Relationship between amount of ester applied and separation. Values were obtained by applying a series of amounts of two different ratios of uroporphyrin I and III esters to several chromatograms each run in kerosene–dioxan = 4.0:1.0. O Uro I ester : uro III ester = 50:50; ● uro I ester:uro III ester = 25:75.

separation by basing calculations upon the total ester eluted (I + III positions) rather than upon the total applied to the paper. The importance of this observation will become apparent in the next section.

Variation in ratio of uro I and III esters applied

Runs were made on a range of mixtures of uro I and III esters using a kerosene–dioxan ratio of 4.0:1.0. Each mixture was applied over a range of quantities and the spots in the I and III position were eluted and measured in the usual way. Fig. 2 relates the apparent isomer distribution to the amount of known mixture applied in the case of two mixtures of standards (I:III = 25:75 and I:III = 50:50 respectively). Whilst there is some evidence of interdependence, this is unimpressive. When, however, the proportion of apparent uro I was plotted against the total ester

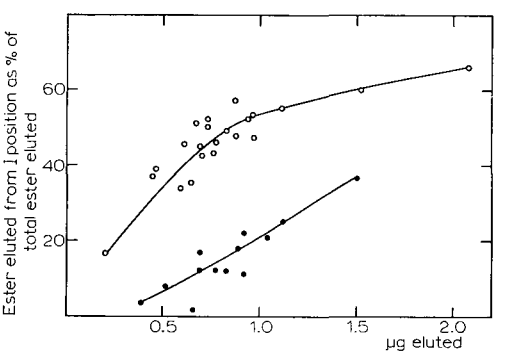

Fig. 3. Relationship between amount of ester eluted and separation. Values were obtained from the same chromatograms as those for Fig. 2. "μg eluted" refers to the sum of the amounts eluted from the I and III positions. O Uro I ester : uro III ester = 50:50; ● uro I ester : uro III ester = 25:75.

eluted from the paper, as in Fig. 3, a clearly defined relationship was discernible, for each of these two mixtures. When comparison was made of a range of ratios of uro I and III esters, it became apparent that with increase in the proportion of the I isomer in the mixture applied quantitative separation was increasingly less dependent on the amount of ester eluted.

As there exists a different optimal amount to be eluted for each ratio of I to III,

TABLE III

RELATIONSHIP OF A, B AND C IN EXPRESSION (2) TO THE TOTAL μg OF ESTER ELUTED

x = total μg of ester (I + III) eluted,
$A = 30.77 - 10.51x - 21.49x^2 + 16.04x^3$
$B = 0.7509 - 0.3755x + 0.5997x^2 - 0.5619x^3$
$C = -0.000999 + 0.005780x - 0.004834x^2 + 0.004361x^3$

x*	A	B	C
0.40	24.2	0.661	0.00082
0.42	23.8	0.657	0.00090
0.44	23.4	0.654	0.00098
0.46	23.0	0.650	0.00106
0.48	22.6	0.647	0.00114
0.50	22.2	0.643	0.00123
0.52	21.8	0.639	0.00131
0.54	21.4	0.635	0.00140
0.56	21.0	0.630	0.00149
0.58	20.6	0.625	0.00158
0.60	20.2	0.620	0.00167
0.62	19.8	0.615	0.00177
0.64	19.5	0.609	0.00186
0.66	19.1	0.603	0.00197
0.68	18.7	0.596	0.00207
0.70	18.4	0.589	0.00218
0.72	18.1	0.582	0.00229
0.74	17.7	0.574	0.00240
0.76	17.4	0.565	0.00252
0.78	17.1	0.556	0.00264
0.80	16.8	0.547	0.00277
0.82	16.6	0.536	0.00290
0.84	16.3	0.526	0.00303
0.86	16.1	0.514	0.00317
0.88	15.8	0.502	0.00332
0.90	15.6	0.489	0.00347
0.92	15.4	0.475	0.00362
0.94	15.2	0.461	0.00379
0.96	15.1	0.446	0.00395
0.98	14.9	0.430	0.00413
1.00	14.8	0.413	0.00431
1.02	14.7	0.396	0.00450
1.04	14.6	0.377	0.00469
1.06	14.6	0.357	0.00489
1.08	14.6	0.337	0.00510
1.10	14.6	0.316	0.00532
1.12	14.6	0.293	0.00554
1.14	14.6	0.270	0.00577
1.16	14.7	0.245	0.00601
1.18	14.8	0.220	0.00626
1.20	14.9	0.193	0.00651

* Linear interpolation may be used for intermediate values of x, but the table should not be extended beyond 0.40 and 1.20.

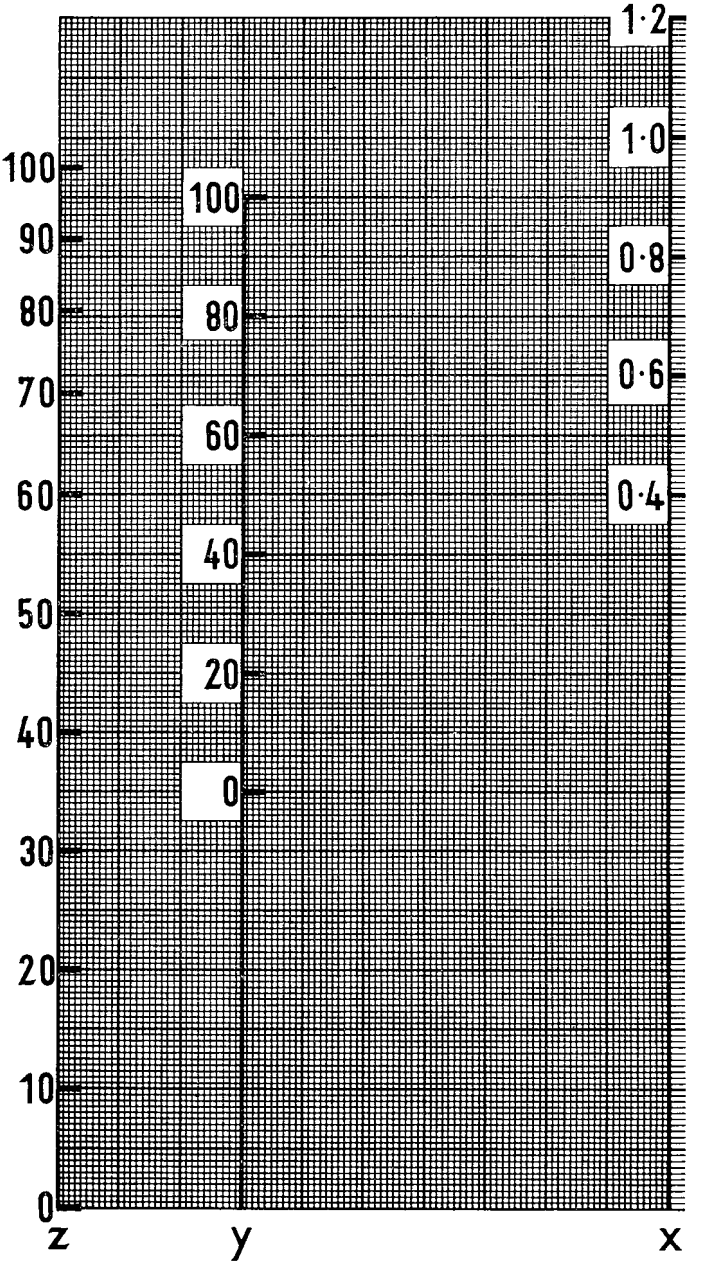

Fig. 4. Nomograph for correction of ratios of uro isomers found by elution. Connect "μg of total uro ester (I + III) eluted" (scale x) with "apparent percentage of uro I isomer found by elution" (scale y) and read off "true percentage of I isomer" (scale z).

it is necessary to correct the ratio found against the amount eluted. This may be done by use of the expression:

$$z = A + By + Cy^2 \qquad (2)$$

where z = the true percentage of the I isomer;

y = the apparent percentage of the I isomer found by elution,

and A, B and C are constants related to the total μg of ester eluted (x) and which may be found by reference to Table III.

For derivation of expression (2) see APPENDIX. Alternatively, correction may be made by use of the nomograph illustrated in Fig. 4, where x, y and z have the same meaning as in eqn. (2) and Table III.

Table IV sets out the results from several chromatograms of the separation of a range of isomer ratios; both the elution figures and the corrected values are included. It can be seen that when between 17.5 and 100 % of uro I is present in a mixture of I and III, it may be detected and estimated within an accuracy of 7 % (mean error = 2.2 %) by use of eqn. (2). The nomograph presents a more simple method of correction but its efficacy falls off when between 90 % and 100 % of the I isomer is found by elution. Since when $y = 0$, $z = A$ in eqn. (2), erroneous results would be obtained in attempts to correct a zero value. In practice, therefore, if no spot is visible in the I position it may simply be concluded that between 0 and 17.5 % of uro I is present. Quantities less than 17.5 % cannot be detected by elution, but 12.5–17.5 % of the I isomer may be detected visually by the tailing of the spot in the III position onto, but not into, the I position.

When a sample of chromatography paper other than Whatman No. 1, namely "Ederol No. 202", was tested, variation in the pattern of separation occurred such that mathematical correction did not yield a theoretical result. It seems, therefore, that the relationship governed by eqn. (2) breaks down with change of paper.

Fig. 5 has been prepared from a typical chromatogram run on 1 μg each of ratios of uro I and III esters ranging between 0:100 and 100:0.

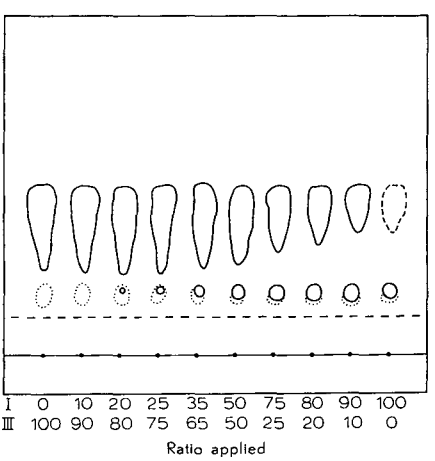

Fig. 5. Paper chromatogram of a series of ratios of uroporphyrin I and III esters. 1.0 μg of each ratio was applied (kerosene–dioxan = 4.0:1.0). Dotted lines mark positions of spots after the first run, solid lines their positions after final development and dashed line the position of a very weak spot after final development. The bottom of the paper was cut off along the horizontal dashed line after the first run.

TABLE IV

SEPARATION OF VARYING AMOUNTS OF A RANGE OF RATIOS OF URO I AND III ESTERS IN KEROSENE–DIOXAN (4.0:1.0)

Ester mixture applied				*Ester eluted*					*Apparent ratio (1:III) corrected*	
Ratio I:III	*I (μg)*	*III (μg)*	*I + III (μg)*	*I position (μg)*	*III position (μg)*	*Sum of I + III positions (μg)*	*Recovery (%)*	*Apparent ratio I:III*	*By nomograph*	*By eqn. (2)*
0:100	0.00	1.00	1.00	0.00	0.55	0.55	55	0:100		
0:100	0.00	1.00	1.00	0.00	0.62	0.62	62	0:100		
0:100	0.00	1.00	1.00	0.00	0.66	0.66	66	0:100		
0:100	0.00	1.00	1.00	0.00	0.67	0.67	67	0:100		
0:100	0.00	1.00	1.00	0.00	0.68	0.68	68	0:100		
0:100	0.00	1.00	1.00	0.00	0.69	0.69	69	0:100		
0:100	0.00	1.00	1.00	0.00	0.70	0.70	70	0:100		
0:100	0.00	1.00	1.00	0.00	0.79	0.79	79	0:100		
0:100	0.00	1.25	1.25	0.00	0.97	0.97	77	0:100		
0:100	0.00	1.50	1.50	0.00	1.04	1.04	69	0:100		
0:100	0.00	1.50	1.50	0.00	1.08	1.08	72	0:100		
0:100	0.00	2.00	2.00	0.14	1.32	1.46	73	10:90		
0:100	0.00	2.00	2.00	0.51	0.96	1.47	73	35:65		
10:90	0.10	0.90	1.00	0.00	0.53	0.53	53	0:100		
10:90	0.10	0.90	1.00	0.00	0.69	0.69	69	0:100		
10:90	0.15	1.35	1.50	0.00	0.73	0.73	49	0:100		
10:90	0.10	0.90	1.00	0.00	0.77	0.77	77	0:100		
10:90	0.15	1.35	1.50	0.00	1.02	1.02	68	0:100		
10:90	0.20	1.80	2.00	0.57	0.97	1.54	77	37:63		
17:83	0.175	0.825	1.00	0.03	0.66	0.69	69	5:95	22:78	22:78
20:80	0.20	0.80	1.00	0.03	0.71	0.74	74	4:96	20:80	20:80
20:80	0.20	0.80	1.00	0.05	0.71	0.76	76	6:94	21:79	21:79
20:80	0.30	1.20	1.50	0.04	0.79	0.83	55	5:95	19:81	19:81
20:80	0.25	1.00	1.25	0.09	0.82	0.91	73	10:90	20:80	21:79
25:75	0.125	0.375	0.50	0.02	0.37	0.39	78	5:95	27:73	27:73
25:75	0.188	0.563	0.75	0.04	0.49	0.53	70	9:91	27:73	26:74
25:75	0.25	0.75	1.00	0.02	0.64	0.66	66	3:97	20:80	20:80
25:75	0.25	0.75	1.00	0.12	0.57	0.69	69	17:83	30:70	29:71
25:75	0.25	0.75	1.00	0.08	0.61	0.69	69	12:88	26:74	26:74
25:75	0.25	0.75	1.00	0.06	0.66	0.72	72	8:92	23:77	23:77
25:75	0.25	0.75	1.00	0.06	0.67	0.73	73	8:92	23:77	23:77
25:75	0.25	0.75	1.00	0.10	0.67	0.77	77	13:87	25:75	24:76
25:75	0.375	1.125	1.50	1.10	0.73	0.83	55	12:88	24:76	23:77
25:75	0.313	0.938	1.25	0.16	0.73	0.89	71	18:82	26:74	26:74
25:75	0.313	0.938	1.25	0.20	0.72	0.92	74	22:78	29:71	28:72
25:75	0.375	1.125	1.50	0.11	0.82	0.93	61	12:88	21:79	21:79
25:75	0.375	1.125	1.50	0.22	0.82	1.04	69	21:79	25:75	25:75
25:75	0.375	1.125	1.50	0.28	0.84	1.12	75	25:75	26:74	25:75
25:75	0.50	1.50	2.00	0.56	0.95	1.51	75	37:63	Beyond limits	
30:70	0.30	0.70	1.00	0.10	0.53	0.63	63	16:84	31:69	30:70
30:70	0.375	0.875	1.25	0.22	0.78	1.00	80	22:78	27:73	26:74
35:65	0.35	0.65	1.00	0.15	0.54	0.69	69	22:78	34:66	33:67
35:65	0.438	0.813	1.25	0.28	0.66	0.94	75	30:70	34:66	33:67

(continued on p. 151)

TABLE IV (*continued*)

Ester mixture applied				*Ester eluted*					*Apparent ratio (I: III) corrected*	
Ratio I: III	*I (μg)*	*III (μg)*	*I + III (μg)*	*I position (μg)*	*III position (μg)*	*Sum of I + III positions (μg)*	*Recovery (%)*	*Apparent ratio I: III*	*By nomograph*	*By eqn. (2)*
40:60	0.40	0.60	1.00	0.21	0.51	0.72	72	29:71	38:62	37:63
40:60	0.50	0.75	1.25	0.32	0.66	0.98	73	33:67	35:65	33:67
50:50	0.15	0.15	0.30	0.03	0.17	0.20	67	15:85	Beyond limits	
50:50	0.25	0.25	0.50	0.16	0.28	0.44	88	36:64	50:50	49:51
50:50	0.50	0.50	1.00	0.18	0.28	0.46	46	39:61	51:49	50:50
50:50	0.50	0.50	1.00	0.20	0.39	0.59	59	34:66	45:55	43:57
50:50	0.50	0.50	1.00	0.28	0.33	0.61	61	46:54	53:47	52:48
50:50	0.50	0.50	1.00	0.22	0.42	0.64	64	34:66	44:56	43:57
50:50	0.50	0.50	1.00	0.35	0.32	0.67	67	52:48	55:45	55:45
50:50	0.50	0.50	1.00	0.31	0.37	0.68	68	46:54	50:50	50:50
50:50	0.75	0.75	1.50	0.30	0.40	0.70	70	43:57	49:51	48:52
50:50	0.50	0.50	1.00	0.38	0.35	0.73	73	52:48	54:46	54:46
50:50	0.50	0.50	1.00	0.37	0.37	0.74	74	50:50	53:47	52:48
50:50	0.75	0.75	1.50	0.33	0.43	0.76	51	43:57	47:53	46:54
50:50	0.375	0.375	0.75	0.35	0.42	0.77	102	45:55	49:51	49:51
50:50	0.50	0.50	1.00	0.41	0.42	0.83	83	49:51	50:50	50:50
50:50	0.50	0.50	1.00	0.50	0.37	0.87	87	57:43	55:45	55:45
50:50	0.625	0.625	1.25	0.49	0.45	0.94	75	52:48	50:50	50:50
50:50	0.50	0.50	1.00	0.51	0.45	0.96	96	53:47	50:50	50:50
50:50	0.50	0.50	1.00	0.46	0.52	0.98	98	47:53	45:55	45:55
50:50	0.50	0.50	1.00	0.62	0.49	1.11	111	56:44	49:51	49:51
50:50	0.75	0.75	1.50	0.92	0.61	1.53	102	60:40	Beyond limits	
50:50	1.00	1.00	2.00	1.37	0.71	2.08	104	66:34	Beyond limits	
75:25	0.75	0.25	1.00	0.48	0.17	0.65	65	74:26	74:26	75:25
75:25	0.75	0.25	1.00	0.57	0.19	0.76	76	75:25	71:29	73:27
75:25	0.75	0.25	1.00	0.65	0.19	0.84	84	77:23	80:20	76:24
75:25	1.125	0.375	1.50	0.76	0.14	0.90	60	84:16	78:22	81:19
75:25	1.125	0.375	1.50	0.97	0.23	1.20	80	81:19	75:25	73:27
80:20	0.80	0.20	1.00	0.45	0.12	0.57	57	79:21	81:19	79:21
80:20	0.80	0.20	1.00	0.47	0.12	0.59	59	80:20	81:19	80:20
80:20	0.80	0.20	1.00	0.59	0.15	0.74	74	80:20	78:22	79:21
80:20	1.20	0.30	1.50	0.71	0.17	0.88	59	81:19	73:27	77:23
80:20	1.20	0.30	1.50	0.85	0.11	0.96	64	89:11	80:20	86:14
90:10	0.90	0.10	1.00	0.60	0.07	0.67	67	90:10	90:10	88:12
90:10	0.90	0.10	1.00	0.66	0.04	0.70	70	94:6	95:5	93:7
90:10	0.90	0.10	1.00	0.65	0.06	0.71	71	92:8	92:8	91:9
90:10	1.35	0.15	1.50	0.91	0.04	0.94	63	97:3	90:10	95:5
90:10	1.35	0.15	1.50	0.92	0.11	1.03	69	89:11	80:20	86:14
100:0	1.00	0.00	1.00	0.60	0.00	0.60	60	100:0	108:0	99:1
100:0	1.00	0.00	1.00	0.71	0.00	0.71	71	100:0	103:0	99:1
100:0	1.00	0.00	1.00	0.74	0.00	0.74	74	100:0	102:0	99:1
100:0	1.00	0.00	1.00	0.77	0.00	0.77	77	100:0	100:0	99:1
100:0	1.00	0.00	1.00	0.82	0.00	0.82	82	100:0	98:2	99:1
100:0	1.25	0.00	1.25	1.04	0.00	1.04	83	100:0	90:10	99:1
100:0	1.50	0.00	1.50	1.18	0.00	1.18	79	100:0	88:12	99:1
100:0	2.00	0.00	2.00	1.62	0.00	1.62	81	100:0	Beyond	limits

TABLE V

R_F VALUES OF ESTERS OF PORPHYRINS WITH 2–8 CARBOXYL GROUPS RUN IN KEROSENE–DIOXAN (4.0:1.0)

Ester	*Source*	R_F
Uroporphyrin I	Standard reference	0.00
Coproporphyrin I	Standard reference	0.04
Uroporphyrin III	Standard reference	0.23
Coproporphyrin III	Standard reference	0.66
Protoporphyrin IX	Standard reference	0.74
Uroporphyrin I	Enzymic	0.00
Uroporphyrin III	Enzymic	0.21
Heptacarboxylic porphyrin III?	Enzymic	0.40
Hexacarboxylic porphyrin III?	Enzymic	0.50
Pentacarboxylic porphyrin III?	Enzymic	0.58
Coproporphyrin III	Enzymic	0.66

Separation of esters of porphyrins with 2–8 carboxyl groups

Table V sets out the R_F values of esters of uroporphyrin, coproporphyrin and protoporphyrin run in a kerosene–dioxan mixture of 4.0:1.0. In addition, the table shows R_F values of three spots found between uro III and copro III positions when the uro fraction obtained from enzymic incubation of porphobilinogen is chromatographed (see Fig. 6). These three spots are assumed to be the esters of porphyrins belonging to the III series with seven, six and five carboxyl groups per molecule respectively (see DISCUSSION).

DISCUSSION

When dioxan from which peroxides had not been removed was used, very poor elution yields of the esters were obtained and separation was far from quantitative; hence

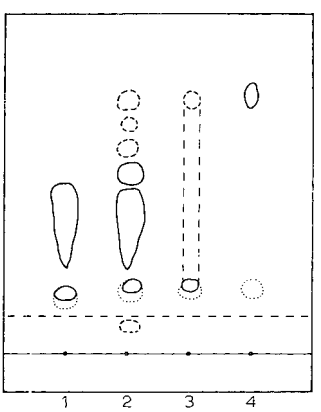

Fig. 6. Paper chromatogram relating chromatographic behaviour of uroporphyrin ester obtained enzymically to standard porphyrin esters (kerosene–dioxan = 4.0:1.0). (1) 50:50 mixture of uroporphyrin I and III esters; (2) material obtained by separation and esterification of uroporphyrin fraction after enzymic incubation of porphobilinogen; (3) coproporphyrin I ester; (4) coproporphyrin III ester. Dotted lines mark positions of spots after the first run, the solid lines their positions after final development and the dashed lines the positions of weak spots after final development. The bottom of the paper was cut off along the horizontal dashed line after the first run.

the need for peroxide-free dioxan was established. FALK AND BENSON[1] used a kerosene–dioxan ratio of 4.0:1.5 but pointed out that the optimal ratio required for separation of mixtures of uroporphyrin I and III esters varied with different batches of dioxan. Thus, in the work reported here the optimal ratio found (4.0:1.0) was relatively low with respect to dioxan as compared with the FALK AND BENSON ratio, obviously as a result of the higher purification of the dioxan.

It seems that the amount of uro I found in the I position after running a chromatogram depends on several factors, including:

(a) the amount of uro I applied,
(b) its partial loss during chromatography,
(c) the amount of uro III applied with it.

In the same way, the amount of uro III present in the III position depends on:

(a) the amount of uro III applied,
(b) its partial loss during chromatography,
(c) the amount of uro I applied with it.

There can be no doubt that some form of molecular association occurs between the two isomers (NICHOLAS AND RIMINGTON[7], EDMONDSON AND SCHWARTZ[2] and BOGORAD AND MARKS[11]). This is further revealed by the present investigation for whereas all the uro I applied alone remains in the I position, yet the presence of uro III will cause some of the I to move up to the III position. Similarly, the addition of uro I to uro III will cause some III to stay down. Contribution by each of the above factors results in a very delicate equilibrium being established which governs the separation of a given mixture of the isomers.

The complexity of the equilibrium is further demonstrated by the following observation: under conditions where a 50:50 mixture separated such that equal amounts were eluted from the I and III positions, several applications of such mixture were run and the ester eluted from all the I positions combined and that from all the III positions combined. When these were each re-chromatographed and estimated under conditions identical with those above, corrected separations of 65:35 (I:III) and 35:65 (I:III) respectively were noted.

Since the way in which a given ratio separates depends not only on that ratio but also on the amount of ester present on the paper, it follows that there is an optimal amount of ester which should be present so that the correct ratio will be found on elution. As the optimal amount was found, however, to vary with the ratio, for example 0.8 μg for a 50:50 mixture and 1.1 μg for a 25:75 (I:III) mixture, inevitable "under-" or "over-loading" in practice necessitates a correction of the ratio found on elution in order to arrive at the true ratio.

Our data have shown, moreover, that the "optimal amount" relates to the total quantity of ester elutable from the paper rather than to the amount applied. Recoveries by elution varied over a range of 50–100 %, most generally being of the order of 70 %: it was noted, however, that variation in recovery of a given amount of ester was reflected in the separation and that such consequent irregularities in separation could be corrected by a formula which contained a term for the amount eluted. It is, therefore, assumed that low recovery results from a loss of ester early in the chromatographic process, perhaps during the first run, before separation has taken place, otherwise such a relationship between separation and amount eluted would not be apparent. 0.5 μg of a 50:50 mixture chromatographed with 88 %

recovery gave essentially the same result as 1.0 μg chromatographed with 46% recovery. That the relationship between separation and amount eluted is not due to a greater relative stability of one isomer is shown by the fact that the same range of yield was found for all ratios of I to III.

No explanation of the physicochemistry of the chromatographic behaviour of the mixed isomers can be offered, but even without such understanding it is considered that with the aid of the empirically-derived formula (2), which relates the equilibrium set up on the paper by a given amount of mixed isomers to their true ratio, the ratio of uro I to uro III in a given mixture may be determined with 7% accuracy.

The formula was found to give accurate results for all ratios of I to III when between 0.4 and 1.2 μg of total uro ester was eluted. Since recovery lay between 50 and 100%, then 1.0 μg of any mixture of uro esters is the most suitable amount to be applied. Uro esters derived from biological sources or from biochemical reactions often contain porphyrin esters other than uro and their proportion had to be determined roughly by a preliminary dioxan chromatogram before an amount of the sample was known which contained approximately 1 μg of uro itself: for example, if the preliminary chromatogram showed approximately 30% of fluorescent material other than uro then 1.5 μg of the sample was applied.

The esters other than uro obtained from enzymic incubation and present in the uro fraction are seen on chromatograms as one to four spots above the uro III position. The most mobile coincides with standard copro III and is only present in very faint traces, if at all, when copro has been separated from uro prior to chromatography. The three spots lying between uro III and copro III are assumed to be the esters of porphyrins of the III series with seven, six and five carboxyl groups per molecule respectively (see Table V and Fig. 6). That they do belong to the III series is deduced from the fact that under these chromatographic conditions uro I and copro I have R_F values of 0.00 and 0.02 respectively and it seems most likely that any esters of the I series with a number of carboxyl groups intermediate between eight and four would also have very low R_F values. The materials being considered have R_F values, however, 0.40, 0.50 and 0.58 respectively. That they contain seven, six and five carboxyl groups per molecule, respectively, is deduced from their positions between uro III and copro III and relative to each other. Those spots provisionally ascribed to penta- and hexacarboxylic porphyrin esters were always very faint, but the spot assumed to contain heptacarboxylic porphyrin ester was often of very significant concentration. The porphyrin responsible for this spot has been designated pseudouroporphyrin by FALK[20], FALK, DRESEL, BENSON AND KNIGHT[19] and FALK AND DRESEL[21]. This spot was eluted at the same time as the uro ester spots and its concentration, as a percentage of the uro, determined with only a small error by using the same $E^{1\%}_{1cm}$ and k values as for uro, (eqn. (1)). It was the intensity of this spot which needed to be allowed for in assessing the amount of sample required to be applied such that the spot would contain approximately 1 μg of uro. It was, of course, always necessary when working with unknowns to run standard mixtures on the same chromatogram and to ascertain that they gave theoretical ratio values on elution and corrected calculation. 100% uro I ester, 100% uro III ester and a 50:50 mixture of these were routinely used in the present investigation.

The method has been used, and found to yield highly reproducible results, in separating the I and III isomers of uro derived from enzymic incubations of por-

phobilinogen. When the uro samples contained a mixture of uro I and III esters, but very little or no pseudouroporphyrin, results by dioxan chromatography were found to agree very closely with those obtained by chemical decarboxylation followed by lutidine chromatography of the copro formed. (The ratio of copro I to copro III after development of lutidine chromatograms was assessed by visual examination of their fluorescence in ultraviolet light. The two spots, being of similar size and shape, may be compared with respect to their relative intensity.) When the pseudouroporphyrin concentration was high, it was found that the ratio of uro I to (uro III + pseudouroporphyrin) agreed closely with results by decarboxylation and lutidine chromatography. This can be taken as further evidence that the spot found above uro III and known as pseudouroporphyrin is a compound which decarboxylates to yield copro III.

After the first run (kerosene–chloroform) of samples derived from enzymic incubation, two spots appear on the paper. The faster-moving spot gives rise after the second run to the characteristic spots of uro I, uro III, and pseudouroporphyrin together with traces of copro III and those materials provisionally designated hexacarboxylic and pentacarboxylic porphyrins. The slow-moving spot, which occurs only in traces is assumed to be either partially esterified porphyrin or a degradation product of the esterification. It is not completely unesterified porphyrin since that material stays firmly on the base line when run as a standard marker whereas our slow-moving material moves a short, but significant, distance from it (see Fig. 6). In practice, the spot is removed when the paper is cut after the first run. When, however, such material, collected after running several heavy applications, was bulked and run in kerosene–dioxan, it moved to the same position as uro III ester, but did not form the characteristic fan-shaped spot. Therefore, if it had been present in large amount and had not been removed completely by cutting the paper after the first run, this unknown material would have contributed to the uro III ester fraction. In practice, the risk of error from this source is negligible.

Although elution figures do not reveal 100 % recovery of the esters applied, but rather a percentage recovery which is generally of the order of 70 %, it is felt that the values obtained for standard isomer solutions by use of the correction formula are sufficiently reproducible to present a method by which the ratio of I and III isomers in a mixture may be estimated within 7 % accuracy. Agreement between the method and the results from decarboxylation and lutidine chromatography supports the efficacy of this means of estimating the ratio of uro I : uro III : pseudouroporphyrin by dioxan chromatography.

APPENDIX

Derivation of eqn. (1)

$$\text{Concentration uro III ester (g/100 ml)} = \frac{P_{405-6}}{E^{1\%}_{1cm}}$$

$$= \frac{2D_{405-6} - (D_{380} + D_{430})}{k \times E^{1\%}_{1cm}} \quad \text{(RIMINGTON AND SVEINSSON[13])}$$

$$\mu g = \frac{2D_{405-6} - (D_{380} + D_{430})}{k \times E^{1\%}_{1cm}} \times \frac{10^6 \times V}{100}$$

where:

D = extinction at wave-length indicated by subscript,
V = volume of solution (ml),
$E^{1\%}_{1cm}$ = 2300 (RIMINGTON[17]),
k = 1.6172 (derived by method of RIMINGTON AND SVEINSSON[13]).

Thus:

$$\mu g \text{ uro III ester} = \frac{[2D_{405-6} - (D_{380} + D_{430})] \times 10^6 \times V}{1.6172 \times 2300 \times 100}$$

$$= [2D_{405-6} - (D_{380} + D_{430})] \times 2.689 \times V$$

Similarly:

$$\mu g \text{ uro I ester} = \frac{[2D_{405-6} - (D_{380} + D_{430})] \times 10^6 \times V}{k \times E^{1\%}_{1cm} \times 100}$$

$$= \frac{[2D_{405-6} - (D_{380} + D_{430})] \times 10^6 \times V}{1.6051 \times 2264 \times 100}$$

$$= [2D_{405-6} - (D_{380} + D_{430})] \times 2.752 \times V$$

Therefore, in the mean:

$$\mu g \text{ uro esters} = [2D_{405-6} - (D_{380} + D_{430})] \times 2.721 \times V \qquad (1)$$

Derivation of eqn. (2)

Let x = total μg of uro ester (I + III) eluted,
y = uro ester eluted from the I position, as a percentage of the total uro ester eluted,
z = true percentage of uro I ester.

A relationship between x, y and z was required in the form, $z = f(x, y)$.

From Table IV, y was plotted against x, for z = 20 %, 25 %, 50 %, 75 %, 80 % and 90 %. 0 % and 10 % were omitted, as were 30 %, 35 % and 40 % which only had two values of x and y each. It was found that for values of x between 0.4 and 1.2, the relationship was linear. The values of a and b in the equation $y = a + bx$ were then determined for each value of z.

Using the set of equations, $y = a + bx$, values of y, for fixed values of x between 0.30 and 1.20, were found for each value of z.

y was then plotted against z for each value of x, and it was found that y and z were best related by a quadratic equation:

$$z = A + By + Cy^2 \qquad (2)$$

where A, B and C were all functions of x. z = 80 % was omitted at this stage as it did not fit in with the other figures.

Values of A, B and C were then determined for values of x between 0.30 and 1.20. Graphs of A, B and C *vs.* x were made and it was found that for values of x between 0.4 and 1.2, A, B and C were all best related to x by the following cubic equations:

$$A = 30.77 - 10.51x - 21.49x^2 + 16.04x^3$$
$$B = 0.7509 - 0.3755x + 0.5997x^2 - 0.5619x^3$$
$$C = -0.000999 + 0.005780x - 0.004834x^2 + 0.004361x^3$$

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SUMMARY

A detailed study of the chromatographic separation of uroporphyrin I and III esters by the dioxan method shows such separation to be highly sensitive to several factors. Among these, the quantity of material applied and that subsequently eluted from the paper are very important.

Technical improvements have raised the recovery by elution to about 70 % (limits 50 % and 100 %). Application of a mathematical correction to the results leads to greater accuracy in the analysis of mixtures of isomers.

Using the method now described, it is possible to detect and estimate ratios of the isomers ranging between 17:83 and 100:0 (I:III) within 7 % accuracy.

ADDENDUM

After preparing this communication, it was noted that recovery by elution was markedly improved by minimizing the time of drying the chromatograms. They are now dried at 110° for 3 min after the first run and for 7 min after the second; recoveries of between 90 % and 100 % are thus routinely obtained. Similar values may also be obtained by drying with a strong air blast at 50° for 10 min and 12 min respectively.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

III. THE CORRELATION OF COMPLEX AND SIMPLE MOLECULES. THE CALCULATION OF R_M VALUES FOR TOCOPHEROLS, VITAMINS K, UBIQUINONES AND UBICHROMENOLS FROM R_M(PHENOL). EFFECTS OF UNSATURATION AND CHAIN BRANCHING

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INTRODUCTION

It was shown in the previous communication[1] that MARTIN's equation can be extended to accommodate constitutive effects in molecules. If sufficient R_M data are available from the study of relevant compounds, many constitutive effects can be analysed in terms of ΔR_M parameters, which can then be used to calculate R_M values. Thus, previously[1], the R_M values of a large number of low molecular weight phenols, alkoxyphenols, coumaranols and chromanols were correlated with their structure and with the R_M value of a standard compound, which, in all these investigations, we have taken as phenol. The aim of the investigations described here was to correlate the chromatographic behaviour of more complex molecules, such as the tocopherols, ubiquinones and ubichromenols, with their chemical structure, and to relate them also to the same standard reference compound, phenol. Several new problems must be solved before this can be done: they largely, but not entirely, spring from the experimental limitation that, since these substances are of fairly high molecular weight, they cannot be chromatographed in any system suitable for the low molecular weight phenols. The most complex phenol it was possible to chromatograph in System 1 contained fourteen carbon atoms[1], whereas α-tocopherol contains twenty-nine and ubiquinone 50 fifty-nine carbon atoms. In order to correlate such compounds with phenol, therefore, the following general procedure must be followed. First, every additive group and every constitutive effect in the required complex molecule must be analysed chromatographically and its ΔR_M parameter determined in a suitable system. Secondly, the complex molecule must be chromatographically correlated with the simple molecule by studying compounds intermediate in structure and molecular weight in a progressive series of chromatographic bridging systems. By these means, the ΔR_M parameters can be calculated for the series of systems and from them R_M values for progressively more complex molecules. These values can then be incorporated in the calculation of the R_M value of the required molecule.

Before it was possible to proceed with confidence, it appeared necessary to answer several pertinent questions. Although it was shown previously[1] that MARTIN's equation is obeyed with respect to several atomic and group ΔR_M parameters, when

a series of related compounds are chromatographed in one system, it was not certain that this would be so for other systems. LEDERER[2] has summarised considerable evidence to show that $\Delta R_M(CH_2)$ is not only constant when a number of different chemical series are run in the same system but that MARTIN's equation continues to be obeyed when the same series is run in several different systems. However, as we have already discussed[1], it has on occasion been suggested that the value of ΔR_M for a group may vary according to the remainder of the molecule—that is, even in the absence of constitutive interaction, ΔR_M in a large molecule might be different from ΔR_M in a small molecule. In view, therefore, of the large differences between the molecular weights of the compounds used in this investigation and the diverse nature of the systems employed, it was essential to investigate the constancy of ΔR_M for groups other than CH_2 and also for constitutive effects.

To this end, a number of new compounds were synthesised and chromatographed in several reversed phase partition systems.

PREPARATION OF COMPOUNDS

(*a*) *Mono-ethers of hydroquinones (alkoxyphenols)*

These were made by the general procedure described in the preceding paper[1], except where stated. A number have been described previously[3]. The following were new compounds: *p-n-tetradecyloxyphenol*, m.p. 84°; *p-n-hexadecyloxyphenol*, m.p. 88°; *p-n-octadecyloxyphenol*, m.p. 91.5°; *p-(hex-4-enyloxy)-phenol*, characterised as the *p-nitrophenylurethane*, m.p. 150°; *p-(1-methylbutoxy)-phenol*, b.p. 110–120°/0.5 mm; *p-(2-methylbutoxy)-phenol*, 80–100°/0.15 mm; *p-(1-methylpentyloxy)-phenol*, b.p. 120–140/0.2 mm; *p-(1-ethylbutoxy)-phenol*, b.p. 110–130°/0.15 mm; *p-(1-ethylpentyloxy)-phenol*, b.p. 105°/0.1 mm; *p-(1-propylbutoxy)-phenol*, b.p. 120°/0.15 mm; *p-(1-methylheptyloxy)-phenol*, b.p. 110–120°/0.5 mm; *p-citronellyloxyphenol*, b.p. 150°/0.2 mm; *p-dihydrocitronellyloxyphenol*, b.p. 135–145°/0.6 mm; *p-hexahydrofarnesyloxyphenol*, b.p. 162°/0.1 mm, n_D^{20} 1.4965; *p-dihydrophytyloxyphenol*, b.p. 184°/0.1 mm, n_D^{19} 1.4890; *p-(6-cyclohexylhexyloxy)-phenol*, m.p. 56–58°.

p-(Hexa-2,4-dienyloxy)-phenol (*p-sorbyloxyphenol*) and *p-geranyloxyphenol* were oils that could not be purified by distillation since they are allylic ethers and undergo thermal rearrangement. However, the former compound analysed correctly after chromatography, whilst the latter was characterised as the *p-nitrophenylurethane*, m.p. 117–118°.

5-Dihydrophytyl-4-methoxy-2-methylphenol was prepared by hydrogenation of the previously described 4-methoxy-2-methyl-5-phytylphenol[4], and had b.p. 180°/0.05 mm.

(*b*) *Alkoxyphenyl benzoates*

Most of the benzoates used in this study are known compounds and were prepared by normal methods from the phenols and alkoxyphenols. The following three benzoates were new compounds and analysed correctly: *p-n-butoxyphenyl benzoate*, had m.p. 71.5°; *p-sec.-butoxyphenyl benzoate* had m.p. 53°; *p-tert.-butoxyphenyl benzoate* had m.p. 92.5°.

(*c*) *Tocopherol ethers*

These were prepared by Williamson synthesis. *Tocol allyl ether* was a pale yellow oil and distilled in a short-path still at 160–170° (bath)/$5 \cdot 10^{-3}$ mm (Pirani); λ_{max} 295 mμ

($E^{1\%}_{1cm} = 81.5$); λ_{min} 255 mμ. *β-Tocopherol allyl ether* was obtained similarly; λ_{max} 292.5 mμ ($E^{1\%}_{1cm} = 72.0$); λ_{min} 260 mμ. *δ-Tocopherol allyl ether* was obtained similarly; λ_{max} 295 mμ ($E^{1\%}_{1cm} = 82.5$); λ_{min} 260 mμ.

(d) Ubiquinones and ubichromenols

Ubiquinones 30 and 50 were the generous gift of Hoffmann-La Roche Laboratories, Basle, Switzerland. Perhydroubiquinone 50 has been described[5]. *Dodecahydroubiquinone 30* was prepared in an analogous fashion by reduction of ubiquinone 30, followed by re-oxidation to the quinone; it was not further characterised. Ubiquinol 30 and dodecahydroubiquinol 30 were obtained by reduction of ubiquinone 30 and perhydroubiquinone 30 respectively with potassium borohydride. Hexahydroubiquinone 20 was prepared by the method of SHUNK *et al.*[6]. Hydrogenation followed by re-oxidation to the quinone gave *octahydroubiquinone 20*, which was not further characterised. Hexahydroubichromenol 20 and hexahydroubichromanol 20 have been previously described[6,7].

PAPER CHROMATOGRAPHIC METHODS

Several new chromatographic systems were used to study the higher molecular weight substances. Increasing strengths of aqueous ethanol were used as the mobile phase. Ethyl oleate—used previously with the simple phenols[1]—was now no longer suitable as stationary phase, being too soluble in these increased concentrations of ethanol. It was replaced by olive oil, whose properties are similar to those of ethyl oleate but which is almost insoluble in all but the highest concentrations of ethanol. Olive oil possessed certain advantages over liquid paraffin or petroleum jelly as stationary phase. Being slightly more polar than the latter, ΔR_M increments for carbon were rather smaller and more substances could be run in any one system; this characteristic is especially desirable in structural studies. For compounds with the highest molecular weights, it was necessary to use liquid paraffin for the stationary phase, this substance remaining satisfactory even when pure ethanol was used as the mobile phase. Paraffin/alcohol systems give the largest ΔR_M increments for groups such as CH_2: they are thus especially useful for the discernment of small molecular weight differences between compounds, but have the corresponding disadvantage that the range of compounds run on one chromatogram must be restricted.

Phenols, esters and ethers were visualized on the paper by the methods described previously[1]. The unsaturated long-chain alcohols were visualized by treatment with sulphuric acid. The ubiquinones, ubichromenols, tocopherols, vitamins K and analogous compounds were observed under ultra-violet light as dark spots. All the polynuclear hydrocarbons appeared as dark spots under ultra-violet light, except anthracene, which was brightly fluorescent.

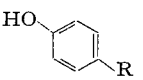
RESULTS WITH SYSTEM 2

System 2 was 70 % (v/v) ethanol against olive oil, and Table I records the R_M values for 54 compounds run in this system. They included phenols, hydroquinone monoethers ranging from *n*-butoxyphenol (No. 5) to *n*-octadecyloxyphenol (No. 16), phenyl benzoates, phenyl nitrobenzyl ethers and several important long-chain isoprenoid alcohols. System 2 provided an important bridge between System 1 (25 %

TABLE I

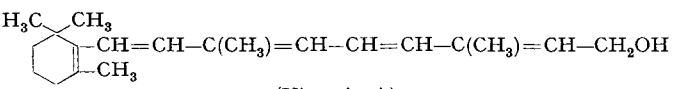
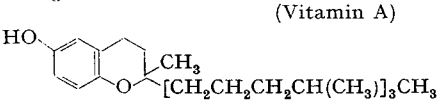
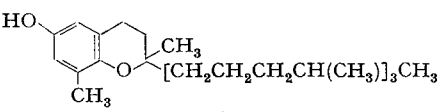
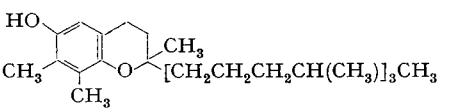
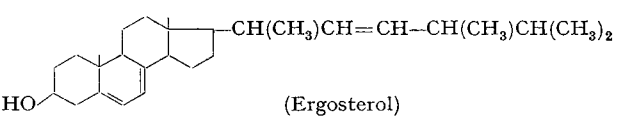
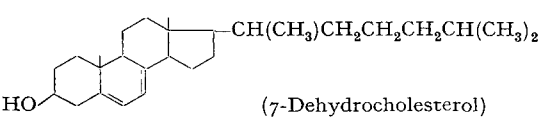
CHROMATOGRAPHY OF PHENOLS, HYDROQUINONE MONO-ETHERS, PHENOL BENZOATES, NITROBENZYL ETHERS AND SOME ISOPRENOID ALCOHOLS IN SYSTEM 2

Stationary phase: Whatman No. 4 paper impregnated with a 5% (v/v) solution of olive oil in light petroleum (40–60°).
Mobile phase: 70% (v/v) ethanol in water.

No.	*Compound*	R_F	R_M
	I. R in structure shown 		
	(a) Phenols		
1	*n*-C_3H_7	0.85	—0.740
2	*n*-C_4H_9	0.80	—0.618
3	*n*-C_5H_{11}	0.75	—0.483
4	$CH_3CH(CH_3)CH_2CH_2$	0.75	—0.483
	(b) Hydroquinone mono-ethers with straight-chain alkoxy groups		
5	*n*-C_4H_9O	0.85	—0.736
6	$CH_3CH(CH_3)CH_2CH_2O$	0.80	—0.600
7	*n*-$C_6H_{13}O$	0.75	—0.476
8	*n*-$C_7H_{15}O$	0.69	—0.347
9	*n*-$C_8H_{17}O$	0.62	—0.215
10	*n*-$C_{11}H_{23}O$	0.40	+0.174
11	*n*-$C_{12}H_{25}O$	0.335	+0.296
12	*n*-$C_{14}H_{29}O$	0.22	+0.554
13	$CH_2=CHCH_2CH_2CH_2O$	0.83	—0.688
14	$CH_3CH=CHCH_2CH_2CH_2O$	0.78	—0.550
15	*n*-$C_{16}H_{33}O$	0.13	+0.811
16	*n*-$C_{18}H_{35}O$	0.08	+1.066
	(c) Hydroquinone mono-ethers with ring-containing alkoxy groups		
17	Phenyloxy	0.81	—0.638
18	Benzyloxy	0.83	—0.678
19	Cyclopentyloxy	0.86	—0.799
20	Cyclohexyloxy	0.83	—0.678
21	6-Cyclohexylhexyloxy	0.37	+0.228
	(d) Hydroquinone mono-ethers with branched alkoxy groups		
22	$CH_3C(CH_3)(CH_3)—CH_2—CH(CH_3)—CH_2—CH_2O$	0.625	—0.225
23	$H[CH_2C(CH_3)=CHCH_2]_2O$ (Geranyloxy)	0.64	—0.255
24	$(CH_3)_2C=CHCH_2CH_2CH(CH_3)CH_2CH_2O$ (Citronellyloxy)	0.60	—0.180
25	$H[CH_2CH(CH_3)CH_2CH_2]_2O$ (Dihydrocitronellyloxy)	0.56	—0.100
26	$H[CH_2CH(CH_3)CH_2CH_2]_3O$ (Hexahydrofarnesyloxy)	0.27	+0.431
27	$H[CH_2CH(CH_3)CH_2CH_2]_4O$ (Dihydrophytyloxy)	0.10	+0.947
	II. Benzoates of phenols and hydroquinone mono-ethers		
28	Phenyl benzoate	0.49	+0.009
29	*p*-Tolyl benzoate	0.42	+0.140
30	3,4-Dimethylphenyl benzoate	0.35	+0.267
31	3,5-Dimethylphenyl benzoate	0.35	+0.267
32	*p*-Ethylphenyl benzoate	0.35	+0.274

(continued on p. 162)

TABLE I (*continued*)

No.	Compound	R_F	R_M
33	*p-n*-Propylphenyl benzoate	0.28	+0.408
34	*p-tert.*-Butylphenyl benzoate	0.25	+0.474
35	*p-n*-Butylphenyl benzoate	0.23	+0.533
36	*p-n*-Butoxyphenyl benzoate	0.29	+0.400
37	*p-sec.*-Butoxyphenyl benzoate	0.33	+0.316
38	*p-tert.*-Butoxyphenyl benzoate	0.37	+0.204
39	5,6,7,8-Tetrahydro-2-naphthyl benzoate	0.27	+0.428
40	4-Diphenyl benzoate	0.21	+0.574
41	2-Naphthyl benzoate	0.295	+0.380
	III. Phenyl nitrobenzyl ethers		
42	Phenyl *p*-nitrobenzyl ether	0.51	—0.020
43	*p*-Tolyl *p*-nitrobenzyl ether	0.44	+0.107
	IV. Isoprenoid alcohols and tocols		
44	$(CH_3)_2C{=}CHCH_2CH_2C(CH_3){=}CHCH_2OH$ (Geraniol)	0.90	—0.918
45	$(CH_3)_2C{=}CHCH_2CH_2CH(CH_3)CH_2CH_2OH$ (Citronellol)	0.87	—0.837
46	$(CH_3)_2C{=}CHCH_2CH_2C(CH_3){=}CHCH_2CH_2C(CH_3){=}CHCH_2OH$ (Farnesol)	0.75	—0.468
47	$(CH_3)_2C{=}CHCH_2CH_2C(CH_3){=}CHCH_2CH_2C(CH_3)CH{=}CH_2$ (Nerolidol)	0.68	—0.328
48	$H[CH_2CH(CH_3)CH_2CH_2]_3CH_2C(CH_3){=}CHCH_2OH$ (Phytol)	0.38	—0.218
49	 (Vitamin A)	0.52	—0.040
50	 (Tocol)	0.135	+0.806
51	 (δ-Tocopherol)	0.10	+0.941
52	 (γ-Tocopherol)	0.08	+1.074
53	 (Ergosterol)	0.10	+0.947
54	 (7-Dehydrocholesterol)	0.09	+1.016

ethanol against ethyl oleate)[1] and the systems described later for use with more complex substances. The main aims of the study with System 2 were to investigate the ΔR_M parameters studied previously[1], to discover how they changed with the change in the system, and to confirm that they remain constant irrespective of the nature of the molecular series. Several phenols and alkoxyphenols were selected and converted into their benzoates and (in a limited number of cases) into their *p*-nitrobenzyl ethers. The compounds could all be chromatographed together in System 2 and, by this means, it was possible to compare ΔR_M increments in virtually identical molecular surroundings, but in different series of compounds running to different points of the same chromatogram. In addition, ΔR_M parameters were checked for constancy in series varying more widely in structure.

$\Delta R_M(CH_2)$ and ΔR_M(ring-attached CH_2) parameters

$\Delta R_M(CH_2)$ was calculated independently from comparison of compounds in three series; phenols, *p*-alkoxyphenols and their respective benzoates. It was found to be $+ 0.129 \pm 0.005$. The results in Table I confirm that the additivity rule is strictly obeyed over a range of ten carbon atoms (*p*-butoxyphenol to *p*-tetradecyloxyphenol) and that $\Delta R_M(CH_2)$ is constant irrespective of the remainder of the molecule, providing constitutive interactions are absent. The value of ΔR_M (ring-attached CH_2) can be calculated, in the usual way, by comparing (i) the benzoates and (ii) the nitrobenzyl ethers of phenol and *p*-cresol. It also is $+ 0.129 \pm 0.003$. Thus, in contrast to the findings[1] in System 1, the two parameters are identical. This arises from the fact that in System 2, $\Delta R_M(CH_2)$ is small. If there was the same relative difference between the two parameters as there was in System 1, it would only amount to about 0.030 in System 2, which is just about experimental error. (Note, however, that different ΔR_M parameters do not by any means change in constant proportion when the system is changed. It would not even seem to be a theoretical requirement that they should all change in the same *direction* when the system is changed—in the chromatography of amino acids, for instance; "crossover" of spots in different systems is familiar. Nevertheless, for the systems studied here, which were all fairly similar and usually involved only an alteration in the mobile phase concentration, the order of change of the limited number of ΔR_M parameters investigated was indeed similar.)

$\Delta R_M(H)$ parameters and the effect of unsaturation

It follows from the fact that $\Delta R_M(CH_2)$ and ΔR_M(ring-attached CH_2) are virtually identical in System 2 not only that the atomic $\Delta R_M(H)$ parameter is small in this system, but that the differences between the $\Delta R_M(H)$ values for hydrogen α, β, γ, etc. to the ring are much too small to be determined with any accuracy (compare System 1[1]). Thus, the R_M values of the two xylenol benzoates (Nos. 30 and 31) are virtually the same as that of *p*-ethylphenyl benzoate (No. 32) and the ΔR_M increment between phenyl benzoate and *p*-cresyl benzoate (Nos. 28 and 29) is almost identical with the R_M difference between *p*-propylphenyl benzoate and *p*-butylphenyl benzoate (Nos. 33 and 35). Nevertheless, although the *difference* between the $\Delta R_M(H)$ parameters at different positions α, β, γ, etc. from the ring is too small to determine, that it still exists in System 2 is clearly illustrated by the fact that *p-tert.*-butylphenyl benzoate (No. 34), which contains no α-hydrogens, runs a little faster than *p-n*-butylphenyl benzoate (No. 35), the R_M difference being 0.059. (This difference is unlikely to

be due to chain branching in the former substance (see later), since the R_M values for *p*-*n*-amylphenol (No. 3) and *p*-3-methylbutylphenol (No. 4) are identical.)

In System 2, therefore ΔR_M(H) can, for all practical purposes, be considered to be a constant, irrespective of the position of the hydrogen with respect to the ring. Its value, although small, can be determined with reasonable accuracy. For example, the R_M values of *p*-(pent-4-enyloxy)-phenol (No. 13) and *p*-(3-methylbutyloxy)-phenol (No. 6) differ by + 0.088, and the R_M values of *p*-(hex-4-enyloxy)-phenol (No. 14) and *p*-hexyloxyphenol (No. 7) differ by + 0.074. The introduction of a double bond into compounds 6 and 7 therefore decreases their R_M values by about — 0.080. This corresponds to a value of + 0.040 for ΔR_M(H). The same value for ΔR_M(H) is found if the higher molecular weight compounds of the hydroquinone mono-ether series are compared. Thus *p*-geranyloxyphenol (No. 23), *p*-citronellyloxyphenol (No. 24) and *p*-dihydrocitronellyloxyphenol (No. 25), which differ from each other by two hydrogen atoms, differ in R_M by about 0.080. The formation of an alicyclic ring also corresponds formally to the loss of two hydrogen atoms. Chromatographically, therefore, the presence of an alicyclic ring should correspond to a double bond, provided that the ring is not attached to oxygen, when other effects appear (see later). This was shown to be the case by comparing *p*-(6-cyclohexylhexyloxy)-phenol (No. 21) with *p*-*n*-dodecyloxyphenol (No. 11), whose R_M values differ by + 0.068. The same value for the ΔR_M(H) parameter was found in the series of isoprenoid alcohols; here, the R_M values of geraniol (No. 44) and citronellol (No. 45) differed by + 0.081. There is thus ample evidence from Table I that ΔR_M(H) is a chromatographic constant. Hence, in the absence of any interaction with other structures, ΔR_M(double bond) must be constant.

Because the atomic ΔR_M parameters for both carbon and hydrogen are small in System 2, little loss of accuracy is introduced if group $\Delta R_M(CH_2)$ parameters are used for the calculation of R_M values of compounds run in this system. It follows, for instance, that if the R_M value of *n*-octylphenol is to be calculated from R_M(phenol), the error introduced by ignoring the fact that α- and β-CH_2 groups are chromatographically slightly different from subsequent CH_2 groups in the alkyl chain is negligible: as the molecular weights of the compounds increase, the contributions of α- and β-hydrogens become correspondingly less. In all the calculations below, therefore, the CH_2 group parameter has been employed.

ΔR_M parameters for oxygen in ethers

The results in Table I show that the R_M values for *p*-*n*-butoxy-, *p*-*sec.*-butoxy- and *p*-*tert.*-butoxyphenyl benzoates (Nos. 36, 37 and 38) in System 2 decrease in the same order as was found previously[1] in System 1; indicating, therefore, that ΔR_M(O) varies in the same way, according to the nature of the alkyl group to which it is attached.

The *group* ΔR_M parameters for oxygen can be calculated, exactly as described earlier[1], by directly comparing the R_M values for the primary, secondary and tertiary butoxyphenyl benzoates (Nos. 36, 37 and 38) with the R_M value for the corresponding primary butylphenyl benzoate (No. 35). The respective values are as follows: ΔR_M (O in OCH_2R) — 0.133; ΔR_M(O in $OCHR_2$) — 0.217; and ΔR_M(O in OCR_3) — 0.329. (It should be noted that these *group* ΔR_M parameters are slightly in error, for reasons that have been discussed in the preceding paper[1]. Briefly, ΔR_M increments for oxygen should ideally be calculated by the methods of atomic parameters outlined previously, or else they do not take into account the variation of the R_M(H) incre-

ments for hydrogen α, β and γ to the ring in the alkyl group and the absence of such variation in the alkoxy group. However, since in System 2 the differences are known to be small, it is unlikely that any serious effect on calculations would be introduced by using these group oxygen parameters—as would certainly have been the case in System 1.) All the other effects of substitution vicinal to oxygen are the same in System 2 as they were in System 1. Thus *p*-phenyloxyphenol (No. 17) runs slightly *slower* than *p*-benzyloxyphenol (No. 18), although the latter contains one more CH_2 group, because of the "resonance" effect of the benzyl group. The following calculation shows that *p*-cyclohexyloxyphenol (No. 20) is slightly faster in System 2 than required by theory, as it was in System 1.

Calculation of R_M(cyclohexyloxyphenol)

R_M(cyclohexyloxyphenol) = R_M(*n*-hexyloxyphenol) — 2 × ΔR_M(H) — (a correction for cyclohexyloxyphenol being a secondary ether)

The correction for a secondary ether is obtained by subtracting the R_M value of *p-sec.*-butoxyphenyl benzoate from that of *p-n*-butoxyphenyl benzoate. It is *subtracted* in the above calculation, since a secondary ether runs faster than a primary ether.
Thus,

$$R_M(\text{cyclohexyloxyphenol}) = -0.476 - (2 \times 0.040) - (-0.400 - 0.316) = -0.640$$
$$\text{Experimental } R_M = -0.678$$

Similarly, *p*-cyclopentyloxyphenol (No. 19; experimental $R_M = -0.799$) runs slightly faster than required by theory (calculated $R_M = -0.771$). The reason for this has been already discussed[1]. It should be noted that in System 2, the differences are only just outside experimental error. Nevertheless, they are of the same order, compared to the value of $\Delta R_M(CH_2)$, as they were in System 1.

The $\Delta R_M(OH)$ parameter

Since all the compounds in Table I, except the isoprenoid alcohols and vitamin A (Nos. 44–49), are phenols or their derivatives, the ΔR_M parameter for the phenolic OH group is already included in the R_M value for the ground molecule. (It could be calculated, if required, by comparing a suitable series of phenols and hydroquinones, although we have not done this.) It should be noted, however, that the values of ΔR_M(alcoholic OH)—in the series of isoprenoid alcohols—obviously depends on whether the alcohol is primary or tertiary. This is the same effect as occurs with ΔR_M(O) in the hydroquinone mono-ether series. But, in the alcohol series, the primary alcohol farnesol runs *faster* than its tertiary isomer, nerolidol. This is to be expected, since now it is oxygen–hydrogen polarization that is the determining factor, not carbon–oxygen polarization.

The effect of chain branching on R_M values

Many of the higher molecular weight compounds to be studied in later systems, especially the naturally-occurring tocopherols and ubiquinones, are isoprenoid in structure and their molecules contain branched alkyl chains. In System 1, most of the compounds studied were unbranched: the others were low-molecular weight com-

pounds and, if they did contain a single branch, any effect it might have had was obscured by the pronounced effects of α- and β-hydrogen atoms on the R_M values of the compounds. Thus, although *p*-isopropylphenol ran faster than *p*-*n*-propylphenol, and *p*-*tert.*-butylphenol ran faster than *p*-*n*-butylphenol[1], this was solely due to the fact that each new branching at carbon replaced, in effect, an α-hydrogen by a β-hydrogen. In compounds where this could be discounted (that is, where the branching occurred beyond the γ-carbon atom) branching as such did not appear to affect chromatographic behaviour in these simple compounds. Thus, in System 1, *p*-amylphenol was inseparable from *p*-3-methylbutylphenol, and *p*-cyclopentylphenol was inseparable from its isomer, *p*-3-methylbut-2-enylphenol[1].

It was clearly necessary, however, to study the effect of multiple chain branching in more detail. This was done in System 2, and it soon became apparent that the behaviour of compounds containing long-branched chains was anomalous. For example, the R_M value (— 0.100) of *p*-dihydrocitronellyloxyphenol (No. 25) differs considerably from the value for the isomeric *p*-*n*-decyloxyphenol, which should be + 0.038 (readily calculated from the data on alkoxyphenols in Table I). Similarly, the R_M values for *p*-hexahydrofarnesyloxyphenol (No. 26) and *p*-dihydrophytyloxyphenol (No. 27) are + 0.431 and + 0.947 respectively, whereas the calculated value for their corresponding straight-chain isomers would be + 0.682 for *p*-*n*-pentadecyloxyphenol and + 1.328 for *p*-*n*-eicosyloxyphenol. Analysis of all the results in this section of Table I shows that if there are n branchings in a chain, there are n — 1 effects on R_M. In the case of System 2, therefore, there is a new parameter to be considered, ΔR_M(branching). Its mean value can be readily obtained from the data already discussed and is found to be — 0.130. The nature of the branching effect and the reason why it had not been observed in System 1 with the lower-molecular weight compounds proved puzzling for a time. It was considered as a possibility, for example, that the branching effect might only occur in systems in which the mobile phase was relatively non-aqueous; *i.e.* that the emergence of the new parameter was actually caused by the system change. To study this possibility, several lower-molecular weight compounds were converted to benzoates and run together with the higher-molecular weight compounds in System 2. It is clear, however, from comparison of the R_M values of *p*-*n*-butylphenyl benzoate (No. 35) and *p*-*tert.*-butylphenyl benzoate (No. 34) that the small difference between them can only be attributed to the slight effect of the ΔR_M(H) parameter and is not nearly large enough to be due to the branching effect. Other work, not shown here, confirmed that the branching effect was in fact not produced by any particular chromatographic system, although its magnitude (in common with other ΔR_M values) was influenced by the nature of the system. The next possibility to be investigated was that, for some reason, the branching effect only manifested itself in isoprenoid type chains containing at least ten carbon atoms. In order to examine this point, we synthesized *p*-(3,5,5-trimethylhexyloxy)-phenol (No. 22), which contains a 9-carbon chain and two chain branchings. This compound has an R_M value of — 0.225. The R_M value of the unbranched *p*-*n*-nonyloxyphenol, on the other hand, would be — 0.086 (this compound was unavailable, but its R_M value can be easily and accurately calculated from the data on the homologous members of this series). The theoretical R_M value of compound No. 22, subtracting *one* increment for ΔR_M(branching), would be — 0.086 — 0.130 = — 0.216, almost identical with the actual experimental R_M value for this compound. (It is important to

note that although compound No. 22 contains a quaternary carbon atom, from the chromatographic point of view this must only be counted as one branch.) This calculation demonstrates clearly that the branching effect is not a characteristic only of isoprenoid chains. Further confirmation of this point was obtained by comparing the R_M values of ergosterol and 7-dehydrocholesterol (Nos. 53 and 54). Although these substances are quite different from the others in our series, they were used because they are convenient sources of a 9-carbon chain and an 8-carbon chain. Ergosterol was an especially valuable compound to correlate, since it contains two *vicinal* branches. Calculation of the R_M value of ergosterol from 7-dehydrocholesterol is given in Table II.

TABLE II

CALCULATION OF R_M FOR ERGOSTEROL

Constituents	*Increment*	
	+	−
R_M(7-dehydrocholesterol)	1.016	
+ $\Delta R_M(CH_2)$	0.129	
+ ΔR_M(double bond)		0.080
+ ΔR_M(branching)		0.130
Sum of increments	1.145	0.210
Calculated R_M(ergosterol)	= +0.935	
Experimental R_M	= +0.947	

The agreement is excellent and shows that the non-isoprenoid side-chain in ergosterol, containing two vicinal branches, also exhibits only one branching effect on the R_M value, confirming that the "$n-1$ effect" is independent of the relative positions of multiple branchings.

From these experiments, therefore, the nature of the branching effect on chromatography in reversed phase systems can be stated as follows. When a compound contains an alkyl chain with at least two branches, its R_M value is decreased by an increment that is a constant for the system. If there are n branchings, there are $n-1$ increments that reduce the R_M value. The value of this parameter is unaffected by the relative positions of the branchings, their structure, or by the length of the alkyl chain. In System 2, ΔR_M(branching) is equal, but opposite in sign to, $\Delta R_M(CH_2)$; this relationship, however, can be expected to be different in other types of chromatographic system (see the results in System 6). In direct phase systems, branching (after the first) must increase R_M, providing the system is suitable for observing the effect.

The effect of branching on R_M can be related to the fact that, in aliphatic hydrocarbons, branched members have a smaller molar volume than unbranched members. The molar volume of a compound is normally determined in the gas state and is affected by all branchings. In chromatography, however, where substances are studied in the liquid state, entropy effects may play a greater part. It is perhaps due to such a consideration that only $n-1$ branchings are effective in chromatography. Thus, the first branch in any chain can always be considered as terminal and subject to free

rotation. A second branch in the chain must introduce a hindrance to rotation, with a resultant effect on entropy.

If the branching forms part of a ring system, as in *p*-cyclohexylhexyloxyphenol (No. 21), the same rule applies: the ring counts as one branch only. Hence there is no ΔR_M increment in this compound, and its R_M value (+ 0.228) is almost exactly as calculated by subtracting $2 \times \Delta R_M(\mathrm{H})$ from the R_M value of the straight-chain compound, *p*-*n*-dodecyloxyphenol (No. 11).

Calculation of R_M values for System 2

We have not calculated the R_M values of all the fifty-four compounds listed in Table I, as many of them have been used to provide the data for the calculation of the various ΔR_M parameters. Two fairly complex compounds were, however, chromatographed in System 2 in order to test the method of structural analysis in this system and particularly the use of the new ΔR_M(branching) parameter. These were tocol, the parent member of the vitamin E series, and the important naturally-occurring substance, vitamin A alcohol. The calculations for these substances are given below.

(*i*) *Tocol.* This substance (No. 50) is 2-methyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol. Its empirical formula is $C_{26}H_{44}O_2$ and it can be considered as a complex derivative of phenol. Its R_M value can be found from that of phenol, and the R_M values for the other tocopherols can all in turn be calculated from that of tocol (Table III).

(*ii*) *Vitamin A.* The R_M value of vitamin A could be calculated from R_M(ethanol)

TABLE III

CALCULATION OF R_M FOR TOCOL

Constituents	*Increment*	
	+	−
R_M(phenol)*		1.125
$+ \Delta R_M$(O in OCR_3)		0.329
$+ \Delta R_M$(20 carbon atoms + 39 hydrogen atoms) $\simeq$ 20 $\times$ $\Delta R_M(CH_2) - \Delta R_M$(H)**	2.560	
$+ 2 \times \Delta R_M$(branching)		0.260
Sum of increments	2.560	1.714
Calculated R_M(tocol)	= +0.846	
Experimental R_M	= +0.806	

* Phenol itself was not chromatographed in System 2, as its R_F value is rather too high and in fact is likely to be less accurate when found experimentally than can be calculated by extrapolation from the data on the higher phenols (compounds Nos. 1–4). For this reason we have used the latter data to provide R_M(phenol) by simple extrapolation.

** For the validity of this approximation in System 2 see text.

if the necessary data were available. In this study, however, we have not studied a sufficient number of alcohols to determine the value of ΔR_M(primary OH), so have calculated from the R_M value of phytol (No. 48), a long-chain poly-isoprenoid alcohol. The calculation is given in Table IV.

TABLE IV

CALCULATION OF R_M FOR VITAMIN A

Constituents	*Increment*	
	+	—
R_M(phytol)	0.218	
$-10 \times \Delta R_M$(H)		0.400
$-\Delta R_M$(branching) *	0.130	
Sum of increments	0.348	0.400
Calculated R_M(vitamin A)	= —0.052	
Experimental R_M	= —0.040	

* The phytol molecule contains 4 chain branches. Vitamin A contains 2 chain branches and one ring, which (see text) also counts as one branch. The difference between them therefore corresponds to one effective branching unit: since vitamin A has less branches than phytol, the ΔR_M parameter, which is negative, is added.

TABLE V

CHROMATOGRAPHY OF SOME HYDROQUINONE MONO-ETHERS AND ALKYL *p*-NITROBENZOATES IN SYSTEM 3

Stationary phase: Whatman No. 4 paper impregnated with a 5% (v/v) solution of olive oil in light petroleum.
Mobile phase: 50% (v/v) ethanol in water.

No.	*Compound*	R_F	R_M
	Ethers		
5	*p*-Butoxyphenol	0.75	—0.469
6	*p*-(3-Methylbutoxy)-phenol	0.63	—0.224
7	*p*-Hexyloxyphenol	0.49	+0.021
8	*p*-Heptyloxyphenol	0.35	+0.267
9	*p*-Octyloxyphenol	0.235	+0.512
13	*p*-(Pent-4-enyloxy)-phenol	0.70	—0.375
14	*p*-(Hex-4-enyloxy)-phenol	0.58	—0.134
19	*p*-Cyclopentyloxyphenol	0.80	—0.600
20	*p*-Cyclohexyloxyphenol	0.70	—0.380
22	*p*-(3,5,5-Trimethylhexyloxy)-phenol	0.24	+0.498
23	*p*-Geranyloxyphenol	0.26	+0.447
24	*p*-Citronellyloxyphenol	0.20	+0.602
25	*p*-Dihydrocitronellyloxyphenol	0.15	+0.747
55	*p*-(1-Methylbutoxy)-phenol	0.75	—0.469
56	*p*-(2-Methylbutoxy)-phenol	0.63	—0.224
57	*p*-(1-Ethylbutoxy)-phenol	0.63	—0.224
58	*p*-(1-Methylpentyloxy)-phenol	0.63	—0.224
59	*p*-(1-Ethylpentyloxy)-phenol	0.49	+0.021
60	*p*-(1-Propylbutoxy)-phenol	0.49	+0.021
61	*p*-Sorbyloxyphenol	0.63	—0.227
	Esters		
62	Ethyl *p*-nitrobenzoate	0.37	+0.238
63	Propyl *p*-nitrobenzoate	0.24	+0.497
64	Allyl *p*-nitrobenzoate	0.295	+0.380
65	Propargyl *p*-nitrobenzoate	0.36	+0.246

RESULTS WITH SYSTEM 3

System 3 was 50 % (v/v) aqueous ethanol against olive oil. Several compounds that had already been chromatographed in System 2 were run in this system, in order to obtain additional information about the effect of changes in the ethanol concentration of the mobile phase on the ΔR_M parameters. System 3 was also used to study one or two other aspects of the unsaturation and branching effects. The results are given in Table V.

The $\Delta R_M(CH_2)$ parameter

In System 3, $\Delta R_M(CH_2)$, calculated from compounds 5–9, was found to be 0.245 ± 0.001.

Unsaturation

The principle of independent contributions of carbon and hydrogen atoms to R_M suggests that the ΔR_M increment for a triple bond should be calculable in the same way as for a double bond, that is, $\Delta R_M(C{\equiv}C)$ should be equivalent to $\Delta R_M(C{=}C) - 2 \times \Delta R_M(H)$. Propargyl alcohol was a convenient acetylenic compound, but we were unable to prepare propargyloxyphenol for comparison with the other hydroquinone mono-ethers. Propargyl alcohol was therefore studied as its *p*-nitrobenzoate (No. 65) and compared with the *p*-nitrobenzoates of ethanol, propanol, and allyl alcohol. $\Delta R_M(C{=}C)$ was calculated by comparing, in the usual way, the R_M values of pairs of compounds, differing only in the presence of one double bond. Thus from comparison of compounds No. 7 and 14, 23 and 24, 24 and 25, $\Delta R_M(C{=}C)$ is -0.152 ± 0.007. The experimental R_M value for propargyl *p*-nitrobenzoate differs from that of the allyl ester by -0.134, almost exactly that required for the further increment due to loss of two hydrogen atoms. This confirms that the acetylenic function can be calculated in the same way as the olefinic function by the method of atomic ΔR_M parameters. It should be noted, however, that the difference in R_M values between the allyl and propyl esters is only -0.097. It was found previously[1] in System 1 that allyl-substituted phenols ran slightly faster than required by theory and it was suggested that the effect was due to resonance in the allyl group. From the admittedly rather slender evidence of compound 64, it would seem that a similar effect might exist even in the allyl ester; here, although the allyl group is separated from the aromatic ring it is possible for conjugation of the allyl group with the ring to take place through the lone pair of electrons on the oxygen atom of the ester grouping. It follows, moreover, from the fact that the allyl and propargyl compounds can be correlated, that propargyl compounds can also be expected to show the "allyl" effect and run slightly faster than required by theory.

Another question was whether $\Delta R_M(C{=}C)$ remained constant if two or more bonds were conjugated with one another. In order to examine this, *p*-sorbyloxyphenol, which contains two conjugated double bonds, was prepared. Its R_M value was -0.227, and the theoretical R_M value for this compound (derived by calculation from *p*-hexyloxyphenol and *p*-(hex-4-enyloxy)-phenol) is -0.286. Considering the dimension of $\Delta R_M(CH_2)$ in System 3, this cannot be taken as seriously in error. There is evidently no major effect of conjugation on $R_M(C{=}C)$—see also the discussion[1] on propenylphenol in System 1—and this is confirmed by the calculation for vitamin A, which contains five conjugated double bonds.

Branching in ethers

Six new ethers (compounds Nos. 55–60) were prepared and chromatographed in System 3, in order to examine whether the *size* of the branched chain in secondary ethers affected the value of $\Delta R_M(O)$. As seen from Table V, the only primary ether in this group, *p*-(2-methylbutoxy)-phenol (No. 56) runs slower than the isomeric secondary ether (No. 55). The five secondary ethers show a constant homologous $\Delta R_M(CH_2)$ increment of + 0.245, irrespective of the nature of the secondary branching at oxygen. Thus the two isomeric secondary hexyl ethers, compounds 57 and 58, have identical R_M values and so do the two secondary heptyl ethers, compounds 59 and 60.

RESULTS WITH SYSTEM 4

In Table VI the results on 11 compounds run in System 4 (90 % ethanol against olive oil) are given. The system was studied to provide yet another bridge between the low molecular weight phenols and the more complex molecules studied subsequently,

TABLE VI

CHROMATOGRAPHY OF HYDROQUINONE MONO-ETHERS AND TOCOPHEROLS IN SYSTEM 4

Stationary phase: Whatman No. 4 paper impregnated with a 5 % v/v solution of olive oil in light petroleum.
Mobile phase: 90 % v/v ethanol in water.

No.	*Compound*	R_F	R_M
11	*p*-Dodecyloxyphenol	0.70	—0.357
12	*p*-Tetradecyloxyphenol	0.59	—0.155
15	*p*-Hexadecyloxyphenol	0.48	+0.041
16	*p*-Octadecyloxyphenol	0.36	+0.258
26	*p*-Hexahydrofarnesyloxyphenol	0.64	—0.250
27	*p*-Dihydrophytyloxyphenol	0.44	+0.107
50	Tocol	0.50	0.000
51	δ-Tocopherol (8-methyltocol)	0.45	+0.091
66	β-Tocopherol (5,8-dimethyltocol)	0.37	+0.228
52	γ-Tocopherol (7,8-dimethyltocol)	0.37	+0.228
67	α-Tocopherol (5,7,8-trimethyltocol)	0.31	+0.342

it being necessary to ensure that the additivity principle could be applied over the whole range of polarity of the mobile phase. The results again confirm that MARTIN's equation is obeyed: $\Delta R_M(CH_2)$ was constant to well within experimental error right up to *p*-octadecyloxyphenol, and was equal to + 0.103 ± 0.006. Note, however, the branching effects in compounds 26 and 27, as before. Table VII summarizes the data on some important parameters for Systems 2, 3 and 4.

RESULTS WITH SYSTEM 5

In this system, the stationary phase was changed to the non-polar liquid paraffin, which is normally used for the chromatography of the tocopherols, ubiquinones and

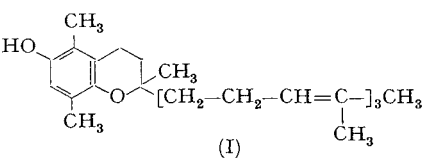
TABLE VII

VARIATION OF ΔR_M PARAMETERS OF SOME GROUPS AND STRUCTURAL FEATURES WITH CHANGE OF ETHANOL CONCENTRATION OF MOBILE PHASE IN SYSTEMS 2, 3 AND 4

Structural unit	ΔR_M *in system*		
	2	*3*	*4*
CH_2	+0.129	+0.245	+0.103
Ring-attached CH_2	+0.130		+0.097
Double bond (—2H)	—0.080	—0.152	
Branching (n—1)	—0.130	—0.255	—0.104

ubichromenols. In the first study, 65 % ethanol was used as mobile phase. Table VIII gives the data on 10 compounds. The relative positions of the substances remain the same as in previous systems, but note the restricted range of the chromatograms, leading to a value for $\Delta R_M(CH_2)$ that is now as large as it was in System 1. Note also the large difference (0.411) between the R_M values of tocol and *p*-dihydrophytyloxyphenol in this system. Although the two substances only differ by 2 hydrogen atoms in their empirical formula (compare the R_M values of compounds 70 and 71, for example, which differ by only 0.204), tocol is a tertiary ether (chromanol) whereas *p*-dihydrophytyloxyphenol is a primary ether.

The data in Table VIII were used for the elucidation of the structure of ε-tocopherol. We have shown elsewhere[8] that natural ε-tocopherol is not a homologue of tocol, as had previously been thought, but in fact has the structure (I).



(I)

This structure can be assigned to ε-tocopherol on chromatographic evidence[9]. Since ε-tocopherol can be hydrogenated to a substance having the same R_M value as β-tocopherol (it is in fact identical with β-tocopherol, as shown by other evidence), the ΔR_M change can be regarded as due to the presence of unsaturation in the former molecule. This value, ΔR_M(β-tocopherol—ε-tocopherol) = + 0.596, is almost exactly the required shift in R_M for three double bonds, which is + 0.612.

RESULTS WITH SYSTEM 6

Study of the high molecular weight ubiquinones, vitamins K and the ubichromenols requires liquid paraffin as stationary phase and 95 % ethanol as mobile phase. The results in this system are given in Table IX. The following sections describe in detail the methods of structural analysis used and show how the R_M values of these complex molecules can be calculated.

TABLE VIII

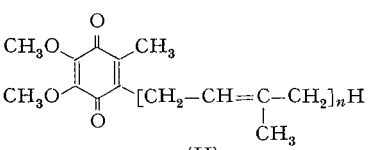
CHROMATOGRAPHY OF HYDROQUINONE MONO-ETHERS AND TOCOPHEROLS IN SYSTEM 5

Stationary phase: Whatman No. 4 paper impregnated with a 5% v/v solution of liquid paraffin in light petroleum.
Mobile phase: 65% (v/v) ethanol in water.

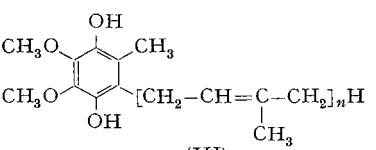
No.	*Compound*	R_F	R_M
50	Tocol	0.65	−0.268
51	δ-Tocopherol	0.425	+0.130
66	β-Tocopherol	0.22	+0.550
52	γ-Tocopherol	0.22	+0.550
67	α-Tocopherol	0.10	+0.956
68	Natural ε-tocopherol	0.53	−0.046
69	Hydrogenated ε-tocopherol	0.22	+0.550
27	*p*-Dihydrophytyloxyphenol	0.42	+0.143
70	4-Methoxy-2-methyl-5-phytylphenol	0.285	+0.398
71	4-Methoxy-2-methyl-5-dihydrophytyl-phenol	0.20	+0.602

Structures of the compounds listed in Table IX

In order to make the structural analyses and calculations more clear, we have depicted below the structures of some of the key compounds of Table IX, with some details of their interrelationships. Ubiquinones 30, 45 and 50 have structure (II) (n = 6, 9 and 10, respectively).

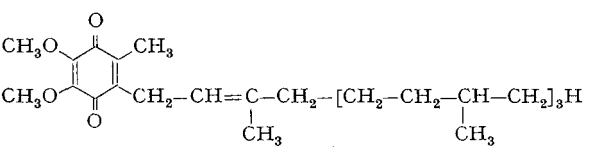


(II)



(III)

The analogous ubiquinols 30, 45 and 50 have structure (III). Dodecahydroubiquinone 30 and dodecahydroquinol 30 are derived from (II) and (III) respectively by reduction of the side-chains. Hexahydroubiquinone 20 (IV) is an allyl-type substituted quinone and octahydroubiquinone 20 is the analogous compound with a saturated side-chain.



(IV)

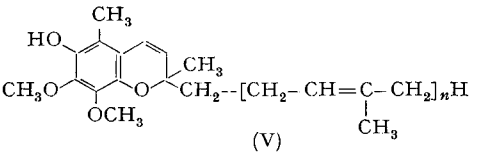
Ubichromenols 20, 30 and 50 have structure (V) (n = 3, 5 and 9, respectively).

TABLE IX

CHROMATOGRAPHY OF UBIQUINONES, UBICHROMENOLS, TOCOPHEROLS, VITAMINS K, PHENYL PALMITATES AND POLYNUCLEAR HYDROCARBONS IN SYSTEM 6

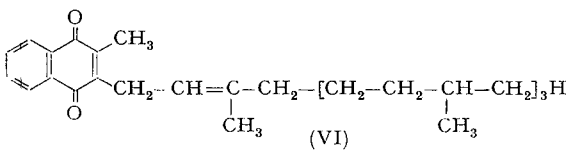
Stationary phase: Whatman No. 4 paper impregnated with a 5% (v/v) solution of liquid paraffin in light petroleum.
Mobile phase: 95% (v/v) ethanol in water.

No.	*Compound*	R_F	R_M
	Tocopherols and their ethers		
67	α-Tocopherol	0.85	−0.746
72	β-Tocopherol allyl ether	0.26	+0.452
73	γ-Tocopherol allyl ether	0.33	+0.301
74	Tocol allyl ether	0.40	+0.167
	Palmitates		
75	Phenyl palmitate	0.58	−0.138
76	*p*-Cresyl palmitate	0.50	0.000
	Hydrocarbons		
77	Anthracene	0.85	−0.770
78	Phenanthrene	0.85	−0.770
79	Benzanthracene	0.75	−0.481
80	Pyrene	0.75	−0.481
	Quinones and quinols		
81	Vitamin K_1	0.425	+0.127
82	Vitamin K_2	0.22	+0.566
83	2-Methyl-5-dihydrophytylbenzoquinone	0.51	−0.046
84	Hexahydroubiquinone 20	0.73	−0.434
85	Octahydroubiquinone 20	0.67	−0.314
86	Ubiquinone 30	0.65	−0.276
87	Dodecahydroubiquinone 30	0.28	+0.418
88	Dodecahydroubiquinol 30	0.72	−0.398
89	Ubiquinol 30	0.92	−1.046
90	Ubiquinone 45	0.25	+0.477
91	Ubiquinone 50	0.16	+0.720
92	Ubiquinol 50	0.555	−0.097
	Chromenols and chromanols		
93	Hexahydroubichromenol 20	0.85	−0.740
94	Hexahydroubichromanol 20	0.81	−0.627
95	Ubichromenol 30	0.79	−0.569
96	Ubichromenol 50	0.27	+0.428
	Hydroquinone mono-ether		
71	4-Methoxy-2-methyl-5-dihydrophytylphenol	0.91	−1.010

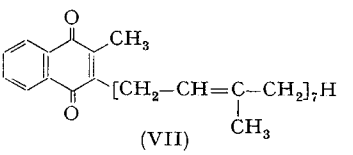


(V)

Although ubichromenol 20 itself was not available, hexahydroubichromenol 20 can be readily prepared from the available quinone and has structure (V) ($n = 3$) with a saturated side-chain. Hexahydroubichromanol 20 is the chromanol derived by reduction of the ring double bond. Vitamins K_1 and K_2 have structures (VI) and (VII), respectively.



(VI)



(VII)

Methods of structural analysis for compounds in System 6

(*a*) *The "isoprene" unit*

The tocopherols, ubiquinones, ubichromenols and vitamins K are all partly isoprenoid in structure and contain branched alkyl chains built up from saturated or unsaturated "isoprene" units. Thus α-tocopherol contains three saturated units, vitamin K_1 three saturated and one unsaturated unit, and ubiquinone 50 ten unsaturated units. For R_M calculations, it was convenient, therefore, to determine two new group parameters, ΔR_M ("isoprene" unit) and ΔR_M (hydrogenated "isoprene" unit). This eliminates the accumulation of small errors introduced when adding large numbers of $\Delta R_M(CH_2)$ and $\Delta R_M(CH)$ values and increments for double bonds, and branching effects. (Note that no "branching effect" error is introduced by this procedure as, in each case, the fusion of the isoprenoid chain with the ring constitutes the first, ineffective branch.) The new parameters were found from two series of compounds that, chromatographically, differ considerably, the ubiquinones and ubiquinols. The values from both series agreed well with each other. They are given in Table X.

TABLE X

ΔR_M PARAMETERS OF VARIOUS STRUCTURAL UNITS IN SYSTEM 6

Structural unit	ΔR_M
"Isoprene" unit in ubiquinones	+0.249
"Isoprene" unit in ubiquinols	+0.238
Hydrogenated "isoprene" unit	+0.366
Ring-attached CH_2 (tocopheryl ethers)	+0.142
Ring-attached CH_2 (aryl palmitates)	+0.138
CH=CH—CH=CH fused to an aromatic ring	+0.289
Double bond	—0.121
Branching effect*	—0.334
OCH_3 group vicinal to C=O in ubiquinones	—0.134

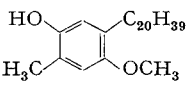
* It was assumed that ΔR_M (ring-attached CH_2) = ΔR_M (CH_2) = + 0.140 (see text).

(b) ΔR_M(ring-attached CH_2)

Many of the compounds contain nuclear-substituted methyl groups, and the parameter, ΔR_M(ring-attached CH_2), must be found for System 6. The tocopherols themselves, which differ in ring methyl groups and can therefore provide this parameter, run rather fast in this system, so three tocopheryl ethers were used. Because of our previous demonstrations that $\Delta R_M(CH_2)$ is strictly additive, we were confident that the value obtained from the ethers would be identical with that in the hydroxy compounds. To provide an additional check, however, the parameter was calculated independently from a comparison of the R_M values of phenyl and *p*-cresyl palmitates, which were synthesised for this purpose. The agreement between the two series was excellent, as shown in Table X.

(c) ΔR_M(OCH_3 ortho to OH)

The ubiquinols contain methoxyl groups *ortho* to their two hydroxy groups. The calculation of this important parameter is described below: it was obtained from the R_M values of the ubiquinol series and the key ether, 4-methoxy-2-methyl-5-phytylphenol (VIII).



(VIII)

(d) ΔR_M (double bond)

This was found by comparing the R_M values of the ubiquinols with those of their perhydro compounds.

(e) ΔR_M(branching)

This parameter was calculated by comparing the R_M values of ubiquinones and the phenyl palmitates, as shown below. (Since no independent determination of $\Delta R_M(CH_2)$ was made in System 6, we have assumed that it has the same value as ΔR_M(ring-attached CH_2). This is certainly valid for this system, in which differences in the values for various ΔR_M(H) parameters must be insignificant.)

The mean value for $\Delta R_M(CH_2)$ from Table X is + 0.140. Hence,

ΔR_M for CH_2—CH_2—CH_2—CH_2—CH_2 = 5 × 0.140 = 0.700
Experimental ΔR_M(saturated "isoprene" unit) = +0.366

Therefore,

ΔR_M(branching) = +0.334

(In this system the ratio of ΔR_M(branching) to $\Delta R_M(CH_2)$ is nearly twice as large as it was in the olive oil systems—see Table VII.)

(f) ΔR_M(OCH_3 ortho to C=O)

This parameter was obtained from the R_M data on perhydroubiquinone 20 and 2-methyl-5-dihydrophytylbenzoquinone.

$$R_M(\text{perhydroubiquinone 20}) = R_M(\text{2-methyl-5-dihydrophytylbenzoquinone}) + 2 \times \Delta R_M(OCH_3 \textit{ ortho to } C{=}O)$$

Therefore,

$$\Delta R_M(OCH_3 \textit{ ortho to } C{=}O) = \frac{-0.314 + 0.046}{2} = -0.134$$

(g) $\Delta R_M(CH{=}CH{-}CH{=}CH)$

This is a new group ΔR_M parameter and is of value in the calculation of vitamins K. The latter are all alkylated naphthaquinones and can be correlated with the ubiquinones through the formal fusion of a new aromatic ring to the existing quinonoid structure in the latter. (Note that, as discussed previously[1], if polarizations in molecules are not pronounced, R_M values can be calculated from these formal structural differences, and they are not influenced by the chemical or electronic changes involved in "aromaticity".)

It is possible, without introducing any serious error, to calculate the new parameter independently from $\Delta R_M(CH_2)$ itself.
Thus

$$\Delta R_M(CH{=}CH{-}CH{=}CH) \simeq 4 \times \Delta R_M(CH_2) + 2 \times \Delta R_M(\text{double bond}) \simeq +0.560 - 0.242 = +0.318$$

However, when dealing with a new ΔR_M parameter, it is preferable, if possible, to check it unequivocally, since an unforeseen interaction can never be ruled out. To do this, we chromatographed a series of polynuclear aromatic hydrocarbons in System 6 and calculated as follows.

$$\Delta R_M(CH{=}CH{-}CH{=}CH) = R_M(\text{benzanthracene}) - R_M(\text{anthracene}) = +0.289$$

It will be seen that the value is indeed very close to the approximation calculated directly from $\Delta R_M(CH_2)$ above. (Note that anthracene and phenanthrene on the one hand and benzanthracene and pyrene on the other are chromatographically indistinguishable, confirming our views on the irrelevance of pure energy characteristics (in the absence of other effects) on chromatographic behaviour.)

Calculations of R_M values for complex molecules in System 6

Calculations of R_M(vitamin K_2) from R_M(ubiquinone 50) and from R_M(vitamin K_1)

These calculations are given in Tables XI and XII. The excellent agreement between these two calculations provides further evidence of the precise convertibility of R_M data from series to series.

Calculation of R_M(ubichromenol 50) from R_M(α-tocopherol) and hence from R_M(phenol)

This is the most extensive calculation we have attempted. It demonstrates the importance of evaluating every new constitutive effect in a molecule. Ubichromenol 50 has a molecular weight of 862.

1st calculation. To calculate the R_M value of ubichromenol 50 from that of α-tocopherol, the effect of the following molecular modifications, in terms of ΔR_M parameters, must be known.

1. Subtracting two CH_3 groups from the ring.
2. Adding two OCH_3 groups to the ring, one *ortho* to the hydroxy group.
3. Adding one double bond to convert from a chromanol to a chromenol.

TABLE XI

CALCULATION OF R_M(VITAMIN K_2) FROM R_M(UBIQUINONE 50)

Constituents	*Increment* +	*Increment* −
R_M(ubiquinone 50)	0.720	
$-2 \times \Delta R_M(OCH_3)$	0.268	
$+ \Delta R_M$(fused ring)	0.289	
$-3 \times \Delta R_M$("isoprene" unit)		0.747
Sum of increments	1.277	0.747
Calculated R_M(vitamin K_2)		$= +0.530$
Experimental R_M		$= +0.556$

TABLE XII

CALCULATION OF R_M(VITAMIN K_2) FROM R_M(VITAMIN K_1)

Constituents	*Increment* +	*Increment* −
R_M(vitamin K_1)	0.127	
$+3 \times \Delta R_M$("isoprene" unit)	0.747	
$+3 \times \Delta R_M$(double bond)		0.363
Sum of increments	0.874	0.363
Calculated R_M(vitamin K_2)		$= +0.511$
Experimental R_M		$= +0.556$

TABLE XIII

FIRST CALCULATION OF R_M(UBICHROMENOL 50) FROM R_M(α-TOCOPHEROL)

Constituents	*Increment* +	*Increment* −
R_M(α-tocopherol)		0.746
$-2 \times \Delta R_M$(ring-attached CH_2)		0.280
$+2 \times \Delta R_M(OCH_3)$		0.268
$+6 \times R_M$("isoprene" unit)	1.494	
$+4 \times R_M$(double bond)		0.484
Sum of increments	1.494	1.778
Calculated R_M(ubichromenol 50)	$= -0.284$	
Experimental R_M	$= +0.428$	

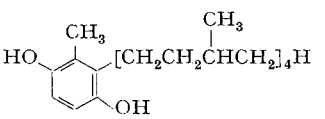
4. Adding a further three double bonds to convert the saturated side-chain to a tri-isoprenoid unsaturated side-chain.

5. Adding a further 6 unsaturated "isoprene" units to increase the chain length.

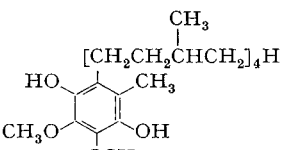
The only ΔR_M parameter whose precise value remained in doubt was ΔR_M-(OCH_3 *ortho* to OH). Because of hydrogen-bonding and the possibility of a pronounced *ortho*-effect it could be expected to be of great importance in the calculation. Before compound 71 was available, this parameter could not be calculated and it was first thought it might be satisfactory to use a value for a similar grouping, *i.e.* the known parameter, given in Table X, for ΔR_M(OCH_3 vicinal to >C=O in quinones). The calculation is given in Table XIII.

It is clear that there is a serious discrepancy between the calculated and experimental R_M values. The error must arise because the interaction between the OCH_3 group and the OH group is, as expected, considerably different from that between the OCH_3 group and the C=O group. The *ortho*-effect between the latter two groups must be large in this system.

2nd calculation. The calculation shown above is in error by an amount equivalent to about four CH_2 groups and it is clear that ΔR_M(OCH_3 *ortho* to OH) must be determined with much greater accuracy. This could be done, by the normal procedure of formal structural analysis, by comparing the R_M values of two suitably substituted phenols, one of which must contain suitably orientated OCH_3 groups. Amongst the range of compounds considered as possibly being available were 2-dihydrophytyl-3-methylhydroquinone (IX) and 2-dihydrophytyl-5,6-dimethoxy-3-methylhydroquinone (X).



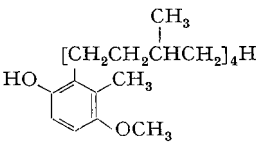
(IX)



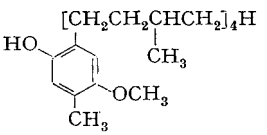
(X)

The orientation of these two compounds is very similar to that in tocopherol and ubichromenol respectively. The difference in R_M between the two compounds would be due only to the two OCH_3 groups, and ΔR_M(IX—X) would be equal to twice ΔR_M(OCH_3 *ortho* to OH). There were two practical difficulties, however. First, even if the two compounds could be prepared, they would be unlikely to chromatograph in System 6, since they each contain two OH groups. This could be overcome by preparing the 1-methyl ether of (IX) and (X) respectively and the resulting ethers would have the further advantage of resembling tocopherol and ubichromenol (both of which are cyclic mono-ethers) even more closely. Secondly, however, although (X) was available through the reduction of the corresponding octahydroubiquinone 20 (No. 85), (IX) could not be readily synthesised since entry of the phytyl group in the 3-position is sterically hindered. The problem was solved in the following manner.

(*i*) *Hypothetical R_M values for (IX) and (X) in System 6.* Although the required ether of (IX), 2-dihydrophytyl-4-methoxy-3-methylphenol (XI), is difficult to synthesise, its isomer, 6-dihydrophytyl-4-methoxy-3-methylphenol (XII) was readily obtained by condensation of toluquinol 1-methyl ether and phytol, followed by hydrogenation of the phytyl group.



(XI)



(XII)

This compound (No. 71) was prepared and had an R_M value of — 1.010 in System 6. Previous work[1] had already shown that differences in the orientation of alkyl groups do not affect R_M values of alkoxyphenols. Therefore it could be safely assumed that, if it were available (XI) would also have an R_M value of — 1.010 in System 6. If the ΔR_M increment were now known for the change involved in converting an OH group to an OCH_3 group, it would be possible to calculate the *hypothetical* R_M value for compound (IX) from the R_M value of (XII). This increment was obtained by comparing the R_M values of α-tocopherol and β-tocopheryl allyl ether, as follows:

(ii) Calculation of R_M(β-tocopherol) from R_M(α-tocopherol)

$$R_M(\beta\text{-tocopherol}) = R_M(\alpha\text{-tocopherol}) - \Delta R_M(\text{ring-attached } CH_2)$$
$$= -0.746 - 0.140 = -0.886$$

(iii) Calculation of R_M(β-tocopheryl methyl ether) from R_M(β-tocopheryl allyl ether)

$$R_M(\text{methyl ether}) = R_M(\text{allyl ether}) - 2 \times R_M(CH_2) - R_M(\text{double bond})$$
$$= +0.452 - 0.280 + 0.121 = +0.293$$

Therefore,

$$\Delta R_M(\text{effect of methylating OH group}) = R_M(\beta\text{-tocopheryl methyl ether}) - R_M(\beta\text{-tocopherol})$$
$$= +0.293 + 0.866 = +1.179$$

(Note: if the methyl ether of β-tocopherol had been available, the difference could have been found directly by chromatographing it with β-tocopherol. This calculation illustrates the interconvertibility of R_M data among related series of compounds.)

(iv) Calculation of R_M values for (IX) and (X)

$$R_M(\text{IX}) = R_M(\text{XI}) - 1.179$$
$$= -2.189$$

which would be the R_M value of (IX) if it could be run in System 6, and R_M(X) can now be calculated from R_M(dodecahydroubiquinol 30), (No. 88), by the usual method, as follows:

$$R_M(\text{X}) = R_M(\text{dodecahydroubiquinol 30}) - 2 \times \Delta R_M(\text{hydrogenated "isoprene" unit})$$
$$= -0.398 - 0.732 = -1.130$$

which would be the R_M value of (X) if it could be run in System 6.

(v) Calculation of ΔR_M(OCH_3 ortho to OH)

$$\Delta R_M(OCH_3 \textit{ ortho to } OH) = \frac{R_M(\text{X}) - R_M(\text{IX})}{2} = +0.530$$

(vi) Calculation of R_M(ubichromenol 50). The calculation is as previously, using the new parameter for OCH_3 (Table XIV).

The agreement is good, considering the lengthy procedure involved. By similar methods it is possible to correlate the R_M values of all the tocopherols, tocotrienols, vitamins K, ubiquinones, ubichromenols and members of related series of compounds. For example, R_M(ubichromenol 20) can now be calculated from R_M(ubichromenol

TABLE XIV

SECOND CALCULATION OF R_M(UBICHROMENOL 50) FROM R_M(α-TOCOPHEROL)

Constituents	*Increment*	
	+	—
R_M(α-tocopherol)		0.746
— 2 × R_M(ring-attached CH_2)		0.280
+ 4 × ΔR_M(double bond)		0.484
+ ΔR_M(OCH_3)		0.134
+ ΔR_M(OCH_3 *ortho* to OH)	0.530	
+ 6 × ΔR_M("isoprene" unit)	1.494	
Sum of increments	2.024	1.644
Calculated R_M(ubichromenol 50)	= +0.380	
Experimental R_M	= +0.428	

50) by subtracting the R_M increment for six unsaturated units (1.494). The calculated value is found to be — 0.751, in excellent agreement with the experimental R_M value of this compound, which is — 0.740. It is clear that, with adequate chromatographic data and with a certain amount of information about the functional groups present, the R_M values of some of these complex molecules can be calculated to within a small fraction of a carbon atom.

DISCUSSION

In principle it should now be possible to accept MARTIN's postulate as to the constancy of ΔR_M values in any molecule and in any system, providing that constitutive effects do not occur. If these do occur, they can often be adequately accounted for, as we have shown here and previously[1]. It is thus possible to calculate the R_M values of many complex molecules from data derived from relatively simple compounds, providing that chromatographic conditions are near-ideal and have been shown to yield accurate R_M values[1]. The recent work of HOWE[10] must be considered in this connection since this author, after his most extensive study on over 100 organic acids in several series, did not find agreement with MARTIN's equation. Two points, however, arise from HOWE's study. First, in some of his series, R_F values rapidly approached a limiting value after 8 carbon atoms. Since this value was about 0.80, this is strongly indicative of the non-ideal conditions that exist near the moving front of chromatograms due to excessive evaporation and other factors. As we have already suggested[1], R_F values of this order are likely to be subject to considerable error under tank con-

ditions and with certain systems, an R_F value of about 0.80 may appear to be the limiting R_F obtainable irrespective of the homologous increment. It is important, therefore, to stress that, providing the system is chosen so that R_F values fall within the workable range, there is apparently no limitation on MARTIN's postulate with respect to homologous addition. Thus HOWE was able to chromatograph dicarboxylic acids up to 10 carbon atoms in length and obtain a linear plot of R_M when the maximum R_F value was 0.53. As we have shown here, the homologous increment ΔR_M-(CH_2) is constant up to a chain length of 18 carbons (octadecyloxyphenol) and we have been able to calculate the R_M values of compounds containing branched side-chains of up to 50 carbon atoms. The second conclusion from HOWE's work was that $\Delta R_M(CH_2)$ varies from one homologous series to another. We regard this as primarily due to the nature of his series. As BARK AND GRAHAM[11] have shown, the paper chromatography of organic acids can be profoundly influenced by adsorption of the functional group on paper. In our own (unpublished) studies we have found that this is true *even in reversed phase systems*, where there is an inert stationary phase over the paper. It must be considered, therefore, that HOWE's results may have been affected in this manner and adsorption could account for the lack of constancy that he found for $\Delta R_M(CH_2)$. It is clear that where a possibility of adsorption exists, MARTIN's equation may not be precisely obeyed, even in homologous series.

The calculation of the R_M value for ubichromenol 50 illustrates that hypothetical R_M values can be calculated for compounds that could not be run in the system for which they have been calculated. These hypothetical R_M values can be dealt with arithmetically, as are real R_M values.

There are obvious advantages in being able to calculate the R_M values of complex molecules. We have already shown elsewhere[9] how such calculations can be used to determine unsaturation in molecules by purely chromatographic methods. They can also be used to obtain information about the structure of an unknown compound, even when it is available only in small amounts or is impure. It is often possible to eliminate alternative structures, such as might be proposed for a new or unknown compound of natural origin.

It may be possible, in the future, to choose a limited series of standard chromatographic system and determine, with accuracy, the values for all the important atomic group and constitutive ΔR_M parameters met with in simple series of compounds. Providing that the chromatographic systems and the conditions of running were both rigorously standardised, it might even be possible for this data to be used by different workers without the necessity of their frequent re-determination in individual laboratories. Reversed phase systems should be chosen as standard wherever possible and the mobile phase be restricted to one of two solvents, such as aqueous ethanol or acetone, which have exceptionally wide scope. With the exception of sugars and amino acids which, for structural analysis purposes as opposed to pure identification purposes, can in any case be handled as their derivatives, such reversed phase systems can deal with most classes of organic compound.

SUMMARY

Series of phenols, hydroquinone mono-ethers, esters, ethers, alcohols, tocopherols, quinones and chromenols were run in five chromatographic systems. Chromatographic

constancy was shown for ΔR_M increments due to the following groups and structural changes: H, CH_2, ring-attached CH_2, double bond, branching, oxygen in ethers, and the "isoprene" unit in long chains. MARTIN's equation was obeyed in all the systems studied. Methods of structural analysis are demonstrated by which the chromatographic behaviour of complex molecules can be accurately predicted from data derived from simple compounds and knowledge of the ΔR_M parameters.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

IV. INTRAMOLECULAR HYDROGEN BONDING

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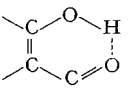
INTRODUCTION

In a previous communication[1], we discussed some of the structural factors in molecules that are responsible for constitutive effects on R_M. One of these was considered to be internal hydrogen bonding and in this communication we wish to present a more detailed study of this phenomenon. The fact that hydrogen bonding or chelation influences chromatographic behaviour was observed for the first time by BATE-SMITH AND WESTALL[2], who noted that, where it occurred, there was a deviation from MARTIN'S equation[3]. We have already discussed[1] the relation of internal hydrogen bonding to the *ortho*-effect, in which it may play a prominent part. Such *ortho*-effects, predominantly of the hydrogen bond type, can be observed in the chromatographic data of many workers: it is sufficient to quote FRANC[4] on the amino-anthraquinones and hydroxy-anthraquinones and LATINAK[5] on hydroxy-naphthalene derivatives, whose work gives many illustrations of the phenomenon.

Although, therefore, there are many empirical observations on the effect of internal hydrogen bond on R_M, there does not appear to have been any more theoretical or quantitative approach to the subject. In order to study the effect of internal hydrogen bonding it is essential—as in other types of chromatographic study—to isolate it from other simultaneous and complicating factors. This is not always easy, as hydrogen bonding between two groups must partly depend on their steric interaction. In aromatic systems in particular, internal hydrogen bonding may affect and be affected by electronic factors. If it were possible, however, to isolate the effect of hydrogen bonding in a series of compounds in which the strength of the bond is known to vary in a predictable manner, it might then be possible to study the quantitative relationship between the degree of bonding and R_M. Since internal hydrogen bonding increases the solubility of organic compounds in non-polar solvents and decreases their solubility in polar solvents the relationship can be studied to advantage in a reversed phase system, where problems of adsorption (which might profoundly influence hydrogen bonding) are minimized.

The classic, and in many ways the simplest and definitive, case of hydrogen bonding occurs in the 6-atom system shown schematically below (I). This system is present in the enolic form of β-diketones and in *o*-substituted aromatic hydroxy-aldehydes and hydroxy-esters. It has been shown by several workers that the strength of chelation in such a system is dependent on the existence of a high degree of conju-

gation in the chelate ring. Under these conditions, stabilization of the chelate ring through resonance forms is possible—a concept first put forward in different terms by ROBINSON[6] and confirmed and illustrated by the work of BAKER[7] on the resacetophenones. More recently, HUNSBERGER and his co-workers[8–11] were able to demonstrate that a roughly quantitative correlation exists between the degree of hydrogen



(I)

bonding in *o*-substituted hydroxy-aldehydes, hydroxy-ketones and hydroxy-esters and the double bond character of the double bond bearing the two chelated groups. They did this by studying the relative shift of the (C=O) bond in the infra-red spectra of the hydroxy compounds, with and without the second *ortho*-substituent. They found that the magnitude of this shift, $\Delta\nu$(C=O), increased as the double bond character of the double bond increased. One way, therefore, of investigating the quantitative effect of internal hydrogen bonding on R_M would be to study a series of compounds such as was used by HUNSBERGER, in which the constitutive ΔR_M effects could be related to the double bond character of the double bond bearing the chelated groups. A series of hydroxy-aldehydes, hydroxy-esters, and parent phenols and aldehydes were chromatographed for this investigation.

EXPERIMENTAL

Compounds

These were either obtained commercially or prepared according to the methods of HUNSBERGER[8–11].

Chromatography

Sheets of Whatman No. 4 paper, 23 × 57 cm, were impregnated with a 10% (v/v) solution of olive oil in light petroleum (40–60°). The mobile phase was dry methanol. The chromatograms were developed by the descending technique for 4 h, during which time the front migrated about 42 cm. Chromatography was also carried out by the tankless horizontal method of GREEN AND MARCINKIEWICZ[12], which gave rather better results with volatile compounds such as salicylaldehyde. The R_M values obtained by the two methods did not differ by more than ± 0.01 and the results in Table I represent mean values from three runs by the descending method and one run by the horizontal method.

Visualization

Phenols were located by spraying with aqueous sodium carbonate (5% w/v) and then diazotised *o*-dianisidine.

RESULTS AND DISCUSSION

Table I gives the R_F and R_M values of a series of phenols and the corresponding *o*-hydroxy-aldehydes derived from them. Table II gives results for three hydroxy-esters. ΔR_M(CHO) and ΔR_M($COOCH_3$) are the increments due to the introduction of either

TABLE I

R_F* AND R_M VALUES OF PHENOLS AND *o*-HYDROXY-ALDEHYDES AND CALCULATED ΔR_M(CHO) INCREMENTS

Parent phenol and o-hydroxy-aldehyde	R_F	R_M	ΔR_M(CHO)	*Relevant bond in corresponding hydrocarbon*
Phenol	0.81	−0.628		
Salicylaldehyde	0.77	−0.523	+0.105	Benzene
1-Naphthol**	0.73	−0.420		
1-Hydroxy-2-naphthaldehyde	0.54	−0.074	+0.346	1,2 in naphthalene
2-Naphthol**	0.73	−0.420		
2-Hydroxy-1-naphthaldehyde	0.51	−0.020	+0.400	1,2 in naphthalene
2-Naphthol**		−0.420		
2-Hydroxy-3-naphthaldehyde	0.69	−0.347	−0.073	2,3 in naphthalene
9-Phenanthrol	0.62	−0.208		
9-Hydroxy-10-phenanthraldehyde	0.27	+0.438	+0.646	9,10 in phenanthrene
3,4-Xylenol	0.75	−0.486		
6-Hydroxy-3,4-xylaldehyde	0.66	−0.290	+0.196	4,5 in *o*-xylene
5-Indanol	0.73	−0.420		
6-Hydroxy-5-indanecarboxaldehyde	0.62	−0.202	+0.218	5,6 in indane
6-Tetralol	0.69	−0.342		
3-Hydroxy-5,6,7,8-tetrahydro-2-naphthaldehyde	0.59	−0.156	+0.186	6,7 in tetralin

* R_F values were calculated from the R_M values[1].
** No *ortho*-effect in the naphthols was observed in this system.

the CHO group or the $COOCH_3$ group into each phenol. It will be seen that these ΔR_M values vary greatly, depending on the nature of the phenol and, in particular, the nature of the double bond bearing the chelate ring. In Fig. 1, therefore, ΔR_M(CHO) and $\Delta R_M(COOCH_3)$ are plotted against $\Delta\nu$(C=O) as determined for the infra-red shift of the carbonyl band in the same series of compounds by HUNSBERGER and co-workers[8–11].

TABLE II

R_F* AND R_M VALUES OF AROMATIC HYDROXY-ESTERS AND PHENOLS AND CALCULATED $\Delta R_M(COOCH_3)$ INCREMENTS

Compound	R_F	R_M	$\Delta R_M(COOCH_3)$	*Relevant bond in corresponding hydrocarbon*
Phenol	0.81	−0.628		
Methyl salicylate	0.50	0.000	+0.628	Benzene
1-Naphthol	0.73	−0.420		
Methyl 1-hydroxy-2-naphthoate	0.32	+0.335	+0.755	1,2 in naphthalene
2-Naphthol	0.73	−0.420		
Methyl 2-hydroxy-3-naphthoate	0.425	+0.133	+0.553	2,3 in naphthalene

* R_F values were calculated from the R_M values[1].

With the exception of the ΔR_M(CHO) values for 6-tetralol and 3,4-xylenol, the chromatographic data fit the infra-red data well, and this applies not only to the hydroxy-aldehydes (Fig. 1a) but also to the three aromatic hydroxy-esters (Fig. 1b). The ΔR_M(CHO) values for 6-tetralol, 5-indanol and 3,4-xylenol are in fact all similar and the difference between them is not much greater than experimental error. These results are remarkably similar to those for the infra-red shift, as shown by HUNSBERGER *et al.*[11], leaving little doubt that essentially the same phenomenon is under study. Their conclusions, in agreement with ours, were that there is a negligible amount of bond fixation in the ground-state of either indane or tetralin, indicating that there is no appreciable MILLS-NIXON effect in these molecules. However, the ΔR_M(CHO) values for 6-tetralol, 5-indanol and 3,4-xylenol are somewhat greater than ΔR_M(CHO) for phenol itself. This seems to indicate that the intramolecular hydrogen bond may

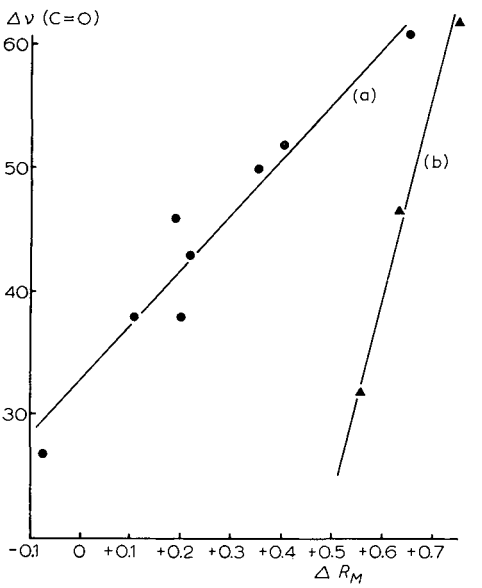


Fig. 1. Relationship between (a) ΔR_M(CHO) and (b) $\Delta R_M(COOCH_3)$ and $\Delta\nu$(C=O) in the infra-red spectra of *o*-substituted hydroxy-aldehydes and hydroxy-esters.

be at least slightly affected by alkyl substitution in the aromatic ring (the two methyl groups in *o*-xylene are formally similar to the α-CH_2 groups in tetralin). On the basis of chromatographic and infra-red evidence, however, it is not possible to decide whether this difference is due to different bond orders and free valencies in alkylated benzenes or to a difference in charge distribution (inductive effect of alkyl groups).

In Fig. 2 we have plotted the ΔR_M(CHO) and $\Delta R_M(COOCH_3)$ values against the bond order of the relevant individual bond in the corresponding hydrocarbon (given in Tables I and II), calculated by the molecular orbital method (L.C.A.O. approximation[13]). The values for indane, tetralin and *o*-xylene are not included, as their bond orders cannot be calculated. The close approach to linearity in both series of compounds provides the strongest evidence (a) that the partition coefficient of these *o*-hydroxy compounds varies directly with the bond order of the bond bearing the

chelate ring, and (b) *in extenso*, that intramolecular hydrogen bonding is directly related in such compounds to relevant bond order. It will be observed that the slope of the plot for the hydroxy-esters is greater than that for the aldehydes. This indicates that chelation is rather stronger in the aldehydes: this could be expected, in view of the greater electronic density on the carbonyl oxygen in these compounds.

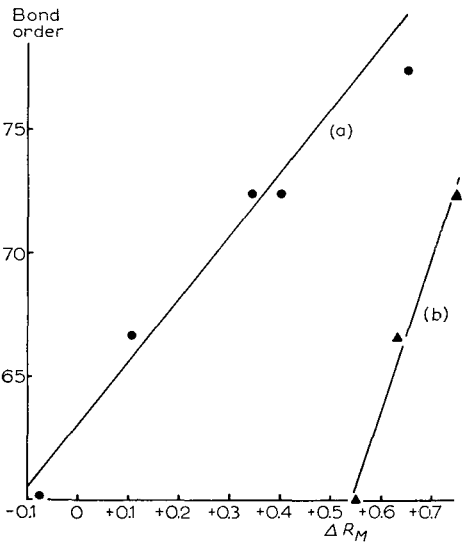
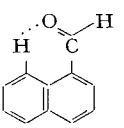


Fig. 2. Relationship between (a) ΔR_M(CHO) and (b) ΔR_M($COOCH_3$) and relevant bond order in corresponding hydrocarbon for *o*-substituted hydroxy-aldehydes and hydroxy-esters.

The difference between the ΔR_M(CHO) values for 1-naphthol and 2-naphthol is 0.054. A somewhat analogous difference was also observed in the infra-red studies of HUNSBERGER[8], who obtained $\Delta\nu$(C=O) = 50 and 52 respectively for the two compounds. What accounts for the chromatographic difference (and hence the difference in hydrogen bonding) in these two substances, which are both substituted on the same double bond? HUNSBERGER *et al.*[11] postulated that a slight difference in hydrogen bonding was produced by "a steric facilitation of chelation due to the proximity of the *peri*-CH in the naphthalene nucleus". On this hypothesis, the *peri*-CH group is assumed to exert a greater steric effect on the bulkier CHO group in the 1-position than it does when the OH group is in this position. MARCINKIEWICZ AND GREEN[14] showed by a molecular orbital treatment that the difference might be due, not to a steric factor,



(II)

but to the different free valencies in positions 1 and 2 of the naphthalene nucleus. That this might be so is further supported by the chromatographic data. 1-Naphthal-

dehyde and 2-naphthaldehyde were run in the same system as the hydroxy-aldehydes and had R_M values of -0.143 and -0.217, respectively. The difference between them is almost identical with that between the two hydroxy-aldehydes. Now, since in this system the steric *ortho*-effect seems to be negligible (*cf.* the two naphthols, which have identical R_F values), the R_M difference between 1- and 2-naphthaldehydes seems to be due to another reason. This may be competitive intramolecular hydrogen bond formation between the CHO group and the *peri*-CH group, as shown above (II), a process that would be facilitated by the difference in the free valencies of the carbon atoms at positions 1 and 2 in naphthalene.

SUMMARY

The constitutive effects of intramolecular hydrogen bonding on the chromatography of aromatic *o*-hydroxy-aldehydes and *o*-hydroxy-esters have been studied. It is shown that the degree of hydrogen bonding between the two *ortho*-groups as indicated by the shifts $\Delta\nu$(C=O) in the infra-red spectra of the compounds can be quantitatively correlated with the values of ΔR_M(CHO) or $\Delta R_M(COOCH_3)$ found by comparing the R_M values of the compounds with those of the parent phenols. Both the chromatographic and infra-red effects can be related to the bond order of the double bond (in the parent hydrocarbon) bearing the two chelating groups. Intramolecular hydrogen bonding is an important factor affecting the precise theoretical calculation of R_M. This study provides an example of how such a constitutive effect can be evaluated.

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QUANTITATIVE GLYKOGEN- UND ZUCKERBESTIMMUNG MIT HILFE DER PAPIERCHROMATOGRAPHIE*

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(Eingegangen den 2. Juli 1962)

Zur quantitativen Bestimmung des Glykogens wurde bisher im allgemeinen die auf PFLÜGER[1] zurückgehende Methode, die vielfach modifiziert wurde[2], angewandt. Hierbei wird das Gewebe zunächst mit 20%iger Natronlauge mazeriert und dann das Glykogen aus heisser Lösung mit Alkohol gefällt. Nach mehrmaligem Auswaschen lässt sich dann entweder die colorimetrisch arbeitende Anthron-Methode[3] anwenden, oder das Glykogen wird einer saueren Hydrolyse unterworfen und danach die entstandene Glukose nach HAGEDORN-JENSEN titrimetrisch ermittelt.

Bei unseren Untersuchungen über die Glykogen- und Polysaccharidveränderungen während der Postembryonalentwicklung von Insekten (z.B. Ameisen) zeigte sich, dass die üblichen Fällungsmethoden viel zu niedrige und keine konstanten Werte ergaben. JOHN[4] wies bereits bei ihren Untersuchungen über den Glykogengehalt der Biene darauf hin, dass ein Auswaschen des Niederschlages mit destilliertem Wasser erhebliche Glykogenverluste zeitigte. Das Glykogen soll in den Zellen häufig als lockere Eiweiss-Glykogen-Verbindung in zwei Formen vorliegen, dem löslichen Lyo- und dem unlöslichen Desmo-Glykogen[5]. Die beiden Glykogene sind mehr oder minder einheitlich in der Zusammensetzung und haben verschiedene Molekulargewichte. Da sich unsere bisherigen Kenntnisse hauptsächlich auf Erfahrungen mit Säugetier-Glykogen stützen, besteht die Möglichkeit, dass bei der Vielzahl der Insekten weitere bisher noch unbekannte Faktoren hinzukommen, die eine Glykogenbestimmung in üblicher Form erschweren.

Nach der wenig erfolgreichen Anwendung der erwähnten Fällungsmethode waren wir gezwungen, nach einer nicht sehr aufwendigen Mikromethode zu suchen, die eine zuverlässige und reproduzierbare Glykogen- und Zuckerbestimmung erlaubte. Mit Hilfe der Papierchromatographie erhielten wir befriedigende Ergebnisse.

BEKANNTE PAPIERCHROMATOGRAPHISCHE BESTIMMUNGSVERFAHREN

Polysaccharide lassen sich papierchromatographisch nur nach Hydrolyse in Form ihrer Bausteine quantitativ ermitteln. In den letzten 10 Jahren sind eine Reihe von papierchromatographischen Bestimmungsmethoden für Zucker bekannt geworden. Zur quantitativen Bestimmung wird entweder ihr reduzierender Charakter oder ihre Furfurolisierbarkeit ausgenutzt. Prinzipiell wurden drei Verfahren beschrieben:

* Die Untersuchungen wurden in dankenswerter Weise von der Deutschen Forschungsgemeinschaft durch eine Sachbeihilfe unterstützt.

** Vorstand: Prof. Dr. K. GÖSSWALD.

1. Nach Chromatographie Anfärbung auf dem Papier und direkte Photometrie des Farbfleckens sowie Planimetrie der erhaltenen Kurvenflächen.

2. Nach Chromatographie Anfärbung auf dem Papier, dann Elution des Farbstoffs und Messung der Extinktion im Spektralphotometer bei bestimmter Wellenlänge.

3. Nach Chromatographie Elution der Zucker, dann Farbstoffbildung *in vitro* und Ermittlung der Extinktion wie bei 2.

Das erste Verfahren erfordert zweifelsohne den geringsten Arbeitsaufwand. Weiterhin sind die durch eine Elution entstehenden Fehlermöglichkeiten ausgeschlossen. Durch direkte Photometrie der Farbflecken ist prinzipiell jede Farbbildung auswertbar. Die zweite Methode erlaubt nur die Verwendung eluierbarer Farbstoffe nach einer vollkommenen Trennung der Substanzen. Ähnlich können im dritten Verfahren nur solche Nachweisreagentien verwendet werden, die lösliche Farbstoffe ergeben. FLOOD und Mitarbeiter[6] umgehen die Farbstoffbildung und führen nach dem Herauslösen der Zucker aus dem Papier eine titrimetrische Mikromethode durch. Die Papierchromatographie wird lediglich als Reinigungsverfahren benutzt. Ähnliche Verfahren liegen besonders aus den letzten Jahren vor[7].

MCFARREN, BRAND UND RUTKOWSKY[8] verwendeten zur quantitativen Bestimmung von Zuckern auf Filtrierpapier wohl als erste ammoniakalisches Silbernitrat. Die nach Besprühen entstehenden Silberflecken sind nicht eluierbar und lassen sich nur direkt auf dem Papier photometrieren. FISCHER UND DÖRFEL[9] bezeichneten diese Reaktion deshalb als ungünstig. Die Bestimmung auf dem Papier soll angeblich umständlicher und ungenauer sein. Weiterhin soll eine mangelhafte Proportionalität zwischen Zuckermenge und Silberschwärzung bestehen. Sie ziehen die Reaktion mit Triphenyltetrazoliumchlorid vor, eluieren das entstandene Formazan und bestimmen die Extinktion mit einer maximalen Streuung der Messwerte von 4–9 % bei 12 Einzelbestimmungen. Der mittlere Fehler betrug 2–4 %. Aber auch hier wurde kein stöchiometrischer Verlauf der Farbreaktion gefunden. Sowohl bei der direkten Photometrie der Flecken auf dem Papier als auch mittels der Elutionsmethode war es nicht möglich, einmal festgelegte Eichkurven zu verwenden. WALLENFELS und Mitarbeiter[10] benutzten beide Methoden, gaben jedoch der direkten Photometrie den Vorrang, während LÜDECKE UND STANGE[11] die TTC-Reaktion bevorzugten und den Farbstoff eluierten. WOHNLICH[12] wendete die direkte Photometrie mit Erfolg auf die Triphenyltetrazoliumchlorid-, Benzidin- und Molybdatophosphorsäure-Reaktion an. Das Lambert-Beer'sche Gesetz soll für einen um so grösseren Konzentrationsbereich Gültigkeit haben, je kurzwelliger die untersuchte Farbe auf dem Papier ist. Auch ROBERTS[13] wertete die Farbreaktion der Zucker mit Benzidincitrat direkt auf dem Papier aus. Die Tatsache, dass Zucker unter bestimmten Bedingungen furfurolisieren und dann mit einer cyclischen Base einen Farbkomplex bilden, nutzten weiterhin BAAR[14], V. CZARNOWSKI[15], DATE[16], ALBERS UND FREISKORN[17], WILSON[18], PHILIPPU[19], COLOMBO und Mitarbeiter[20] sowie HIMES und Mitarbeiter[21] zur quantitativen Zuckerbestimmung aus. Die meisten Autoren[14,17,18,20] verwendeten die Phthalsäure–Anilin-Reaktion. Der entstandene Farbstoff wurde entweder eluiert und dann kolorimetriert[14,16,18] oder direkt auf dem Papier gemessen[15,17,20,21]. Für eine quantitative Bestimmung müssen bei dieser Farbreaktion wenigstens 20 Chromatogramme angefertigt werden, von denen die 12 besten Flecken ausgewertet werden[17]. Dann soll nach Angaben der Autoren ein mittlerer Fehler von $\pm$ 1 % er-

reicht werden. Die italienischen Autoren[20] mussten allerdings 12 Chromatogramme mit je zwei Testflecken und fünf Vergleichsflecken anfertigen, um einen mittleren Fehler von ± 2–4 % zu erreichen. Bei direkter Photometrie auf dem Papier erhalten die Autoren zwischen Extinktion und Konzentration auf semi-logarithmischem Papier eine Gerade. Eine lineare Beziehung wurde auch gefunden zwischen den planimetrisch bestimmten Extinktionsflächen und der Quadratwurzel der Zuckerkonzentration[17] sowie zwischen der Extinktionsfläche und dem Logarithmus der Zuckerkonzentration[22]. Nach Elution des Farbstoffes und Photometrie wurde eine Proportionalität zur Menge zwischen 10–125 μg festgestellt[18]. Wird der Zucker zunächst eluiert und die Farbstoffbildung *in vitro* durchgeführt, erhält man Fehlergrenzen von 0.8–5.4 %, die von der Zuckerkonzentration abhängig sind[19]. Bei höherer Zuckermenge ist der Fehler geringer. ATHENSTEDT[23] fand nach Anwendung der Anthron-Reaktion einen mittleren Fehler von ± 3 %.

Viele Arbeiten, die sich mit der quantitativen papierchromatographischen Zuckerbestimmung beschäftigen, sind wenig kritisch durchgeführt worden. Einige Bestimmungsverfahren sind nach unseren Erfahrungen zu wenig zuverlässig. In keinem Falle war es möglich, eines der beschriebenen chromatographischen Verfahren zu unserer Zufriedenheit anzuwenden.

ERARBEITUNG EINER NEUEN CHROMATOGRAPHISCHEN METHODE

In zahlreichen Vorversuchen haben wir die verschiedenen im Schrifttum angeführten Bestimmungsmethoden unter Verwendung von Reinsubstanzen wie Glukose und Glykogen der Firma Merck (beide Substanzen enthielten 7–8 % Wasser) geprüft. Durch Kombination bekannter Verfahren und verschiedene Abänderungen in der Methodik gelang es uns, ein neues einfaches und zuverlässigeres Verfahren zur Polysaccharid- und Zuckerbestimmung zu entwerfen.

Zur Chromatographie der Zucker erwiesen sich Schleicher & Schüll-Papiere Nr. 2043b Mgl gegenüber den dünneren Whatman Nr. 1-Papieren als vorteilhafter; das dickere Papier lässt sich vor allem beim Wässern besser handhaben. Wir erhielten mit Silbernitrat leicht auswertbare, runde bis ovale Flecken auf weissem Grund. Praktisch jedes Chromatogramm war auswertbar, sobald die Konzentration günstig gewählt war. Hierzu ist jeweils ein Vorversuch notwendig, indem neben bekannten Mengen die zu ermittelnden unbekannten Konzentrationen aufgetragen, chromatographiert und angefärbt wurden. Leider konnten wir wiederholt feststellen, dass Papiere aus verschiedenen Fertigungsserien mitunter unterschiedliche Laufzeiten hatten, die vorher jeweils ermittelt werden mussten. Die Whatman-Papiere sind in ihrer Laufgeschwindigkeit wesentlich konstanter. Mit dem von FISCHER UND DÖRFEL[9] verwendeten Laufmittel (Äthylacetat–Pyridin–Wasser, 40:11:6) erzielten wir im absteigenden Verfahren (Durchlaufchromatogramm) eine Laufgeschwindigkeit für Glukose von *ca.* 15 cm in 9–15 Stunden bei 25° und gute quantitative Ergebnisse.

Zur Ermittlung der Lage der Glukoseflecken auf dem Papierchromatogramm verwendeten wir Silbernitrat, Triphenyltetrazoliumchlorid[9,10], Anilin–Phthalsäure-Reagens[24], Anilin–Phosphorsäure-Reagens[25], *p*-Aminohippursäure[26], und Benzidin–Trichloressigsäure[27]. Das Triphenyltetrazoliumchlorid wird in alkalischer Lösung durch reduzierende Substanzen zum tiefroten Triphenylformazan hydriert. Selbst unter vollständigem Lichtausschluss war es nicht möglich, einen weissen Papier-

grund zu erhalten. Wir fanden stets tiefrote Flecken auf nicht gleichmässig rötlich gefärbtem Papier, wie auch in der Literatur angegeben wird[9]. Dies erschwerte eine direkte photometrische Auswertung auf dem Papier, die unseres Erachtens am wenigsten aufwendig ist. Wir waren deshalb bestrebt, letzteres Verfahren auszunutzen. Die Färbung mit Anilin–Phthalsäure ist relativ schwach mit leichter Papiergrundfarbe. Es ist nötig, grössere Zuckermengen aufzutragen. Dies ist jedoch insofern von Nachteil, weil dann gewöhnlich keine vollständige Reaktion der Zucker mit dem Reagens erreicht wird, was sich nachteilig auf die photometrische Auswertung und quantitative Bestimmung auf dem Papier auswirkt. Weiterhin ist diese Reaktion nur etwa zu 60% zuverlässig reproduzierbar. Auch ALBERS UND FREISKORN[17] erhielten nach dieser Anfärbung einen hohen Prozentsatz an Chromatogrammen, die nicht auswertbar waren. Ähnlich verhielten sich die Färbungen mit Anilin–Phosphorsäure, *p*-Aminohippursäure und Benzidin–Trichloressigsäure.

Da wir verlangten, dass die Farbreaktion auf dem Papier möglichst vollständig verläuft und eine hohe Extinktion ergibt, fiel unsere Wahl auf die Silbernitrat-Färbung. Diese Reaktion ist sehr empfindlich, so dass nur geringe Zuckermengen zur Chromatographie benötigt werden. WALLENFELS, BERNT UND LIMBERG[10] haben verschiedene Farbreagentien auf ihre Empfindlichkeit hinsichtlich der Reaktion mit Zuckern geprüft und gelangten auch zu dem Ergebnis, dass die Reaktion der Zucker mit Silbernitrat am empfindlichsten ist und die höchste Extinktion bei Verwendung von gleichen Zuckermengen ergibt. Die Extinktion steigt mit zunehmender Zuckermenge steil an, wodurch der Absolutfehler verringert wird.

Entgegen den wenig ermutigenden Literaturangaben[9] bezüglich der Auswertbarkeit der Silberflecken für quantitative Zuckerbestimmungen, fanden wir mit dieser Methode die beste Reproduzierbarkeit. Allerdings arbeiteten wir nicht mit ammoniakalischem Silbernitrat, sondern zogen das Chromatogramm sehr gleichmässig durch eine Silbernitrat-Lösung in Aceton (siehe S. 202). Nach dem Trocknen erfolgte ein beiderseitiges Besprühen mit äthanolischer Natronlauge[28]. Die dunkelbraunen bis schwarzen Silberflecken erscheinen kurz danach bei Raumtemperatur. Damit die vorhandene Glukose möglichst quantitativ mit dem Sibernitrat reagiert, wird stets nach einigen Minuten nochmals mit äthanolischer Natronlauge besprüht. Das Papier ist dann auch bei Tageslicht nur leicht bräunlich gefärbt und kann durch anschliessendes Baden der Chromatogramme in Fixiersalz-Lösung bis auf die Silberflecken völlig entfärbt werden[10]. Es ist nur darauf zu achten, dass eine möglichst wenig benutzte, noch farblose Fixiersalz-Lösung verwendet wird. Das Salz wird durch Spülen in Leitungswasser wieder entfernt.

Nach dem Trocknen der Chromatogramme werden die Flecken direkt auf dem Papier mittels eines Extinktionsschreibers photometriert. Die schwarzen Silberflecken gestatten es, bei geringer Empfindlichkeit des Apparates zu arbeiten. Dies hat den Vorteil, dass die Grundlinie der Extinktionskurve, ermittelt aus dem Papierwert, nahezu eine Gerade darstellt und mit relativ grosser Genauigkeit festgelegt werden kann. Die bei hoher Empfindlichkeit stark hervortretenden optischen Unebenheiten des Papiers, wodurch die Festlegung der Grundlinie erschwert und die Genauigkeit der Bestimmung herabgesetzt wird, entfallen. Da die Höhe der Extinktion von vielen sehr schwer zu standardisierenden Faktoren abhängig ist, war es unbedingt erforderlich, die Flecken unbekannter Menge mit solchen bekannter Konzentration nebeneinander auf demselben Papierbogen, wie in Fig. 1, aufzu-

tragen und sie während des ganzen Prozesses möglichst gleich zu behandeln[8]. Ein Vergleich über denselben Papierbogen hinaus mit Hilfe einer generellen Eichkurve ist nicht möglich.

Die photometrierten Flecken liefern Kurvenflächen, deren Grösse planimetrisch

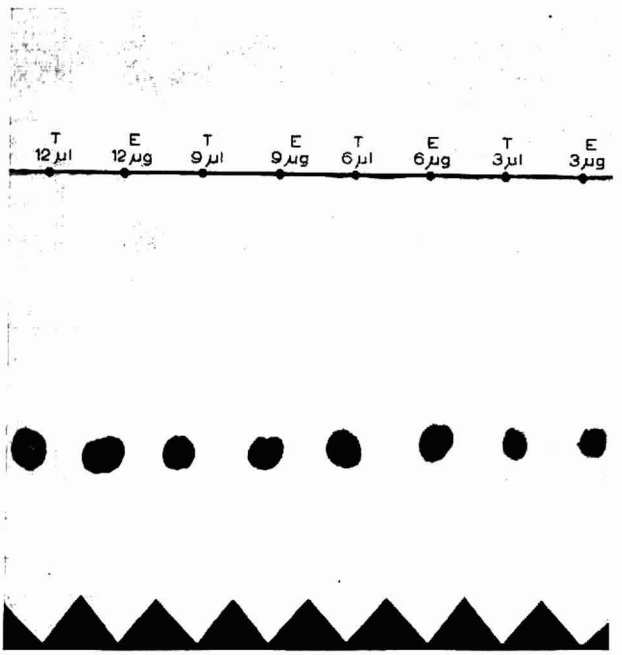

Fig. 1. Muster eines verwendeten Chromatogrammbogens, absteigend. E = Eichfleck; T = Testfleck, aufgetragen: bei 75° hydrolysiertes Glykogen.

ermittelt wird. Die Flächengrösse wird mit der der mitgewanderten Flecken bekannter Konzentration verglichen, und daraus werden die unbekannten Mengen graphisch ermittelt.

ZUR GÜLTIGKEIT DES LAMBERT-BEER'SCHEN GESETZES BEI DIREKTER PHOTOMETRIE

Das Lambert-Beer'sche Gesetz fordert eine geradlinige Abhängigkeit der Extinktion von der Konzentration eines absorbierenden Stoffes. Nach GRASSMANN UND HANNIG[29] hat es bei direkter Photometrie auf dem Papier jedoch nur dann Gültigkeit, wenn eine gleichmässige Verteilung eines Farbstoffes über die ganze Spaltbreite gewährleistet ist. Dies ist normalerweise in der Papierchromatographie nicht der Fall, so dass man bei direkter Photometrie stets Kurven erhält, die mit zunehmender Konzentration auf der Abszisse nach unten gekrümmt sind (Fig. 2). Wie erwähnt, ist die Steilheit der Kurven abhängig vom verwendeten Farbstoff. Eine mathematische Berechnung der Kurven soll nur dann möglich sein, wenn das Filtrierpapier vorher transparent gemacht wurde. Aufgrund der bei Verwendung von Silbernitrat nur wenig abweichenden Kurven haben wir für unsere Zwecke zur besseren Handhabung auf ein vorheriges Transparentmachen verzichtet.

Auffallend ist, dass Literaturangaben vorhanden sind, die trotz direkter Photometrie der Flecken dem Lambert-Beer'schen Gesetz für einen bestimmten Bereich Gültigkeit zuschreiben, obgleich Extinktionsschreiber bei nicht monochromatischem Licht normalerweise immer Kurven mit mehr oder weniger starker Krümmung

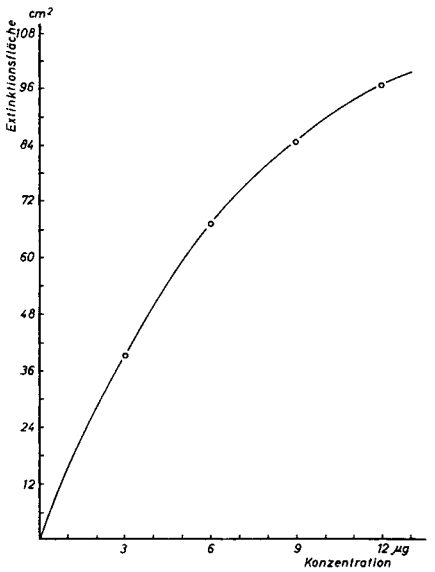

Fig. 2. Beziehung zwischen planimetrierter Extinktionsfläche und Glukose-Konzentration nach Anfärbung mit Silbernitrat.

liefern. Ihr Verlauf ist abhängig vom verwendeten Filter, der benutzten Lichtquelle sowie von der Extinktionskurve des verwendeten Farbstoffs. WALLENFELS und Mitarbeiter[10] photometrierten Silberflecken mit monochromatischem Licht und auf transparentem Papier. Sie fanden dann eine Linearität zwischen Extinktionsfläche und Konzentration bis zu 25 μg.

Bei direkter Photometrie des Farbfleckens auf dem Papier hat das Lambert-Beer'sche Gesetz unter Verwendung von nicht monochromatischem Licht und nicht transparent gemachtem Papier demnach keine Gültigkeit. Trotzdem ist eine quantitative Auswertung der Flecken unter Beachtung der vorstehenden Ausführungen möglich und liefert gute Ergebnisse, wenn ein Bereich gewählt wird, in dem die

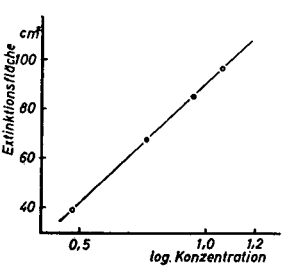

Fig. 3. Abhängigkeit der Extinktionsfläche von der Glukose-Konzentration im halblogarithmischen Koordinatensystem nach Anfärbung mit Silbernitrat.

Kurve möglichst steil verläuft. Dies ist bei geringen Konzentrationen der Fall. Glukoseflecken bis zu 15 μg sind für eine quantitative Auswertung brauchbar. Bei höheren Konzentrationen verlaufen die Kurven auch bei anderen Farbmethoden so flach, dass der absolute Fehler grösser und die Bestimmung ungenauer wird.

Wir fanden für die vorliegende Methode eine lineare Abhängigkeit von Extinktionsfläche und dem Logarithmus der Konzentration (Fig. 3). Diese Proportionalität ist in der Literatur bereits für andere in der Zuckerchromatographie verwendete Farbstoffe angegeben worden[20,22]. Auch mit Hilfe dieser Darstellung ist durch Interpolation eine quantitative Bestimmung möglich.

HYDROLYSE VON GLYKOGEN UND WEITERE PRÄPARATION ZUR CHROMATOGRAPHIE

Glykogen zerfällt nach saurer Hydrolyse über Dextrine und Maltose in Glukose, die dann, wie beschrieben, ermittelt werden kann. Wir strebten eine möglichst optimale Hydrolyse des Glykogens an und haben deshalb zunächst die Wirkung der die Spaltung beeinflussenden Faktoren geprüft. Zur Hydrolyse benutzten wir Salzsäure, da sie sich leichter entfernen lässt als beispielsweise Schwefelsäure. 1 *N* Salzsäure ergab während einer Zeitdauer von 15 Stunden die höchste Glukoseausbeute. Zum Vergleich führten wir Versuche mit 1/10, 1/2, 1 und 2 *N* Salzsäure durch.

Weiterhin war der Einfluss der Temperatur zu prüfen. Bekanntlich hat die Temperatur einen beträchtlichen Einfluss auf den Hydrolyseverlauf. Nach Fig. 4

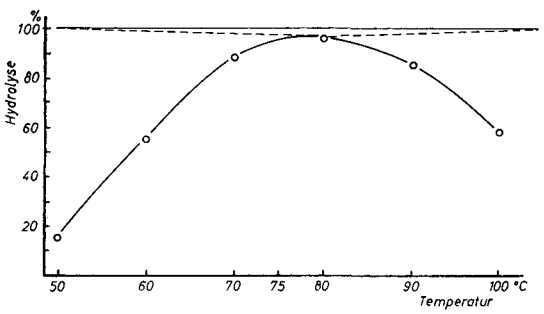

Fig. 4. Einfluss auf die Hydrolyse und Zersetzung von Glykogen mit 1 *N* Salzsäure; Dauer: 15 Stunden; Werte papierchromatographisch ermittelt.

liegt das Optimum unter unseren Bedingungen zwischen 70 und 80°. Bereits bei 90° tritt eine leichte Verfärbung des Hydrolysats ein, die auf eine schon eingetretene Zerstörung von Glukose hindeutet. Wir führten unsere Hydrolysen deshalb bei einer konstanten Temperatur von 75° im zugeschmolzenen Röhrchen durch. Wie chromatographisch nachgewiesen werden konnte, wurde unter diesen Bedingungen eine nahezu vollständige Spaltung des Glykogens in Glukose erreicht. Zwischenprodukte waren nicht mehr nachweisbar (vgl. Fig. 1).

Vor der Chromatographie ist es notwendig, die Säure weitgehend zu entfernen. Auch Salzsäure zersetzt beim Auftragen der Lösung das Filtrierpapier. Eine einfache Neutralisation lieferte uns quantitativ schlecht auswertbare Flecken. Die bei Verwendung von Schwefelsäure gewöhnlich durchgeführte Entfernung der Sulfationen mittels Bariumhydroxyd[17,30] ist aufwendig und birgt Fehlermöglichkeiten

in sich. Die von uns benutzte Salzsäure konnten wir bequem entfernen, wenn unter wiederholtem Hinzufügen von wenig Wasser mittels eines Vakuum-Rotationsverdampfers[31] etwa dreimal bis zur Trockne eingeengt wurde. Danach liess sich eine einwandfreie Chromatographie der Glukose durchführen.

FEHLERMÖGLICHKEITEN UND IHRE BERECHNUNG

Hydrolysefehler

Zur Abschätzung des durch die Hydrolyse bedingten Fehlers wurden vier Glykogenproben bei 80° an verschiedenen Tagen hydrolysiert. Nach Entfernung der Säure wurden auf einen Chromatogrammbogen (30 × 30 cm²) je zweimal nebeneinander von jedem Hydrolysat 8 μg aufgetragen und chromatographiert. Dies wurde achtmal wiederholt. Nach Photometrie der Silberflecken und Planimetrie der Kurvenflächen wurde für jedes Hydrolysat und Chromatogramm der Index *a/b* ermittelt und eine Fehlerberechnung durchgeführt (Tabelle I).

TABELLE I

BERECHNUNG DES GESAMTFEHLERS OHNE BERÜCKSICHTIGUNG DES HYDROLYSEFEHLERS (GLYKOGENHYDROLYSATE)

Chrom. Nr.	*Indices*			
	1	*2*	*3*	*4*
1	0.942	0.965	——	——
2	1.000	1.001	1.005	0.927
3	0.940	1.046	0.998	0.988
4	0.930	0.962	0.967	0.947
5	0.940	0.965	1.060	1.049
6	1.058	0.973	1.043	0.963
7	1.030	1.012	0.975	1.036
8	1.000	0.998	1.032	1.111
Mittlere Fehler	1 ± 0.019	1 ± 0.011	1 ± 0.014	1 ± 0.025
%-Fehler	± 1.9	± 1.1	± 1.4	± 2.9
Maximale Abweichung (%)	—4.2	—2.6	+2.9	+5.3

Tabelle I zeigt die Reproduzierbarkeit des chromatographischen Vorgangs (Auftrage-, Anfärbe-, Photometrier- und Planimetrierfehler) ohne dabei den auf die Hydrolyse entfallenden Fehler zu berücksichtigen. Da auf jedem Chromatogramm für diesen Zweck nur zwei vergleichbare Flecken vorhanden sind, war es notwendig, die Indices zu berechnen, um den Fehler, der durch Verwendung von mehreren Chromatogrammen entsteht, auszuschalten und dann über das Chromatogramm hinaus Vergleiche vornehmen zu können. Ausserdem sind die einzelnen Extinktionsflächen eines Hydrolysats der verschiedenen Chromatogramme insofern nicht vergleichbar, da die Empfindlichkeit des Chromatometers jeweils so reguliert wurde, dass etwa gleich grosse Extinktionsflächen erhalten wurden. Die Berechnung ergab einen Maximalfehler von ± 4–5 %; der grösste errechnete mittlere Fehler betrug ± 2.5 %.

TABELLE

BERECHNUNG DES GESAMTFEHLERS

Chrom. Nr.	Hydrolysate								Hydrolysate $\frac{a+b}{2}$			
	1		2		3		4					
	a	b	a	b	a	b	a	b	1	2	3	4
I	35.3	37.5	37.2	38.6	36.9	—	—	—	36.4	37.9	36.9	—
II	40.5	40.5	40.0	39.9	38.2	38.0	35.3	38.1	40.5	40.0	38.1	36.7
III	38.1	39.5	39.2	37.5	39.3	39.4	36.1	36.5	38.8	38.4	39.3	36.3
IV	40.0	43.0	42.8	44.5	41.3	42.7	39.6	41.9	41.5	43.5	42.0	40.8
V	39.2	41.7	40.2	41.6	43.6	41.2	36.5	34.8	40.4	40.9	42.4	35.7
VI	37.4	35.3	36.0	37.0	38.8	37.2	36.0	37.4	36,4	36.5	38.0	36.7
VII	40.2	39.0	39.3	38.8	39.8	40.9	42.1	40.6	39.6	39.1	40.3	41.3
VIII	38.6	38.6	39.8	39.9	41.3	40.0	41.6	37.4	38.6	39.8	40.6	39.5

Wird der Hydrolysefehler mit in die Berechnungen einbezogen, wie in Tabelle II, so ist zu fordern, dass der Gesamtfehler sich erhöht, wenn durch die zu verschiedenen Zeiten durchgeführte Hydrolyse der Fehler vergrössert wird. Nach Tabelle II beträgt die maximale Abweichung (Mittelwert) einschliesslich der Hydrolyse etwa $\pm$ 4% und ist damit nicht höher als bei der in Tabelle I gezeigten Berechnungsweise. Der durch Hydrolyse verursachte Fehler liegt also innerhalb des gesamten Chromatographierfehlers. Er fällt praktisch nicht ins Gewicht und ist zu vernachlässigen. Dies bedeutet, dass die Herstellung einer geeichten Vergleichslösung unter den angegebenen Hydrolysebedingungen genügt.

Auftragefehler

Der durch das Auftragen der Substanz auf das Papier entstehende Fehler ist stark abhängig von der Konzentration der verwendeten Lösung. Bei Verwendung einer zu konzentrierten Lösung ist die aufzutragende Menge zu gering und der Fehler wird vergrössert. Bei stark verdünnten Lösungen wird die Auftragezeit übermässig lang. Wir benutzten 0.1 %ige Glykogen- bzw. Glukoselösungen.

Auch die zum Auftragen verwendete Pipette ist für die Fehlerentstehung von grosser Wichtigkeit. Wir erzielten unter Verwendung möglichst ein und derselben Elphor-Pipette die grösste Reproduzierbarkeit. Eine Agla-Spritze war wegen ihres relativ grossen temperaturempfindlichen Volumens weniger geeignet.

Anfärbefehler

Um diesen Fehler möglichst niedrig zu halten, ist ein zweimaliges gleichmässiges Besprühen des Chromatogramms mit alkoholischer Natronlauge erforderlich, damit die Reaktion der Glukose mit dem Silbernitrat vollständig erfolgt. Jedoch dürfte gerade dieser Fehler sehr gering zu halten sein.

Photometrierfehler

Während sich die beiden vorstehenden Fehler nicht ohne weiteres quantitativ erfassen lassen, ist dies bei der Photometrie leicht zu erreichen. Da es nicht möglich ist,

II

EINSCHLIESSLICH HYDROLYSEFEHLER

Mittelwert M	*Abweichung von M*				*Abweichungen (%)*			
	1	*2*	*3*	*4*	*1*	*2*	*3*	*4*
37.1	—0.7	+0.8	—0.2	—	—1.9	+2.15	—0.54	—
38.8	+1.7	+1.2	—0.7	—2.1	+4.38	+3.09	—1.81	— 5.41
38.45	+0.35	—0.05	+0.85	—2.15	+1.01	—0.15	+2.46	— 6.23
41.95	—0.45	+1.55	+0.05	—1.15	—1.07	+3.70	+0.12	— 0.74
39.85	+0.55	+1.05	+2.55	—4.15	+1.38	+2.64	+6.40	—10.4
36.9	—0.5	—0.4	+1.1	—0.2	—1.35	—1.08	+2.98	— 0.54
40.1	—0.5	—1.0	+0.2	+1.2	—1.24	—2.49	+4.98	+ 2.99
39.6	—1.0	+0.2	+1.0	—0.1	—2.52	+5.05	+2.52	— 0.25
Maximalabweichung:					—1.86	+2.54	+2.72	— 4.08

mehr als einen Flecken gleichzeitig zu photometrieren, könnte der zeitlich verschobene Messvorgang beim Vergleich mehrerer Flecken Fehler verursachen, wenn sich die Empfindlichkeit des Apparates mit der Zeit ändert. Es wurde deshalb ein und derselbe Fleck innerhalb einer Stunde und über einen Arbeitstag hinweg jeweils achtmal gemessen. Das Ergebnis war:

Photometrierfehler bei Messdauer von 1 Stunde	± 0.68 %
Photometrierfehler bei Messdauer von 8 Stunden	± 0.76 %
davon Planimetrierfehler	± 0.22 %

Der mittlere Photometrierfehler einschliesslich Planimetrierfehler beträgt etwa 1/3 bis 1/2 des mittleren Gesamtfehlers. Es ist günstig, Flecken, die miteinander verglichen werden sollen, nach Möglichkeit nacheinander zu photometrieren. Die von uns eingestellte Arbeitsweise des Photometers erlaubte, etwa acht Flecken in einer Stunde durchzumessen.

Gesamtfehler bei Benutzung verschiedener Zuckermengen

In diesen Versuchen sollte die Frage geprüft werden, ob die aufgetragene Zuckermengen einen Einfluss auf den Gesamtfehler hat. Zu diesem Zwecke wurden von einer Probe hydrolysierten Glykogens verschiedene Mengen von 3, 6, 9 und 12 μg auf ein Chromatogramm wie in Fig. 1 aufgetragen. Dies wurde auf elf verschiedenen Chromatogrammbögen wiederholt, die nach verschiedenen Gesichtspunkten ausgewertet wurden. In Tabelle III wurden zur Ermöglichung eines Vergleichs über die einzelnen Chromatogrammbögen hinweg wiederum von den als Test- und Eichflecken bezeichneten Spots gleicher Konzentration die Indices gebildet und die mittlere Abweichung berechnet.

Nach Tabelle III ist der mittlere Fehler bei kleineren Auftragemengen etwas höher als bei grösseren. Dies ist wohl darauf zurückzuführen, dass der durch die Festlegung der Grundlinie nach der Photometrie entstehende Fehler bei kleineren Extinktionskurven stärker ins Gewicht fällt als bei grösseren. Die beste Reproduzier-

barkeit wird demnach zwischen Auftragemengen von 8–12 μg erreicht, obgleich sich in diesem Bereich bereits ein deutlicher Abfall der Extinktionskurve bemerkbar macht (Fig. 3). Es wurde in diesem Bereich ein maximaler mittlerer Fehler von $\pm$ 2.6 % gefunden.

TABELLE III

VERGLEICH DER MITTLEREN ABWEICHUNGEN BEI VERWENDUNG VERSCHIEDENER KONZENTRATIONEN EINER HYDROLYSIERTEN GLYKOGENPROBE

Chrom. Nr.	*Indices Eichfleck/Testfleck für*			
	3 µg	*6 µg*	*9 µg*	*12 µg*
Gly I	1.13	1.03	1.09	0.83
Gly II	1.16	1.27	0.89	1.01
Gly III	0.84	0.92	0.94	0.98
Gly IV	1.09	0.88	0.99	1.02
Gly V	1.02	1.07	0.98	1.04
Gly VI	0.95	0.93	0.99	0.94
Gly VII	1.15	1.01	0.87	0.99
Gly VIII	0.99	0.85	1.08	0.99
Gly IX	1.13	1.33	1.00	1.02
Gly X	0.95	1.04	0.94	0.91
Gly XI	1.05	1.15	0.85	1.08
Mittlere Abweichung	1 $\pm$ 0.036	1 $\pm$ 0.049	1 $\pm$ 0.026	1 $\pm$ 0.018
%-Fehler	$\pm$ 3.6	$\pm$ 4.9	$\pm$ 2.6	$\pm$ 1.8

Um zu prüfen, wie gross die mittlere Abweichung bei Verwendung verschiedener Konzentrationen ist, wenn jeweils zwei Chromatogramme mit je acht Flecken verglichen werden, wurde jedes Chromatogramm, dass zur Ermittlung der in Tabelle III eingetragenen Indices verwendet wurde, mit jedem anderen kombiniert und für die jeweils erhaltenen 8 Indices die mittlere Abweichung errechnet. Man erhält dann 55 Werte, deren arithmetisches Mittel nach Tabelle IV bei $\pm$ 4.3 % Abweichung liegt.

TABELLE IV

MITTLERE FEHLER NACH KOMBINATION ALLER VERFÜGBAREN CHROMATOGRAMME; ERRECHNUNG DES ARITHMETISCHEN MITTELWERTES UND DER MITTLEREN ABWEICHUNG

	Gly I	*Gly II*	*Gly III*	*Gly IV*	*Gly V*	*Gly VI*	*Gly VII*	*Gly VIII*	*Gly IX*	*Gly X*
Gly II	0.0543	—	—	—	—	—	—	—	—	—
Gly III	0.0402	0.0512	—	—	—	—	—	—	—	—
Gly IV	0.0372	0.0488	0.0324	—	—	—	—	—	—	—
Gly V	0.0332	0.0459	0.0278	0.0233	—	—	—	—	—	—
Gly VI	0.0342	0.0466	0.0286	0.0245	0.0180	—	—	—	—	—
Gly VII	0.0421	0.0518	0.0367	0.0332	0.0296	0.0300	—	—	—	—
Gly VIII	0.0387	0.0499	0.0341	0.0320	0.0255	0.0266	0.0351	—	—	—
Gly IX	0.0568	0.0650	0.0538	0.0516	0.0488	0.0495	0.0544	0.0526	—	—
Gly X	0.0356	0.0476	0.0310	0.0363	0.0230	0.0218	0.0315	0.0283	0.0503	—
Gly XI	0.0437	0.0543	0.0406	0.0370	0.0330	0.0339	0.0408	0.0385	0.0566	0.0352

Arithmetisches Mittel aus 55 Werten: 0.0432
Mittlere Abweichung: $\pm$ 0.0017

Nach Tabelle IV liegen 2/3 aller für je 8 Indices berechneten mittleren Abweichungen zwischen ± 4.15–4.49 %. Die Methode ist also in den meisten Fällen mit einem Fehler von ± 4–4.5 % belastet. Bei Addition aller Fehler wird die Reproduzierbarkeit bis auf ± 6.5 % erhöht; im günstigsten Falle wurde ein Fehler von ± 1.8 % ermittelt. Eine Verringerung des Fehlers kann durch Verwendung günstiger Konzentrationen und durch Eliminierung schlecht auswertbarer Flecken bzw. stark abweichender Indices erfolgen, was in Tabelle IV nicht geschehen ist.

VERGLEICH GLEICHER MENGEN GLUKOSE UND GLYKOGEN AUF DEM CHROMATOGRAMM SOWIE ERMITTLUNG DES WÄHREND DER HYDROLYSE ZERSTÖRTEN ANTEILS

Der Vergleich einer bestimmten Glukosemenge mit dem nach Hydrolyse aus einer gravimetrisch gleichen Glykogen- oder anderen Polysaccharidmenge entstandenen Glukoseanteil erschien insofern interessant, da hierdurch die Möglichkeit gegeben wird, mit Hilfe eines ermittelten Faktors Glukosemengen in Glykogen umzurechnen und diese neben Polysaccharidmengen relativ leicht ohne Abtrennung zu bestimmen. Der während der Säureeinwirkung verlustig gehende Glukoseanteil ist bei der Berechnung entsprechend zu berücksichtigen.

Zur Ermittlung des Umrechnungsfaktors wurden mehrere Chromatogramme angefertigt, auf denen Glukose mit hydrolysiertem Glykogen verglichen wird. Zur Bestimmung der während der Hydrolyse durch Säureeinwirkung auftretenden Verluste an Glukose wurde reine Glukose unter gleichen Temperaturbedingungen (75°) mit *N*-Salzsäure über 15 Stunden behandelt und dann papierchromatographisch mit einer unbehandelten Glukoseprobe gleicher Konzentration verglichen. Da die zeitliche Hydrolyse des Glykogens asymptotisch verläuft und anfangs die grösste Spaltung auftritt, scheint uns dieser Vergleich bei einer Hydrolysedauer von 15 Stunden gerechtfertigt zu sein. Unter Berücksichtigung der Hydrolyse bei der Säurebehandlung des Glykogens wird in diesen Fällen die Zerstörung noch etwas geringer anzunehmen sein als bei der hier zum Vergleich herangezogenen mit Säure behandelten Glukose. ALBERS UND FREISKORN[17] fanden bei der Schwefelsäurehydrolyse bei einer Versuchstemperatur von 100° einen Verlust von 8 % Glukose.

Nach Literaturangaben[32] entspricht 1 g Glukose 0.925 g Glykogen. Nach Hydrolyse entsteht demnach aus 1 g Leberglykogen 1.081 g Glukose, wenn die Hydrolyse vollständig ist und keine Verluste an entstandener Glukose auftreten. Unter Berücksichtigung der methodischen Fehlergrenzen von ± 4.5 % war unter unseren Hydrolysebedingungen kein Glukoseverlust feststellbar. Damit dürften unsere Hydrolysebedingungen schonender sein als die von ALBERS UND FREISKORN[17]. Jedoch war andererseits die Hydrolyse nicht vollständig, da wir aus unseren Glukoseproben nach Hydrolyse immer etwas weniger Glukose erhielten als dem Umrechnungsfaktor entspricht.

Glykogen (Merck)	1 g ergibt
Glukose, theoretisch	1.081 g
Glukose, gefunden	1.033 g (Mittel aus 4 Einwaagen nach Hydrolyse

Wir fanden demnach für unsere Methode einen Umrechnungsfaktor von 0.968. Es werden unter unseren Bedingungen etwa 4.5 % Glykogen nicht erfasst. Eine voll-

ständigere Spaltung ohne merkliche Zerstörung könnte durch eine Erhöhung der Hydrolysezeit erreicht werden.

METHODISCHE AUSFÜHRUNGEN

Hydrolyse und Entfernung der Säure

Jeweils genau 25 mg Glykogen der Firma Merck, Darmstadt (mit 7–8% H_2O) wurden direkt in die zur Hydrolyse verwendeten kleinen Reagensgläser (*ca.* 10 ml) eingewogen, mit 1 ml *N*-Salzsäure versetzt und zugeschmolzen. Die nun folgende Hydrolyse erfolgte in einem Thermostaten bei 75° ± 1° über 15 Stunden. Danach wurde das Hydrolysierröhrchen in einem 50 ml Schliffrundkolben (NS 14.5 mm) zerbrochen und die am Kolbenhals hängengebliebenen Glas- und Substanzreste mit einigen ml destilliertem Wasser in den Kolben gespült. Mittels eines Vakuum-Rotationsverdampfers wurde bis zur Trockne eingedampft. Zur möglichst weitgehenden Entfernung der Salzsäure wurde dann der Rückstand im Kolben noch zweimal mit einigen ml destilliertem Wasser aufgenommen und dieses wiederum, wie vorher, abgedampft. Der verbliebene Rückstand wurde nun in genau 25 ml destilliertem Wasser gelöst und diese Lösung zur Chromatographie verwendet.

Chromatographie

Wir verwendeten Papier der Firma Schleicher & Schüll 2043b Mgl, Format 30 × 30 cm^2. Auf einer Startlinie wurden in Abständen von 3.5 cm acht Flecken pro Chromatogrammbogen mit einer Elphor-Pipette in Konzentrationen von 2–12 μg, besser 8–12 μg aufgetragen. Zur Erreichung einer möglichst grossen räumlichen Nähe wurden bei Vergleichen Test- und Eichfleck jeweils nebeneinander aufgetragen. Absteigendes Durchlaufchromatogramm; Laufmittel: Äthylacetat–Pyridin–Wasser, 40:11:6. Nach einer Wanderung der Glukose von 10–15 cm auf dem Papier (Laufzeit ist stark von der Papierserie abhängig: 6–15 Stunden) wurde das Chromatogramm zur Entfernung des Laufmittels bei *ca.* 25° getrocknet.

Anfärbung

Die trockenen Chromatogramme werden möglichst gleichmässig durch das in einer flachen Schale sich befindende Silbernitrat-Reagens (1.5 ml einer nahezu gesättigten wässrigen Silbernitrat-Lösung in 100 ml Aceton geben, dann eben so viel Wasser hinzutun, bis der entstandene Niederschlag sich gerade löst) gezogen und dann bei Zimmertemperatur im Abzug wiederum völlig getrocknet. Bei ungleichmässigem und ruckartigem Durchziehen der Chromatogramme durch das Silbernitrat-Reagens ergeben sich helle Streifen, die, wenn sie über die Zuckerflecken zu liegen kommen, zu Verfälschungen der Extinktionswerte führen. Nach *ca.* 10 Min. wird das Chromatogramm mit alkoholischer NaOH (10 g NaOH in möglichst wenig H_2O lösen und dann auf 500 ml mit 95 %igem Äthanol auffüllen) beidseitig gleichmässig mittels eines N_2-Stromes besprüht. Dieser Prozess wird nach einigen Minuten wiederholt, damit die Reaktion quantitativ erfolgen kann. Sogleich nach dem Abtrocknen des Alkohols (*ca.* 5–10 Min.) im Abzug wird das Chromatogramm in ein für die Photographie verwendetes Fixierbad gelegt. Nach Entfärbung des Untergrundes (*ca.* 5–10 Min. im frischen Fixierbad) sowie Entfernung des überschüssigen $AgNO_3$ gelangt es in ein fliessendes Wasserbad zur einstündigen Wässerung; anschliessend Trocknung bei etwa 70°.

Photometrie

Wir benutzten ein Chromatometer zur direkten Photometrie der erhaltenen Silberflecken auf dem Papier der Firma Dr. B. Lange, Berlin-Zehlendorff. Die Flecken wurden senkrecht zur Laufrichtung photometriert. Hierdurch und durch das Ausmessen von wenigstens vier Flecken eines Chromatogrammbogens hintereinander wird die Festlegung der Grundlinie wesentlich erleichtert. Nach planimetrischer Ermittlung der Kurvenflächen wurden diese in einer graphischen Darstellung in Beziehung zur Konzentration gesetzt. Anhand der erhaltenen Kurve kann der Glukose- bzw. Glykogengehalt unter Beachtung der oben erwähnten Bedingungen graphisch ermittelt werden.

Die Methode ist selbstverständlich auch zur Bestimmung anderer reduzierender Zucker anwendbar.

ZUSAMMENFASSUNG

Nach eingehender Besprechung der im Schrifttum vorhandenen quantitativen papierchromatographischen Verfahren zur Zuckerbestimmung wird eine modifizierte Methode zur Glykogen- und Glukosebestimmung mitgeteilt. Durch absteigende Durchlaufchromatographie (Laufmittel: Äthylacetat–Pyridin–Wasser, 40:11:6) und Anfärbung mit Silbernitrat-Lösung in Aceton/alkoholischer Natronlauge sowie anschliessende Entfärbung in Fixiersalz werden gut auswertbare Silberflecken erhalten. Die quantitative Bestimmung erfolgte durch direkte Photometrie auf dem Papier und Planimetrie der erhaltenen Extinktionsflächen. Es besteht eine lineare Abhängigkeit zwischen der Extinktionsfläche und dem Logarithmus der Konzentration. Das Glykogen wurde vorher mit Salzsäure hydrolysiert. Der optimalste Hydrolysegrad wurde mit 1 *N* Salzsäure nach einer Zeitdauer von 15 Stunden bei 75° erreicht. Reproduzierbarkeit der Methode: Maximalabweichung ± 6.5 %, mittlere Abweichung ± 4–4.5 %. Die Reproduzierbarkeit kann durch Eliminierung schlecht auswertbarer Chromatogramme erhöht werden.

SUMMARY

Earlier work dealing with the quantitative determination of sugars by paper chromatography is reviewed extensively, and a modified method for the determination of glucose and glycogen is given. After one-dimensional descending chromatography (solvent system: ethyl acetate–pyridine–water, 40:11:6), the spots were detected by dipping the chromatograms into an acetone solution of silver nitrate and spraying them twice with sodium hydroxide dissolved in ethanol. In order to reduce the colour of the background the chromatograms were treated with a fixing bath used for photographic purposes. Quantitative estimation was carried out by direct photometry on the paper strip by means of an automatic densitograph. Glycogen was treated with hydrochloric acid before estimation. Hydrolysis could be carried out best with 1 *N* hydrochloric acid for 15 h at 75°. For the quantitative estimation the curves were measured with a planimeter. A linear relation was found between extinction area and the log of the concentration. Reproducibility of the method: maximum deviation ± 6.5 %, standard deviation ± 4–4.5 %. The reproducibility can be increased by eliminating the chromatograms that are difficult to evaluate.

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ION-EXCHANGE PAPER CHROMATOGRAPHY OF LANTHANIDE IONS

I. ANION EXCHANGE OF LANTHANIDE CITRATE COMPLEXES

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The method of paper partition chromatography has been applied to the separation of lanthanide ions by several workers, notably POLLARD, MCOMIE AND STEVENS[1], LEDERER[2], and DANON AND LEVI[3]. While these investigations have been to some extent successful, the separation factors obtained have been low and separation of adjacent lanthanides has not been achieved except in the case of lanthanum and cerium[2d]. In one of the most successful experiments LEDERER[2d] obtained a practical separation factor (ratio of distances moved) of 1.3 between the next-but-one neighbours europium and promethium after five days elution with a mixture of ethanol and dilute hydrochloric acid.

A considerably greater extent of separation is obtained in the well-known chromatographic method using a column of cation-exchange resin and eluting with a solution of a complexing agent. For example MAYER AND FREILING[4] using a column of Dowex 50 obtained, in less than 8 h, a practical separation factor of 3.5 between the next-but-one neighbours terbium and europium, using EDTA solution as eluent. Using citric acid for the elution the corresponding separation factor was 1.6.

In view of the simplicity and convenience of paper chromatography and the superior separating power of the ion-exchange method, it seemed desirable to investigate the use of the ion-exchange papers now available. Some preliminary work on the separation of rare earths using cellulosic cation-exchange papers has been reported by WELLS[5]. Separation of lanthanum, cerium and yttrium on paper impregnated with Dowex 50 was achieved by LEDERER[6] using citric acid as eluent.

In the work reported here cellulosic anion-exchange resins have been used together with citric acid solutions for the elution.

EXPERIMENTAL

Whatman anion-exchange papers were used. There are three types: diethylaminoethyl-cellulose; aminoethyl-cellulose, and "ecteola" cellulose. The first mentioned was used most extensively.

The lanthanides were in the form of radio-tracers, the following nuclides being

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used: ^{144}Ce (carrier-free), ^{147}Pm, ^{152} + ^{154}Eu (100 mC/g) and ^{160}Tb (initially 50 mC/g). Solutions of these (obtained from the Radiochemical Centre, Amersham) were diluted to concentrations of 5–50 μC/ml, 0.01–0.04 ml of the diluted solutions being taken for each chromatogram.

Chromatography was carried out on strips about 3 cm wide using downward flow of eluent. Convenient and simple holders for the paper and solvent were made from ordinary 4 oz. poly(ethylene) bottles. The lower half of the bottle was cut off and the resulting cylindrical pot provided on opposite sides with two horizontal slits about 4 cm long and 0.5 cm high, through which the paper strips could be inserted. These slits were made by cutting the sides and upper length of the rectangle and then bending the resulting strip outwards over a hot glass rod. This produced a protruding lower lip which kept the paper away from the sides of the bottle and this prevented siphoning. The vessel was closed with a stopper through which passed a thistle funnel whose stem passed almost to the bottom of the vessel. The polythene vessel was held in the neck of a gas-jar by means of a larger stopper. This apparatus has the advantage, apart from its negligible cost, that the paper may be placed in position and aligned with the holder empty; the solvent may then be added at any convenient time through the thistle funnel.

The paper strips were pretreated with the eluent to be used and were then air-dried before adding the tracer solution. Chromatograms were evaluated by cutting the dried strips of paper into 1 cm sections and measuring the activity of each section with a Geiger-Müller counter. Strictly therefore, the resulting chromatograms should be drawn as histograms, but for convenience they were drawn as smoothed curves. In most cases chromatograms were run with cerium and one other lanthanide. To confirm the identity of a particular peak, the counts were often repeated with the interposition of an aluminium absorber just thick enough to absorb the β-particles of the lanthanide giving the less energetic β-particles—in practice the one other than cerium (*cf.* ref.[7]).

RESULTS

(*i*) *Diethylaminoethyl-cellulose (DEAC)*

Examples of the chromatograms obtained with this paper are shown in Figs. 1 and 2. It will be seen that the R_F value decreases with increasing atomic number of the lanthanide. This is to be expected, since retention depends on the formation of anionic citrate complexes whose stability should increase with decreasing size of the lanthanide ion.

Chromatograms of this type were carried out using a number of different citric acid concentrations and the effects of adding ethanol to the solvent and of lowering the pH by adding HCl were also investigated. The most convenient eluent was found to be a 0.026 *M* (0.5 %) solution of citric acid in water; the pH of this solution is 2.4.

The results are summarised in Table I.

In order to produce measurable separations it was necessary to choose conditions where R_F values are low, and to continue eluting long after the solvent front had reached the end of the paper. This means that direct observation of the R_F values was not generally possible, and the values reported are based on the estimated "position" of the solvent front obtained by extrapolating time–distance curves for the movement of the solvent front in the early stages of the experiments. (The rate of movement

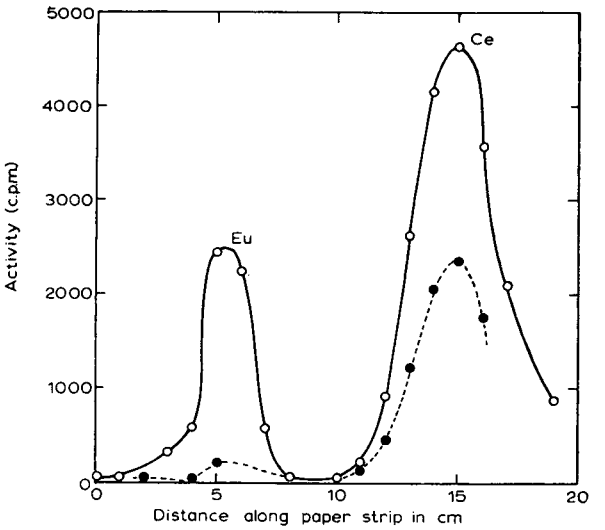

Fig. 1. Separation of ^{144}Ce and ^{152} + ^{154}Eu on DEAC paper by elution with 0.026 *M* citric acid. The dotted curve shows the activity measured through an aluminium absorber of thickness 248 mg/cm^2 which absorbs most of the emission from europium. Elution time 5 h.

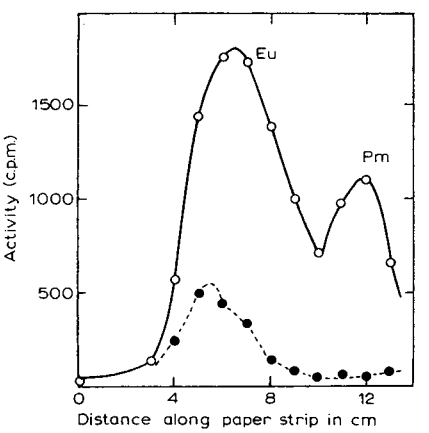

Fig. 2. Separation of ^{147}Pm and ^{152} + ^{154}Eu on DEAC paper by elution with 0.026 *M* citric acid solution containing 40 % of ethanol. The dotted curve shows the activity measured through an aluminium absorber of thickness 41 mg/cm^2 which absorbs all of the emission from promethium. Elution time 30 h.

down the paper is not constant but tends to be faster in the initial stages.) In view of this R_F values are reported for cerium only, but values of apparent separation factors for cerium are given for the other ions used, since the distances moved by the peaks can be measured directly.

It will be seen that the R_F value is increased by increasing the citric acid concentration or by addition of HCl. These may be regarded as two different methods of

TABLE I

SEPARATIONS ON DEAC PAPER WITH CITRIC ACID SOLUTION AS ELUENT

Composition of eluent						
Citric acid molarity	*HCl molarity*	*Ethanol % v/v*	$(R_F)_{Ce}$	d_{Ce}/d_{Eu}*	d_{Ce}/d_{Tb}	d_{Ce}/d_{Pm}
0.026	—	—	0.17	2.6	2.6	2.2
0.010	—	—	0.06		2.2–3.4	
0.0026	—	—	0.006	3		
0.026	—	10		2.4–3.4	2.9	
0.026	—	20	0.04	3.2–3.6		
0.2	—	60	0.03	2.2–3.8		
0.5	—	60	0.4	1.5		
0.010	—	20	0.02	3.2		
0.026	0.026	—		2.0		
0.026	0.04	—	0.6	1.4		
0.026	0.026	20	0.25	1.8		

* The apparent separation factor, (*e.g.* d_{Ce}/d_{Eu}), is the ratio of the distance moved by one ion (*e.g.* Ce^{3+}) to that moved by the other (*e.g.* Eu^{3+}).

lowering the pH and thereby reducing the concentration of anionic complexes. Addition of ethanol on the other hand lowers the R_F value to a marked extent. The apparent separation factors seem generally to be increased by those factors which reduce the R_F values. This makes it advantageous to use eluents containing ethanol. This is shown in the separation factor of 1.8 between the next-but-one neighbours Pm and Eu (Fig. 2) obtained using 0.026 *M* citric acid in 40 % ethanol; in absence of the ethanol the separation factor was 1.3 determined directly, or only 1.2 calculated indirectly from measurements against Ce. On the other hand eluents containing ethanol often gave erratic and unreliable results.

(ii) Aminoethyl-cellulose paper (AEC)

Retention of rare earth–citrate complexes on this ion-exchange paper is greater than that of the DEAC paper discussed above. Thus using 0.026 *M* citric acid, the R_F value for cerium is about 0.006 for AEC paper as compared with the value of 0.17 for DEAC paper. Separations were produced on this paper but these required rather long times (40–70 h) and gave apparent separation factors similar to those found for DEAC paper, *i.e.* $d_{Ce}/d_{Tb} \simeq 3$. The R_F values could be increased and the time shortened by using higher citric acid concentrations, but this lowered the separation factors.

(iii) "Ecteola" paper

This anion-exchange paper gave very high R_F values with the eluents used for the other papers. These values could be reduced by addition of ethanol or by lowering the citric acid concentration. No satisfactory results were obtained on this paper, however. The peaks obtained were very broad and in many cases irregular, as if a variety of small peaks were superimposed upon the main peaks.

DISCUSSION

HUFFMAN AND OSWALT[7] investigated the separation of tracer quantities of rare earths on a column of anion-exchange resin (Dowex A-1) using a 0.0125 *M* solution of citric acid acidified to pH 2.1 with HCl. They report a chromatogram for Eu and Pm which shows an apparent separation factor of 1.3; this is identical with our value using aqueous citric acid and DEAC paper; their separation is nevertheless superior even to that shown in Fig. 2—where the separation factor is 1.8—owing to the greater sharpness of the peaks. Other workers[8,9] have reported separations of lanthanide ions on anion-exchange columns with citric acid as eluent.

The apparent separation factor is of course not a fundamental property of the system. According to simple chromatographic theory (see, *e.g.*, ref.[10]):

$$(R_F)_A = \frac{1}{D_A + 1} \tag{1}$$

where D_A is the distribution ratio of the constituent A between the stationary and the mobile phases. The true separation factor for two constituents A and B is equal to the ratio of their distribution ratios; in ion-exchange terminology this is usually called the selectivity coefficient $K_B{}^A$, so that we may write:

$$K_B{}^A = D_A/D_B \tag{2}$$

It may readily be shown by combining eqns. (1) and (2) that the practical separation factor is given by the following equation:

$$\frac{d_A}{d_B} = (R_F)_A/(R_F)_B = (R_F)_A\,(1 - K_A{}^B) + K_A{}^B \tag{3}$$

When the concentration and pH of the eluent changes the separate R_F values of the two constituents change, but it is possible for their ratio, *i.e.* $K_B{}^A$, to remain constant. This is the case for rare earth elutions where only one type of complex ion is formed; MAYER AND FREILING[4] have pointed out that in this case the true separation factor for two ions is equal to the ratio of the formation constants of the corresponding complex ions.

If this is the case here and $K_{Ce}{}^{Eu}$ is a constant, eqn. (3) predicts that a graph of practical separation factor against the R_F value for cerium should be a straight line of intercept $K_{Ce}{}^{Eu}$ and slope $(1 - K_{Ce}{}^{Eu})$. The results of Table I are plotted in this way in Fig. 3. It will be seen that there is no support for the hypothesis of a constant selectivity coefficient. At low R_F values a value of 3.2 for $K_{Ce}{}^{Eu}$ fits the

results best, but at higher R_F values $K_{Ce}^{Eu} = 2.1$ gives a better fit. Hence the selectivity is reduced by addition of HCl or by using high concentrations of citric acid.

Many studies have been made[11] of the separation of rare earths on cation-exchange columns by elution with citric acid solutions, mostly at pH values close to 3.

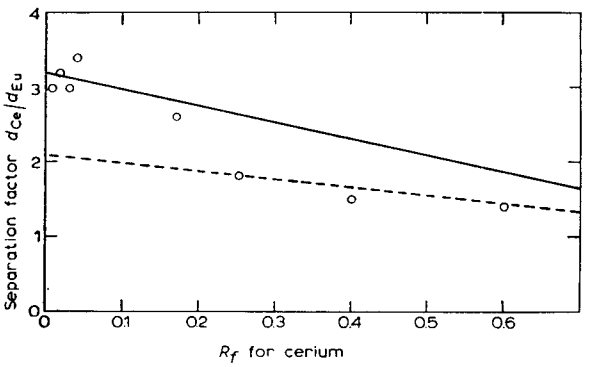

Fig. 3. Test of eqn. (3). Theoretical line for $K_{Ce}^{Eu} = 3.2$ drawn continuous. Theoretical line for $K_{Ce}^{Eu} = 2.1$ drawn dotted.

According to TOMPKINS AND MAYER[12] the complex ions produced are of the type: $La(H_2Cit)_3$, and only at pH values above 3.5 are appreciable amounts of anionic complexes formed. The work described here, and that of other investigators[7-9] shows, however, that on anion exchangers strong adsorption from citrate solutions occurs even at pH values around 2, so that some type of dissociation of the neutral complexes, *e.g.*:

$$Ln(H_2Cit)_3 \rightleftharpoons [Ln(H_2Cit)_2(HCit)]^-$$

must be strongly favoured by the anion-exchange resin.

The equilibrium studies of TOMPKINS AND MAYER predict a separation factor of 7.2 between terbium and cerium at pH 2.88 on a cation-exchange resin. This is far higher than the value of about 3 found in the present work on anion exchange. Consistent with this, TOMPKINS AND MAYER found lower separation factors at higher pH values and attributed this to the formation of the anionic complexes which are essential in the present work.

The practical separation factors obtained[11,13] in separations on cation-exchange columns using citric acid are generally somewhat higher than those found in the present work, but not as high as those predicted by the equilibrium studies of TOMPKINS AND MAYER. It is possible therefore that anion exchange using citrate complexes might prove practically useful, particularly in any case where it would be convenient to have the usual elution order for the lanthanides reversed.

ACKNOWLEDGEMENT

This work is supported by the South African Atomic Energy Board, to whom grateful acknowledgement is made.

SUMMARY

The application of cellulosic anion-exchange papers to the separation of tracer amounts of rare earths has been investigated, using citric acid solutions as eluents. It was found most satisfactory to use diethylaminoethyl-cellulose paper with 0.026 *M* citric acid (pH 2.4) as eluent. Under these conditions a practical separation factor of 2.6 between europium and cerium was obtained. Slightly higher separation factors could be obtained by adding ethanol to the citric acid solutions.

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SEPARATION OF SOME LOWER OXY-ACIDS OF PHOSPHORUS BY ANION-EXCHANGE CHROMATOGRAPHY

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In a previous communication[1], the authors have reported the separation of hypophosphite, phosphite and phosphate using gradient elution anion-exchange chromatography. This scheme has been extended to include the following lower oxy-anions of phosphorus, hypophosphate, diphosphite, pyrophosphite, isohypophosphate, an acid containing three phosphorus atoms and the condensed phosphate, pyrophosphate.

EXPERIMENTAL

Materials

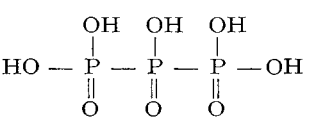
B.D.H. reagent grade sodium hypophosphite, $NaH_2PO_2 \cdot H_2O$ and disodium phosphite, $Na_2HPO_3 \cdot 5H_2O$, were twice recrystallised from aqueous solution. AnalaR grade disodium hydrogen orthophosphate, $Na_2HPO_4 \cdot 12H_2O$ was used without further purification. Disodium hypophosphate, $Na_2H_2P_2O_6$ was prepared by the method of LEININGER AND CHULSKI[2] and three times crystallised from hot water.

Trisodium diphosphite, $Na_3HP_2O_5 \cdot 12H_2O$ was prepared by the hydrolysis of phosphorus trichloride[3,4].

Disodium pyrophosphite $Na_2H_2P_2O_5$ was prepared by heating monosodium phosphite $NaH_2PO_3 \cdot 2.5H_2O$ at 120° under vacuum for 6 h[5]. Iodometric titration of the resultant product showed that a small quantity of phosphite remained[5]. Due to the ease of hydrolysis of pyrophosphite in aqueous solution[6], this solid was used without further purification.

Samples of trisodium isohypophosphate $Na_3HP_2O_6 \cdot 4H_2O$ and tetrasodium pyrophosphate $Na_4P_2O_7 \cdot 10H_2O$ were kindly donated by Albright and Wilson (Mfg.) Ltd. and were used without further purification. The isohypophosphate contained traces of orthophosphate and orthophosphite as impurities and this salt, like pyrophosphite, hydrolyses in aqueous solution[7]

The acid containing three atoms of phosphorus and having the structure[8]:



was isolated as $Na_5P_3O_8 \cdot 14H_2O$ from the oxidation products of red phosphorus by

sodium chlorite solution. This acid will be referred to as ($\overset{4}{P}$–$\overset{3}{P}$–$\overset{4}{P}$) following the nomenclature proposed by BLASER[9].

B.D.H. reagent grade potassium chloride.

Preparation and use of the chromatographic columns

The anion-exchange columns were prepared and used in an identical manner to that described in the previous communication[1]. The conditions are given in Table I. 10 ml fractions of the column effluent were collected, transferred to conical flasks and boiled with 5 ml of bromine water for 30 min and then for a further 30 min after adding 10 ml of concentrated nitric acid. The phosphorus content of each fraction was then determined spectrophotometrically by the phosphovanadomolybdate method[10].

TABLE I

CONDITIONS FOR THE CHROMATOGRAPHIC SEPARATION

Column dimensions (cm × cm)	*KCl concentration in mixing vessel (ml/M)*	*KCl concentration in reservoir (M)*	*Temperature (°C)*	*pH*	*Flow rate (ml/h)*
50 × 1.5	750/0.05	0.20	18	6.8	60

Buffer solution: pH 6.8; 25 ml of 2 *M* ammonium acetate solution per litre of potassium chloride. This pH was selected in order to keep the hydrolysis of pyrophosphite and isohypophosphate at a minimum during the chromatographic separation.

RESULTS AND DISCUSSION

An examination of Fig. 1 and Table II shows that good separations were obtained with the exception of that between diphosphite and pyrophosphite. Elution schemes using more dilute potassium chloride solutions were investigated to try and improve this separation, but without success. Neither could the separation be improved by using eluant solutions buffered at a pH of 9, nor by reducing the column temperature to 2°.

TABLE II

RETENTION VOLUMES OF PHOSPHORUS OXY-ANIONS*

Species	*Retention volume (ml)*
Hypophosphite	150
Phosphate	260
Phosphite	330
Hypophosphate	590
Diphosphite	680
Pyrophosphite	720
Isohypophosphate	890
Pyrophosphate	990
($\overset{4}{P}$–$\overset{3}{P}$–$\overset{4}{P}$)	1180

* Total percentage of phosphorus recovered = 97%.

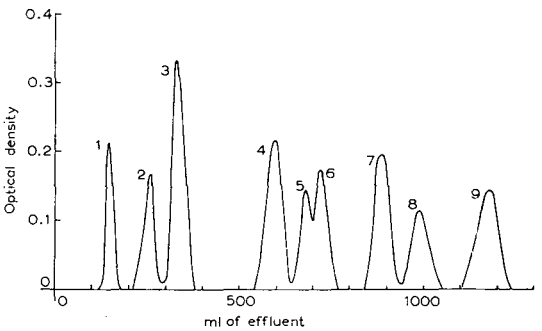


Fig. 1. Elution pattern for the separation of some phosphorus oxy-anions at pH 6.8. (1) Hypophosphite; (2) phosphate; (3) phosphite; (4) hypophosphate; (5) diphosphite; (6) pyrophosphite; (7) isohypophosphate; (8) pyrophosphate; (9) ($\overset{4}{\text{P}}$–$\overset{3}{\text{P}}$–$\overset{4}{\text{P}}$).

No appreciable hydrolysis of pyrophosphite or isohypophosphate appeared to occur during the separation. Maximum separations were achieved when the amount of phosphorus per species was less than 2000 μg.

ACKNOWLEDGEMENTS

We wish to thank Mr. D. L. CRONE of these laboratories for the sample of the acid ($\overset{4}{\text{P}}$–$\overset{3}{\text{P}}$–$\overset{4}{\text{P}}$), and the Department of Scientific and Industrial Research for a Research Studentship to M. T. ROTHWELL.

SUMMARY

Anion-exchange chromatography employing a gradient elution technique has been used to obtain separations of some lower oxy-anions of phosphorus.

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QUANTITATIVE INORGANIC CHROMATOGRAPHY

PART IX. THE ANALYSIS OF ALKALINE-EARTH METALS BY ION-EXCHANGE CHROMATOGRAPHY AND FLAME SPECTROPHOTOMETRY

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In a previous communication[1] a quantitative analysis of the alkaline-earth metals was described using paper chromatography and flame spectrophotometry. After the separation stage, the metals were extracted from the chromatogram and estimated by flame spectrophotometry. The use of ion-exchange chromatography avoids the extraction step necessary in the previous method.

Several workers[2,3] have separated the alkaline-earth metals by means of cation-exchange chromatography using ammonium lactate as an eluting agent at various temperatures. The necessity for the separation of the metals when present together in a sample, has clearly been demonstrated[1] and this method allows samples containing the various alkaline-earth metals, in various concentrations, to be estimated. By use of flame spectrophotometry, a high accuracy of estimation was possible with even low concentrations.

EXPERIMENTAL

This paper describes the procedure recommended for the quantitative separation and estimation of all four alkaline-earth metals. The flame spectrophotometer used was the Unicam SP. 900.

Apparatus and general technique

The cation-exchange material used for the separation was Dowex-50 W, 8 %, D.V.B. cross-linked, 200–400 mesh, and the column was 66 cm long and 0.75 cm in diameter. A glass wool plug was placed at the top of the column such that when loading samples into the column, it remained undisturbed especially in its upper layers. Above the column was the usual gradient-elution system which gave the system a pressure head of about 75 cm from the top of the packed column. A Shandon automatic fraction collector (balance type) was used to collect 5.0 g samples of the eluant. Dropping rate of the column was 0.2 ml/min.

Eluting agents

Magnesium, calcium and strontium were eluted with ammonium lactate solution at pH 7.0. The first two metals were eluted using 1 *M* ammonium lactate, whilst the strontium was removed by gradient elution caused by dropping 3 *M* ammonium

lactate into 500 ml of 1 *M* ammonium lactate in the lower reservoir (in some later samples the 3 *M* ammonium lactate was increased to 5 *M* lactate to decrease the elution time). Barium was removed too slowly from the column using ammonium lactate for the elution, and was eluted using 0.1 *M* disodium ethylenediaminetetracetic acid (ENTA) buffered to pH 10.5.

The lactate solutions were prepared from the calculated quantity of AnalaR lactic acid (103 g/l for 1 *M* solution), neutralising with 0.880 AnalaR ammonia solution to pH 7 and diluting the solution to 1 l.

The 0.1 *M* ENTA was prepared by dissolving 37.23 g of AnalaR disodium ethylenediaminetetracetic acid in water, and adding 0.24% w/v ammonium chloride in 5 *N* ammonium hydroxide solution until the pH 10.5 was reached. The solution was then diluted to 1 l.

Purity of water and components of eluting agents

The water used throughout this work was distilled in an all-glass apparatus having ground-glass joints. The distilled water was then passed through a mixed Zeocarb resin column about 100 cm long and 5 cm diameter contained in a hard-glass apparatus. The demineralised water was stored in hard-glass or plastic aspirators, the alkaline-earth content was below the level of detection of the flame spectrophotometer.

The alkaline-earth content of the eluting agents was found to be negligible as the eluant was diluted at least twenty-five times before determination, and the alkaline-earth metal content was below the detection limits of the spectrophotometer. However, all standard solutions of barium used in the estimations were prepared containing the appropriate concentration of ENTA because this organic component considerably enhanced the barium emission.

Solutions

(*a*) *Reference solutions for chromatography and flame spectrophotometry.* Standard barium solution: AnalaR barium chloride $BaCl_2 \cdot 2H_2O$ in water containing 1 mg of barium/ml.

Standard calcium solution: AnalaR calcium carbonate, $CaCO_3$ in 0.01 *N* hydrochloric acid solution containing 1 mg of calcium/ml.

Standard magnesium solution: AnalaR magnesium carbonate $MgCO_3$ in 0.01 *N* hydrochloric acid solution containing 1 mg of magnesium/ml.

Standard strontium solution: Strontium carbonate $SrCO_3$ in 0.01 *N* hydrochloric acid solution containing 1 mg of strontium/ml.

A solution containing a mixture of 1 mg/ml of individual alkaline-earth metals was prepared from the same salts as used for the individual solutions above. These solutions were used for loading the ion-exchange column; 0.50–2.0 ml of the solution being transferred to the column.

(*b*) *Reference solutions for flame spectrophotometry.* The individual solutions prepared in (a) above, were then diluted to give the "working" flame spectrophotometric solutions fresh daily, the ranges were:

Calcium and strontium 0–2 μg/ml of alkaline-earth metal.
Barium and magnesium 0–5 μg/ml of alkaline-earth metal.

All solutions were stored in polythene screw-cap bottles.

Procedure

The resin was converted into the ammonium form by passage of approx. 3 N ammonium chloride solution through the resin and finally washed free of chloride ions by distilled water. The column was then "loaded" with 0.50, 1.0, or 2.0 ml of the 1 mg/ml alkaline-earth solution on to the column. The elution procedure was as follows:

(1) The lower reservoir of the gradient-elution apparatus was filled to contain 500 ml of 1 M ammonium lactate at pH 7.

(2) 100 ml of this solution (20 fractions) was allowed to pass through the column during which time magnesium was eluted between 65–85 ml (fractions 14–17).

(3) After step 2 was completed, the 3 M ammonium lactate (pH 7) was connected to the 1 M ammonium lactate reservoir, and gradient elution allowed to take place using the conditions of the standard practice. Calcium was eluted between a further 25–60 ml (fractions 22–29).

(4) When a further 200 ml of eluant had been collected, gradient elution was stopped. Strontium was eluted between 220–290 ml (fractions 54–60). When 5 M ammonium lactate was used, the strontium appeared about 60 ml (12 fractions) earlier.

(5) The column was washed with 30 ml of distilled water.

(6) The barium was eluted by passage of 50 ml of 0.1 M ENTA solution through the column.

Finally, 1 ml aliquots were taken from each fraction collected, diluted to a suitable concentration and estimated by flame photometry. The type of separation obtained is shown in Fig. 1. A constant eluting concentration is necessary for the sepa-

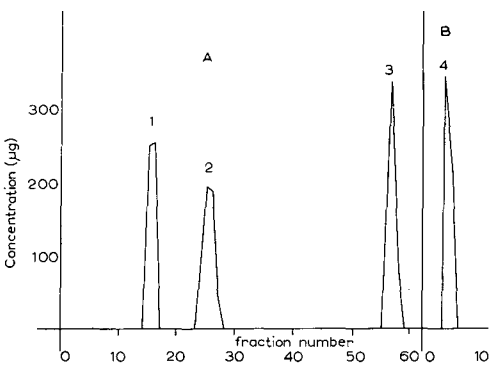

Fig. 1. Separation of alkaline-earth metals. Eluate A: ammonium lactate (pH 7). Eluate B: 0.1 M ENTA (pH 10.5). (1) Magnesium; (2) calcium; (3) strontium; (4) barium. 5 ml fractions.

ration of magnesium and calcium, whilst gradient elution must be employed, otherwise the strontium remains on the column too long and appears as a diffuse band. The resin is regenerated by washing with water, followed by 3 N ammonium chloride solution, and finally with water, after which the column is again ready for use.

Flame spectrophotometry

The instrument used was a Unicam SP. 900 flame spectrophotometer operated under the following conditions:

(1) The fuel gas was B.O.G. commercial acetylene in cylinders. The cylinder

pressure must not be allowed to fall below 50 p.s.i., since there is danger of acetone (solvent for acetylene) being swept into the flame. The presence of acetone in the flame gives enhanced values for the emission of the alkaline-earth metals[4].

(2) Atomising and oxidising gas was normal British Oxygen Company, compressed in cylinders. A cylinder was used until the pressure falls below 200 p.s.i., after which instability of the flame was noticed.

(3) The burner unit was fitted with a jet having 0.028 in. internal bore.

The operating conditions were those suggested by the manufacturers, 30 p.s.i. compressed air pressure, while the acetylene pressure varies with the need for a steady flame, but usually of the order of 3 in. measured on a dibutyl phthalate manometer. After ignition of the flame, deionized water was aspirated through the instrument which was allowed to reach thermal and electronic equilibrium. All results were recorded using the scanning motor and a Sunvic RSP2 high speed potentiometric recorder.

The conditions used for the elements were those given in Table I.

TABLE I

SPECIAL CONDITIONS USED TO RECORD THE FLAME SPECTRA

(Electrical bandwidth: 4; photomultiplier: Mazda 27M3)

	Magnesium	*Calcium*	*Strontium*	*Barium*
Slit (mm)	0.08	0.04	0.04	0.04
Wavelength scan (mμ)	284.5–286.0	420–427	457–467	554–561
Amplifier gain	4.2	4.2	4.2	3.4
Recorder F.S.D. (mV)	0–1	0–10	0–10	0–1

The amplifier gain is given only as a guide, as it will vary from day to day, and instrument to instrument. The shape of the emission peaks for calcium and strontium are symmetrical on a relatively flat baseline, whilst magnesium and barium have the shape discussed in ref.[1]. It was found that by using a 0–1 mV potentiometric recorder, scanning between the wavelengths given above, and allowing the recorder to "settle" it is possible to obtain a linear calibration curve for magnesium and barium which passes through zero. This procedure eliminates with the use of "pseudo peak heights", and allows lower concentrations of magnesium and barium to be determined. Using this method it was noticed that the wavelength of maximum magnesium emission varies slightly with magnesium concentration as reported by DEAN[5].

By reference to standard calibration graphs (prepared for each determination), the concentrations of the alkaline-earth metals in the unknowns were calculated.

Analysis of synthetic mixtures

For this work, a quaternary alkaline-earth mixture was prepared as described containing 1 mg/ml of each of the alkaline-earth metals. Separations and determinations were carried out using 0.5, 1 and 2 ml of this solution. The sensitivity and applicability of this method using small quantities of one alkaline-earth metal in the presence of a large quantity of another, was tested in the analysis of mineral samples. Results for

the analysis of synthetic alkaline-earth mixtures are given in Table II. This shows that results by this method are within 3% accuracy.

TABLE II

ANALYSIS OF SYNTHETIC ALKALINE-EARTH MIXTURES

Present (μg)				*Found (μg)*			
Mg	*Ca*	*Sr*	*Ba*	*Mg*	*Ca*	*Sr*	*Ba*
500	500	500	500	511	516	495	511
1000	1000	1000	1000	1028	1003	981	1034
1000	1000	1000	1000	973	991	1000	1018
—	1000	1000	1000	—	1023	976	1007
2000	2000	—	2000	1985	2074	—	1945
2000	2000	2000	2000	2018	2050	2057	1961

Analysis of mineral samples

Limestone. A mixture of magnesium and calcium carbonates containing 38.79% calcium and 0.421% magnesium by classical chemical methods of analysis.

About 2.5 g of limestone was dissolved in the least possible quantity of dilute hydrochloric acid and made up to 250 ml with de-ionised water. 1 ml of this solution was used for analysis.

Dolomite. A mixture of magnesium and calcium carbonates containing 31.17% calcium and 5.79% magnesium. About 5 g of dolomite was dissolved in dilute hydrochloric acid and made up to 250 ml with water and 1 ml used for analysis.

Barytes. A mixture of magnesium and calcium carbonates plus barium sulphate, containing 16.83% calcium, 0.084% magnesium and 35.39% barium. About 7.5 g were fused with 6 g anhydrous sodium carbonate for 1.5 h, leached with water,

TABLE III

ANALYSIS OF MINERALS

		Chemical analysis				*Found by ion exchange and flame spectrophotometry*			
		Mg	*Ca*	*Sr*	*Ba*	*Mg*	*Ca*	*Sr*	*Ba*
Limestone	(μg)	208	19,198	—	—	212.5	19,359	—	—
	(%)	0.421	38.79	—	—	0.429	39.11	—	—
Dolomite	(μg)	1582	8515	—	—	1612.5	8624.7	—	—
	(%)	5.79	31.17	—	—	5.90	31.55	—	—
Barytes	(μg)	63	1266	—	2663	60	1233	—	2487*
		—	1264	—	2658	—	1256	—	2421*
	(%)	0.084	16.83	—	35.39	0.08	16.38	—	33.04
						—	16.58	—	32.23
Celestine	(μg)	—	50	18,324	386		64	19,676	397.5
	(%)	—	0.124	45.6	0.961		0.159	47.47	0.989

* The low result for barium in barytes has previously been found by paper chromatography by others workers[6].

centrifuged and the residue dissolved in a little dilute hydrochloric acid and made up to 100 ml with water. 1 ml of solution used for analysis.

Celestine. A mixture of the sulphates of calcium, strontium and barium plus calcium carbonate containing 0.124 % calcium, 45.6 % strontium and 0.961 % barium. About 1 g fused as before and made up to 25 ml. 1 ml solution used.

The results given in Table III show the versatility of the method to alkaline-earth mixtures of varying concentrations.

In progress, at present, is work to automate the flame spectrophotometric estimations, and to present the elution patterns directly upon the potentiometric recorder[7].

ACKNOWLEDGEMENT

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SUMMARY

An ion-exchange chromatographic procedure is described for quantitative analysis of μg/mg amounts of barium, calcium, magnesium and strontium. After elution, the metals are determined by flame spectrophotometry.

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HIGH-VOLTAGE PAPER ELECTROPHORESIS OF INORGANIC CATIONS

CONDITIONS FOR CATHODIC MIGRATION AND MEASUREMENT OF MIGRATION RATES*

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INTRODUCTION

It is well known that inorganic cations can be made to migrate to the anode by means of various complexing agents such as halogen acids, organic hydroxy-acids and poly-anions. The result is often a better separation or differentiation where a separation would be difficult or not at all possible in the cationic state. This subject was treated exhaustively in a recent up-to-date publication[1].

In a previous article[2] some examples of separations of anodically migrating cations were given. In fact, almost only the alkali metals in a cationic state could be demonstrated to give such differential migration rates as to allow satisfactory resolution of their mixtures. A comprehensive bibliography of separations by paper electrophoresis was recently published[3]. The use of a complexing agent such as ethylenediaminetetraacetic acid (EDTA) in solutions of varying pH, the dependence of the movement of metallic ions on the degree of dissociation of the anionic complexes formed, on the concentration of ligand and pH conditions, were recently investigated and theoretically discussed[4]. The variable effect on the sign of migration of the concentration of the background electrolyte was also reported briefly but convincingly[5]. Other authors described the strong effect of pH on the migration behaviour of inorganic cations in the presence of organic complexing agents[6].

It was for these reasons that it was decided to search for non-complexing electrolyte solutions less liable to be affected by slight variations in conditions and thus more suitable for routine experiments without the need for frequent rigorous standardization and checking.

EXPERIMENTAL

Choice of background electrolyte

Several guiding principles in the choice of electrolytes or buffer solutions have to be observed. Convenience of preparation, assurance of reproducible pH, steadiness of pH during the experiments, sufficient buffering power, low to medium degree of

* The results of this paper were presented at Corso Estivo di Chimica, Rome, on September 21st, 1962.

dissociation in aqueous solutions, non-toxicity, non-inflammability, economic price of chemicals and low degree of corrosiveness. Several of the points have an enhanced importance in the case of high-voltage electrophoresis requiring large volumes of solution. A strong electrolyte such as HCl or HBr leads to high current density and correspondingly increased heat generation, making satisfactory control of experimental conditions at high voltages difficult.

It has always been the author's policy to find an electrolyte solution that could be made up conveniently and used for more than one group of compounds, irrespective of their organic or inorganic nature. For instance, a 0.05 *M* sodium tetraborate solution giving a pH of 9.2 can be used for the electrophoresis of sugars as well as of amino acids, even two-dimensionally[7]. Another electrolyte found very useful is an approx. 0.1 *M* ammonium carbonate solution of pH 8.9, suitable for alkali metals[2] and inorganic[8] as well as organic acids[9]. A 0.75 *M* solution of formic acid was previously found to be suitable for amino acids[7] and other organic acids. A particularly great advantage of the latter two solutions is that they are capable of being removed from the dried paper by moderate heating.

Some time ago an investigation was initiated to find possible applications in the inorganic field for the formic acid solution, with particular reference to the separation of Ca^{2+} from Mg^{2+}. It was soon discovered that both cations migrated towards the cathode at conveniently high and sufficiently different rates of migration to afford a clean separation. A systematic investigation of a great number of cations showed that nearly all behaved as cations under these conditions, and that they had, with few exceptions, quite interesting mobilities. Those that did not move were usually adsorbed or rather precipitated at the base line.

Measurement of migration rates at pH 2.0

The measurements were carried out using the previously described high-voltage apparatus[10] providing speed, precision and good reproducibility. Several K^+ and Zn^{2+} spots distributed evenly on each sheet were used as reference markers and several sucrose spots as markers for electro-osmotic and hydrodynamic flow effects. Cellophane sleeves interposed between the sheet and the connecting paper wads reduced the electrolyte flow to a minimum, ensuring thus sufficiently steady hydrodynamic conditions in the sheet. All distances were measured from the front of the sucrose spot to the front of the cation spot. In view of the tendency of some of the cations to form irregular or unduly elongated spots, this was considered to be the most reasonable and dependable way of measurement. The deduction due to electro-osmosis was generally small and not more than 1.5 cm. The duration of the experiment at 100 V/cm was 20 min for most cations. The alkali metals were also run for 15 min. The correlation between the results at 15 and 20 min followed a linear relationship. The same applied to the potential–migration rate and the temperature–migration rate relationships, corresponding in the latter case to about +3 % per 1° over the measured range of 6–15°. The mean temperature of the cooling water was taken as the temperature of the paper sheet, a fair assumption with the constancy of current during the experiment as an indication of efficient heat dissipation and steady conditions. The cooling system and electrical energy throughput were so adjusted that the system was underrun rather than taxed to the limit. Paper sheets of 12 in. × 22 1/2 in. (Whatman No. 3 MM), allowing the application without mutual interference of 8–10 streaks or

spots plus several marker spots were used throughout. Most applications were in the form of streaks, 2 cm wide. The reproducibility of results was generally good and amounted to well within 4 % between individual sheets and better than that for replicates on the same sheet. The moisture uptake of the paper sheet at 1.5 lb./sq. in. was 140 % (±3 %), compared with the weight of the dry paper.

The experimental conditions were consequently as follows: pH 2.0 (0.75 *M* formic acid solution), 100 V/cm, 6 mA/cm, 1.5 lb./sq. in., 20 min, 11° (mean temperature of cooling water), 140 % moisture content.

Reagents for detecting heavy and alkaline earth metals were $(NH_4)_2S$ or 8-hydroxyquinoline and NH_3-vapour, or better because of greater sensitivity 8-hydroxyquinoline dissolved in conc. ammonia solution[11], for alkali metals bromophenol blue in ethanol, and for $(NH_4)^+$ ninhydrin, which surprisingly was found to give a strong reaction with K^+ and Na^+ and a weaker one with Li^+ and some of the alkaline earth metals. Tests indicated that this hitherto unreported reaction appeared to be based on some nitrogenous impurities in the paper, as test tube experiments failed to give this otherwise highly reproducible reaction.

The results at pH 2.0 are summarised in order of valency in Table I. All figures are corrected for electro-osmotic and hydrodynamic flow effects.

Although the migration rates of certain alkali metals at pH 8.9 had already been reported[2], it was considered desirable to re-measure more accurately these and the rest of the cations as shown in Table I. The results confirmed more quantitatively the extremely small but real differences between Rb^+, Cs^+ and K^+ and elucidated the fate of the alkaline earth metals in the system of ammonium carbonate solution. The latter was made possible by using several detecting reagents of which ninhydrin (see above) proved very useful. The experiments were run at 80 V/cm (4 kV), *i.e.* below the available capacity of 5 kV, in order to avoid any possible over-heating or temperature rise during the experiment. The results are summarised in Table II.

It is interesting to note that only the mono- and divalent cations possess appreciable mobilities under these conditions.

Relative migration rates of some alkali metals

It is useful to compare the results obtained for Rb^+, Cs^+ and K^+ with those published earlier[2]. Migration rates, relative to $Rb^+ = 1.000$, are shown in Table III.

Considering that different equipment was used and the time interval between the two sets of measurements was about five years, the agreement appears to be quite satisfactory. The present apparatus affords better control of conditions and thus greater precision of measurements.

Mobilities

Since it was established that under the given experimental conditions electrophoretic migration was a linear function of potential and time and independent of the size or design of the apparatus, and that all the significant physico-chemical factors such as temperature, moisture content of paper, conductance, and pH could be maintained constant, it seemed appropriate to calculate absolute mobilities from the migration rates for comparison with corresponding figures quoted in the literature. Table IV illustrates the agreement between mobilities derived from measurements in paper-stabilized

TABLE I

MIGRATION RATES OF INORGANIC CATIONS AT pH 2.0

(Conditions: 0.75 *M* formic acid soln.; 100 V/cm; 1.5 lb./sq.in.; 11°; 20 min)

Cation	*Distance travelled towards cathode (cm)*	M_K* *(relative migration rate)*	*Reagent*	*Colour in visible light*	*Colour in U.V. light*	*Remarks*
Ag^+	6.0	0.157	H.qu**	Brown	Dark (quenching)	
Hg^+	0.0	0.0	H.qu.	Yellow	Dark	Precipitate
Cu^+	17.6	0.461	H.qu.	Yellow	Dark	
Tl^+	35.8	0.937	H.qu.	Brown	Dark	
Cs^+	39.65	1.038	Bromophenol blue	Blue	—	Also ninhydrin blue colour
Li^+	20.2	0.529	Bromophenol blue	Blue	—	Also ninhydrin blue colour
Na^+	27.2	0.712	Bromophenol blue	Blue	—	Also ninhydrin blue colour
$(NH_4)^+$	39.1	1.024	Bromophenol blue	Blue	—	Also ninhydrin blue colour
K^+	38.2	1.000	Bromophenol blue	Blue	—	Also ninhydrin blue colour
Rb^+	39.7	1.039	Bromophenol blue	Blue	—	Also ninhydrin blue colour
Ba^{2+}	26.5	0.694	H.qu.	—	Greenish yellow	
Be^{2+}	16.5	0.432	H.qu.	Pale yellow	Yellow	
Ca^{2+}	27.9	0.730	H.qu.	—	Greenish yellow	
Cd^{2+}	19.9	0.521	H.qu.	Yellow	Yellow	
Co^{2+}	21.9	0.573	H.qu.	Orange	Dark	
Cu^{2+}	16.9	0.442	H.qu.	Bright yellow	Dark	
Fe^{2+}	21.2	0.555	H.qu.	Black	Dark	
Mg^{2+}	23.6	0.618	H.qu.	Pale yellow	Whitish yellow	

Mn^{2+}	21.9	0.573	H.qu.	Orange	Dark	
Ni^{2+}	21.3	0.558	H.qu.	Yellow	Dark	
Pb^{2+}	21.75	0.569	H.qu.	Yellow	Dark	
Sn^{2+}	0.0	0.0	H.qu.	Yellow	Bright yellow	Precipitate
Sr^{2+}	24.9	0.652	H.qu.	—	Greenish yellow	
$(UO_2)^{2+}$	8.95	0.234	H.qu.	Orange-brown	Dark	
Zn^{2+}	21.8	0.571	H.qu.	Yellow	Bright yellow	
Al^{3+}	17.9	0.468	H.qu.	Brownish yellow	Bright yellow	
Bi^{3+}	3.9	0.102	H.qu.	Yellow	Dark	
Ce^{3+}	21.02	0.550	H.qu.	Reddish brown	Dark	
Fe^{3+}	8.45	0.221	H.qu.	Black	Dark	
La^{3+}	22.5	0.589	H.qu.	Brownish yellow	Yellow	
Sb^{3+}	0.0	0.0	H.qu.	Brownish yellow	Dark	Precipitate
Ti^{3+}	10.4 (8.4)	0.272 (0.219)	H.qu.	Brownish yellow	Dark	Brown band develops only after several hours
Ce^{4+}	6.77	0.177	H.qu.	Grey	Dark	Contained also band of Ce^{3+}
Th^{4+}	10.2	0.267	H.qu.	Brownish yellow	Brown	
Ti^{4+}	8.4	0.219	H.qu.	Yellow	Dark	
Zr^{4+}	0.5	0.013	H.qu.	Yellow	Dark	Trailing

* $M_K = \frac{\text{Migration rate of cation}}{\text{Migration rate of potassium}}$

** H.qu. = 8-hydroxyquinoline.

TABLE II

MIGRATION RATES OF INORGANIC CATIONS AT pH 8.9.
(Conditions: approx. 0.1 *M* ammonium carbonate soln.; 80 V/cm; 1.5 lb./sq. in.; 11°; 20 min)

Cation	*Distance travelled towards cathode (cm)*	M_K* *(relative migration rate)*	*Reagent*	*Colour in visible light*	*Colour in U.V. light*	*Remarks*
Ag^+	4.7	0.148	H.qu.**	Brown	Dark (quenching)	
Hg^+	15.6	0.492	H.qu.	Yellow	Dark (quenching)	
Cu^+	1.6	0.050	H.qu.	Yellow	Dark (quenching)	
Tl^+	25.1	0.792	H.qu.	Brown	Dark (quenching)	
Cs^+	32.5	1.025	Bromothymol blue	Blue	—	
Li^+	16.8	0.529	Bromothymol blue	Blue	—	
Na^+	23.1	0.729	Bromothymol blue	Blue	—	
K^+	31.7	1.000	Bromothymol blue	Blue	—	
Rb^+	33.1	1.044	Bromothymol blue	Blue	—	
Ba^{2+}	15.8	0.498	H.qu.	—	Greenish yellow	Some precipitate and trailing to base line
Be^{2+}	0.0	0.0	H.qu.	Pale yellow	Yellow	Ppt. and trail. to anode
Ca^{2+}	13.1	0.413	H.qu.	—	Greenish yellow	Ppt. and trail. to base line
Cd^{2+}	0.0	0.0	H.qu.	Yellow	Yellow	Ppt.
Co^{2+}	4.3	0.130	H.qu.	Orange	Dark (quenching)	Ppt.
Cu^{2+}	0.0	0.0	H.qu.	Yellow	Dark (quenching)	Ppt.
Fe^{2+}	0.0	0.0	H.qu.	Black	Dark (quenching)	Ppt.
Mg^{2+}	15.0	0.473	H.qu.	Pale yellow	Whitish yellow	

Mn^{2+}	5.9	0.186	H.qu.	Orange	Dark	Some ppt. and trail.
Ni^{2+}	5.9	0.186	H.qu.	Yellow	Dark	
Pb^{2+}	0.0	0.0	H.qu.	Yellow	Dark	Ppt.
Sn^{2+}	0.0	0.0	H.qu.	Yellow	Bright yellow	Ppt.
Sr^{2+}	15.2	0.479	H.qu.	—	Greenish yellow	Trail. to base line
$(UO_2)^{2+}$	0.0	0.0	H.qu.	Brown	Dark	Anodic trail.
Zn^{2+}	0.0	0.0	H.qu.	Yellow	Bright yellow	Ppt.
Al^{3+}	0.0	0.0	H.qu.	Yellow	Bright yellow	Ppt. and anodic trail.
Bi^{3+}	0.0	0.0	H.qu.	Yellow	Dark	Ppt.
Ce^{3+}	0.0	0.0	H.qu.	Brown	Dark	Ppt.
Fe^{3+}	0.0	0.0	H.qu.	Black	Dark	Ppt.
La^{3+}	0.0	0.0	H.qu.	Yellow	Dark	Ppt.
Sb^{3+}	0.0	0.0	H.qu.	Yellow	Dark	Ppt.
Ti^{3+}	0.0	0.0	H.qu.	Yellow	Dark	Ppt.
Ce^{4+}	0.0	0.0	H.qu.	Grey	Dark	Ppt.
Th^{4+}	0.0	0.0	H.qu.	Brown	Brown	Ppt. and anodic trail.
Ti^{4+}	0.0	0.0	H.qu.	Yellow	Dark	Ppt.
Zr^{4+}	0.0	0.0	H.qu.	Yellow	Dark	Ppt.

* $M_K = \frac{\text{Migration rate of cation}}{\text{Migration rate of potassium}}$

** H.qu. = 8-hydroxyquinoline.

TABLE III

COMPARISON OF MEASUREMENTS OF SOME RELATIVE MIGRATION RATES

Cation	M_{Rb} *Previous results*	M_{Rb} *Present results*	*Remarks*
K^+	0.962	0.958	Apparatus of similar
Rb^+	1.000	1.000	design but different
Cs^+	0.988	0.982	dimensions and precision

systems under varying conditions and the discrepancies between these figures and those derived from conductivities at infinite dilution.

Although the agreement between previous and present results was fairly good and the discrepancy, due to the tortuosity factor, between mobilities as measured in paper-stabilized systems and in free electrophoresis was of the expected order of magnitude, some figures seemed to be outside the limit of error and consistently higher. It was then discovered that there was a measurable effect due to the machine direction or arrangement of the cellulose fibres in the paper. The effect of this on the flow of solvent in paper chromatography is well known, but it was not suspected to affect the mechanism of paper electrophoresis.

An additional tortuosity effect

It was found that cutting the paper sheet in the machine direction produced under the given conditions consistently higher results when compared with those obtained by cutting the paper across the machine direction. The differences were always significant though varying with the batch of paper, between 4 and 10%. There was also a noticeable difference in the current flowing through the paper, the sheet cut along the machine direction requiring more current. The results in Table V demonstrate the magnitude of the effect found when measuring some inorganic and organic ions of varying mobilities in certain batches of paper after having established the machine direction.

The existence of this migration-retarding effect was only discovered because of the high reproducibility of the mobility measurements. Any discrepancy greater than 3% was suspect and electrical, timing and cooling conditions were checked

TABLE IV

COMPARISON OF IONIC MOBILITIES
(in $cm^2\ V^{-1}\ sec^{-1} \cdot 10^5$)

Ion	*Mobilities (in paper) at 11°*				*Mobilities*[12] *(free electrophoresis) at 18° (from conductivities)*
	Previous results[2,9]		*Present results*		
	pH 2.0	*pH 8.9*	*pH 2.0*	*pH 8.9*	
K^+	—	32.32	31.83	33.02	64.6
Rb^+	—	33.56	33.08	34.48	67.5
Cs^+	—	33.16	33.04	33.84	68.0
Na^+	—	—	22.66	24.06	43.4
Li^+	—	—	16.81	17.5	33.4
Cl^-	32.65	31.6	—	—	65.5

TABLE V

TORTUOSITY EFFECT
(Due to machine direction of paper)

Ion	*pH 2.0* *Conditions: 100 V/cm, 13°, 15 min*						*pH 8.9* *Conditions: 80 V/cm, 13°, 20 min*			
	Paper batch A		*Paper batch B*		*Current mA/cm*		*Paper batch C*		*Current mA/cm*	
	across M.D. (cm)	*along M.D. (cm)*	*across M.D. (cm)*	*along M.D. (cm)*	*across M.D.*	*along M.D.*	*across M.D. (cm)*	*along M.D. (cm)*	*across M.D.*	*along M.D.*
$(NH_4)^+$	31.2	35.5	31.8	33.8	6.0	6.7			8	10
K^+	30.6	34.7	31.3	33.3			31.25	32.0		
Na^+	21.7	24.55	22.4	24.0			22.4	23.1		
Li^+	16.4	18.4	16.7	18.3			16.7	17.3		
Lysine	13.4	15.2	14.0	15.2						
Alanine	8.75	9.75	9.0	9.75						
Glutamic acid	5.95	6.65	6.1	6.65						

immediately. As long as batches of paper were cut by sheer routine or chance in the same way, no discrepancies were bound to appear. But once the routine was reversed or a batch was particularly bad in this respect, the differences called for an investigation.

Whether this effect is simply due to an extended tortuous path caused by a different arrangement of the cellulose fibres with respect to the direction of migration, is at present difficult to decide. Although the trend of the current consumption would support the assumption that the electrical resistance is correspondingly affected, the actual differences appear to be rather too high for a strict correlation.

DISCUSSION

High-voltage paper electrophoresis should be particularly useful for separation of inorganic ions because of their relatively high rates of migration and the avoidance of disturbing diffusion effects during the extremely short runs at high-potential gradients. It should be possible to make use of very small differences in mobilities, provided suitable background electrolytes can be found to give maximal differentiation and thus optimal resolution.

A systematic investigation of background electrolytes, involving the measurement of migration rates of a great number of inorganic cations under the rigorously controlled conditions of a suitable precision high-voltage apparatus[10], established the usefulness of an electrolyte solution consisting of a 0.75 *M* formic acid solution of pH 2.0. Under these conditions most cations migrate to the cathode at varying rates, offering many interesting separation possibilities. Migration rates in this electrolyte and in an approx. 0.1 *M* ammonium carbonate solution of pH 8.9 were measured. Mobilities in paper-stabilized systems for some ions were calculated and compared with theoretical mobilities. An unknown additional tortuosity effect was observed and measured.

It would appear that the potential applicability of the technique of paper electrophoresis to problems of inorganic chemistry is far from exhausted. Higher standards of precision of measurement and better control of experimental conditions

could lead to more examples of exploitation of even very small differences in mobilities. The advantage of the formic acid solution is that the time for some separations has been reduced to one-third, *i.e.* a few minutes.

ACKNOWLEDGEMENTS

Thanks are due to Mr. R. W. BUTTERS for his valuable technical assistance and to the directors of Tate & Lyle Refineries Ltd. for the permission to publish this communication.

SUMMARY

Conditions were investigated under which most inorganic cations are non-complexed and migrate at varying rates towards the cathode. A background electrolyte consisting of a 0.75 *M* formic acid solution of pH 2.0 was found particularly suitable for this purpose. Another electrolyte solution, *viz.* an approx. 0.1 *M* ammonium carbonate solution of pH 8.9 was again thoroughly investigated for the migration behaviour of many cations. The measured migration rates were tabulated and mobilities calculated and compared. A new tortuosity effect was observed. All measurements were made, using a previously described high-voltage apparatus[10] of ample precision ensuring good reproducibility of results.

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Short Communications

The effect of the column tubing composition on the recovery of chlorinated hydrocarbons by gas chromatography

Recent discussions[1,2] on the effect of the type of metal used in gas chromatographic columns for the quantitative analysis of some halogenated compounds prompts us to submit data obtained with columns of different tubing composition including one designed from quartz. Little data was presented to accurately show these effects. The following study was designed to present carefully measured recovery values of microgram quantities of several chlorinated hydrocarbons. The compounds selected were considered relatively difficult to chromatograph and were also considered to be important in certain commercial fields.

All of the metal columns were 6 ft. long and 1/4-in. O.D. The quartz column was 6 ft. long, 6 mm O.D. and 4 mm I.D. Although all of the metal tubings were purchased as 1/4-in. O.D. tubing, it will be noted in Table I that the variation of the I.D. affected

TABLE I

COLUMN PACKING DATA

Tubing	*Copper*	*Stainless steel*	*Aluminum*	*Quartz*
Column length	6 ft.	6 ft.	6 ft.	6 ft.
Column packing	15 g	17 g	19 g	10 g
Packing per column-foot	2.50 g	2.83 g	3.16 g	1.66 g

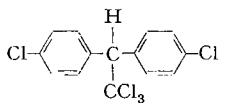
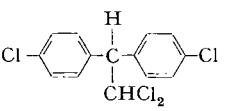
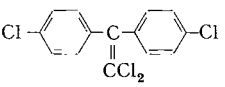
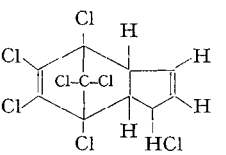
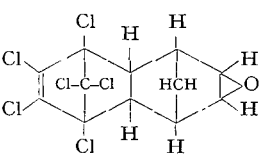
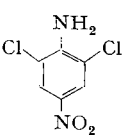
the amount of column packing per foot of column, but had no effect on the amount of recovery of the compound (Table II).

The liquid phase for the chromatographic column was prepared from Dow-Corning No. 11 silicone compound. The silicone was dispersed in ethyl acetate and filtered on a Whatman No. 1 filter paper. The clear filtrate was concentrated in a rotary evaporator, the concentrate was added to absolute ethyl alcohol, and the mixture was refrigerated 16 h. The resultant precipitate, which was water-clear and fluid, was separated from the supernatant layer and washed several times with absolute ethanol, air-dried, and used as the stationary phase for the chromatographic column. Twenty grams of the silicone was dispersed in chloroform and added to 100 g of acid-washed, 40–50 mesh, Chromosorb P. The chloroform was removed from the slurry by a rotary vacuum evaporator, followed by drying the packing with an infrared lamp.

The gas chromatograph was a Dohrmann Model 100 equipped with a pyrolysis train (825°) and a microcoulometric detector. The injection block and column temperatures were each 250°. The nitrogen and oxygen flow rates were 100 ml/min.

TABLE II

GAS CHROMATOGRAPHY RECOVERY DATA

Compound	Common name	Percent recovery			
		Column tubing			
		Copper	Stainless steel	Aluminum	Quartz
	*p-p'*-DDT	53	56	65	77
	Technical DDT*	50	56	66	77
	DDD	78	77	77	85
	DDE	83	87	86	86
	Heptachlor	24	36	68	79
	Endrin	72	77	87	88
	Dicloran	—	—	84	97

* An isomeric mixture, plus related halogenated compounds.

Results and discussion

The data in Table II are characteristic of a number of determinations. With the compounds studied, copper tubing was the least desirable and quartz tubing the most efficient. Exercising extreme care in the sampling and the control of temperature conditions, reproducibility of results on a given day were within a variation range of $\pm 3\,\%$, with the exception of the compound, heptachlor. It should be noted that the effect of the metal on the compound is not the only factor to be considered in the analysis of these compounds. For example, the degree of purity of the compound may not be accurately known. Endrin[3] and DDT[2] are subject to degradation at high temperatures. Another type of stationary phase on the column might possibly be more applicable for a given compound. For example, heptachlor, which is considered to be

relatively stable, gave very erratic results with the various columns (see Table II), suggesting that it could not be efficiently resolved with the column packing that was used.

The aluminum block of the gas chromatograph was "preconditioned" with 100 μg quantities of the halogen compound to be tested, as suggested by the manufacturer of the instrument. The results of such treatment were not conclusive. Subsequently, the block was modified to include a quartz tube insert in the injection port area. However, since the vaporized sample component could still come in contact with sections of the metal block, this modification did not materially improve the degree of recovery of the compound.

Finally, the block and the columns were pretreated with tris-(2-biphenyl) phosphate[2] which has been suggested as an inhibitor of the Friedel-Crafts type of catalysis. Indiscriminate application of this reagent to the chromatographic instrument can produce adverse effects because of its low vapor pressure at 250°. Excess amounts may accumulate in the block and tangibly interfere with the analytical results. None of the above treatments materially improved the analytical results.

In conclusion, our experiences indicate careful sampling is necessary (accuracy is limited by the present type of syringes available), strict temperature control is essential, pin-hole leaks in the chromatographic systems must be avoided, and an all-glass or all-quartz system—including the sample injector area—seem to be preferable.

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An unusual ninhydrin reaction with inorganic cations

Ninhydrin, triketo-hydrindene hydrate, is universally used as a very sensitive reagent for amino-nitrogen in amines, amino acids and peptides.

ZACHARIUS AND TALLEY[1] reported recently that certain non-nitrogenous keto acids in eluates from ion exchange columns were found to react with ninhydrin to give coloured compounds. Although the colour thus obtained was mostly red, the danger of ascribing a colour reaction to the presence of certain nitrogenous compounds was pointed out. Nothing, however, is known about a reaction of ninhydrin with inorganic cations, except $(NH_4)^+$.

It was therefore greatly surprising to discover in a recent investigation of amino acids by high-voltage paper electrophoresis that some inorganic cations present in the sample produced a colour reaction similar to that of amino acids. Since under the chosen experimental conditions[2] the positions of K^+ and Na^+ almost coincided with

those of $(NH_4)^+$ and dimethylamine, the identification of the latter compounds was thereby seriously complicated, if not entirely frustrated.

Fig. 1 illustrates the positions of several alkali and alkaline-earth metals with reference to $(NH_4)^+$ and those of some of the common amino acids, all revealed by application of a ninhydrin spray and heating for 10 min at 80°.

The reaction could only be produced on the paper, and not in solution. This might indicate a mechanism depending on certain impurities in the paper. Extracts from the

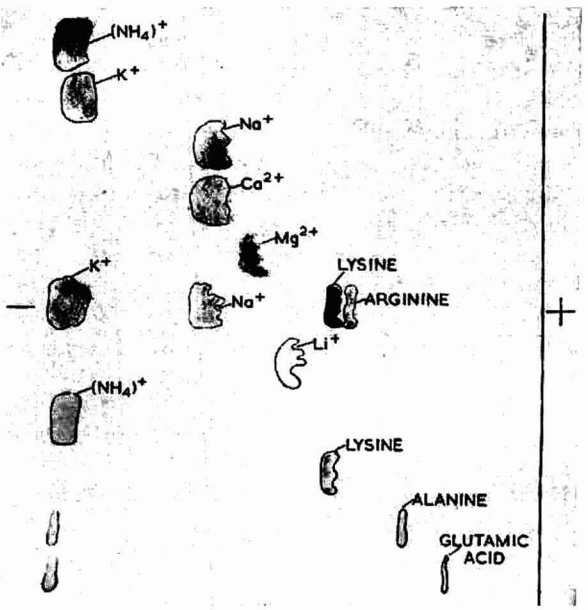

Fig. 1. An unusual ninhydrin reaction. Conditions: 100 V/cm, 6.7 mA/cm, pH 2.0, 0.75 *M* formic acid soln., Whatman No. 3 MM paper, 12°, 1.5 lb./sq. in., 15 min, spraying reagent = 0.1% ethanolic ninhydrin soln.

paper and the presence of shredded paper did not, however, produce a similar reaction in the test tube. The reaction worked on the paper strip not only at pH 2.0 but equally well with an electrolyte of pH 8.9 (ammonium carbonate solution).

Knowledge of this unusual reaction should lead to a more careful identification of $(NH_4)^+$ in the presence of K^+, and of dimethylamine in the presence of Na^+, but may also be utilized for the expedient detection of the presence of several inorganic cations such as K^+, Na^+, Li^+, Ca^{2+} and Mg^{2+} simultaneously with that of accompanying amino acids.

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Chromatography of nucleotides on a new anion-exchange paper

Although it has been known for many years[1,2] that polyethylenimine (poly-EI) and other basic polymer compounds are fixed substantively on cellulose fibers, it was not recognized that poly-EI-cellulose is an effective anion-exchanger which can be used in column[3], thin-layer[4], and paper chromatography.

In this paper the preparation of poly-EI-paper is described, and its usefulness for the separation of nucleotides is demonstrated.

Preparation of the anion-exchange paper

500 ml distilled water are added to 100 g of a 50 % solution of poly-EI in water*. The solution is stirred until all the poly-EI has dissolved. It is then neutralized with concentrated hydrochloric acid and filled up with distilled water to 2,000 ml. Sheets of Whatman No. 1 chromatography paper**, previously washed with distilled water, are dipped for 2 sec in the poly-EI solution and then dried in a stream of warm air. Finally they are washed several times with distilled water and air-dried.

The nitrogen content of the paper can be raised (lowered) by raising (lowering) the concentration of the poly-EI solution.

Chromatography

The samples are applied from a pointed micropipette 2 cm from the edge of the paper sheet. After drying ascending chromatography is carried out in an open beaker, the paper being attached to a glass rod. The development is stopped after the solvent has travelled an appropriate distance. The chromatogram is dried immediately in a stream of warm air. The compounds are detected by means of an ultraviolet lamp (254 mμ).

TABLE I

R_F VALUES OF RIBONUCLEOTIDES ON POLY-EI-PAPER

Solvent: 1.0 *M* NaCl. Distance covered: 10 cm in 25–30 min. n = number of developments. F = compound moves with the solvent front.

Compound	R_F		
	$n = 1$	$n = 2$	$n = 3$
Adenosine-5′-phosphate	0.63	0.83	F
Adenosine-diphosphate	0.37	0.63	0.83
Adenosine-triphosphate	0.12	0.25	0.32
Guanosine-5′-phosphate	0.58	0.80	F
Guanosine-diphosphate	0.25	0.47	0.65
Guanosine-triphosphate	0.07	0.17	0.23
Cytidine-5′-phosphate	0.77	F	F
Cytidine-diphosphate	0.46	0.76	F
Cytidine-triphosphate	0.15	0.30	0.44
Uridine-5′-phosphate	0.86	F	F
Uridine-diphosphate	0.57	0.87	F
Uridine-triphosphate	0.21	0.40	0.59

* A 50 % solution of poly-EI in water ("Polymin P") is obtainable from Badische Anilin- und Sodafabrik, Ludwigshafen a. Rh., Germany.

** H. Reeve Angel, London, E.C.4, England.

Nucleoside mono-, di-, and triphosphates are separated in 10–30 min with neutral electrolyte solutions on the poly-EI-paper, whereas on the commercial DEAE-, AE-, and ECTEOLA-papers* this separation cannot be obtained under these mild conditions.

In some cases repeated chromatography yields better separations: after the first chromatography the paper is washed with distilled water, dried and developed a second time with the same or with a stronger solvent. This procedure can be repeated a third time and so on.

Table I shows the R_F values of ribonucleotides on poly-EI-paper obtained with 1.0 *M* sodium chloride solution.

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Received September 10th, 1962

* H. Reeve Angel, London, E.C. 4, England.

Notes

Separation of the coproporphyrin isomers I and III by thin layer chromatography*

Ascending paper chromatography[1] was used for isomer analysis of coproporphyrin (COPRO), produced by a heme-requiring *Staphylococcus aureus* (JT/52)[2,3]. Though this method gave satisfactory results under standard conditions, it became desirable to develop a procedure that was more rapid, more sensitive, and less subject to disturbances due to slight temperature changes, contaminating salts or organic materials, and heavy sampling. An attempt was made therefore to adapt ERIKSEN's method[1] to thin layer chromatography (TLC). While TLC was suggested for the separation of the tetramethyl esters of COPRO I and III[4], no mention of TLC applied to the chromatographic analysis of *free* porphyrins could be found in the literature.

Materials and methods

Only small glass plates (50 mm × 200 mm) in small, cylindrical developing chambers (58 mm × 230 mm) with plastic closures were used throughout this study. Thus, 3 or 4 samples could be conveniently accommodated while considerable amounts of materials were saved and the equilibration time in the chamber was reduced.

The plates were coated with silica gel G (30 g in 60 ml H_2O) using a 250 micron

* Supported by PHS research grant C-3165, National Cancer Institute.

spreader. The dried and activated plates (110° for 45 min) were stored in a desiccator over $CaSO_4$ at room temperature. It was found that better separation was achieved if the plates were not used immediately after activation but rather after overnight storage in the desiccator or after approximately 1 h exposure to the room air (25°, rel. humid. 50 %).

Reference porphyrins were chromatographically pure COPRO I and COPRO III tetramethyl esters which were hydrolysed in 7 *N* HCl at room temperature in the dark. The bacterial porphyrins were dissolved in 1.5 to 2 *N* HCl. The samples were dried in an air current at room temperature. For application the solvent mixture suggested by ERIKSEN[1] proved most satisfactory: ammonium hydroxide (30 % NH_3) + water + acetone 1 + 2 + 7 volumes respectively. The samples were applied in quantities of 0.002 to 0.004 ml containing from 0.01 *µg* porphyrin to amounts readily visible in daylight.

The developing mixture was 2,6-lutidine (Eastman 95 % Practical) and water. The lutidine–water proportion was found to be very critical. The mixture used in paper chromatography[1] was not satisfactory since the R_F values of the I and III isomers were too similar to give good separation. After many trials the best mixture proved to be 10 ml 2,6-lutidine + 3 ml H_2O. Thus, 20 ml lutidine and 6.0 ml H_2O were poured into the chamber, and thoroughly mixed. Then a small cylindrical glass container (50 mm × 22 mm), filled 2/3 with NH_4OH (30 % NH_3), was placed on the bottom of the jar with the aid of a glass rod. The jar was then allowed to equilibrate for at least 30 min before the plates were inserted. Attempts to omit the ammonia vessel by adding ammonia directly to the water, as suggested for paper chromatography[5], failed.

Results

The technique described is a direct adaptation of ERIKSEN's method[1] to TLC. It has all the advantages of his method along with the additional ones characteristic of TLC: (1) rapidity, (2) sensitivity, (3) reproducibility, and (4) ease of recovery. (1) After 2 h the solvent front has traveled approximately 100 mm and the COPRO I and III isomers are completely separated into two distinct spots (R_F 0.19 and 0.25 respectively) of uniform density. (2) As little as 0.01 *µg* of COPRO I or III can readily be detected with the aid of a U.V. lamp. (3) Though R_F values vary slightly from one plate to another, on the same plate, spots containing minute amounts of porphyrin have exactly the same R_F values as those containing 10 to 100 times that amount. The spots are equally distinct and show no "tailing". (4) The spots can be scraped off easily and quantitatively, extracted with, for example, 2 *N* HCl, and rechromatographed or assayed in a spectrofluorometer. The system does not require special insulation; no disturbances have been observed without such provisions in a normally air-conditioned room at room temperatures of approximately 25°. Furthermore, preliminary studies have shown that metal complexes such as Cu–COPRO III as found in our bacterial system[2] have distinct R_F values, and the spots can be made fluorescent by direct treatment with concentrated H_2SO_4.

The U.V. photograph (Fig. 1) shows a typical chromatogram as it is obtained with the technique described in this communication. It shows from left to right COPRO I, COPRO III, and a mixture of the two isomers. The latter two samples were applied rather heavily in order to show (a) the contamination of the III isomer, which appeared

pure on paper chromatography, with traces of I and (b) the independence of the R_F values from the concentration of the sample.

Since two-carboxyl porphyrins and uroporphyrin were found to follow essentially the paper chromatography pattern, it seems that TLC can be applied with advantage to porphyrin chromatography in general.

Fig. 1. A photograph of a thin layer chromatogram (silica gel G) under U.V. light showing separation of coproporphyrin isomers I and III by 2,6-lutidine-water-NH_3. Running time 130 min. At extreme left 0.01 μg coproporphyrin I sampled.

As far as our own work is concerned[2], this sensitive method confirmed our former findings: the heme-requiring *Staphylococcus* (JT/52), whose porphyrin production is greatly enhanced by the presence of heme in the culture medium, produces only coproporphyrin of the type III isomer, its metal complexes (Zn and Cu) and uroporphyrin. Neither two-carboxyl porphyrins nor porphyrins with more than four and less than eight carboxyl groups could be detected in the medium.

I wish to thank Dr. SAMUEL SCHWARTZ for the gift of reference porphyrins and Mr. ANTHONY M. KUZMA for photographing the chromatograms under U.V. light.

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[3] J. JENSEN, *Bacteriol. Proc.*, (1962) 116.
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A device for transferring small volumes of gas from a vacuum line to a gas chromatograph

Various techniques have been developed for introducing vapour samples into gas chromatographic instruments[1–5]. However, none of these methods is particularly suitable for transferring small volumes of gas from a vacuum line. The apparatus described below has been designed to be used in conjunction with one or more Ward Stills[6] and a gas chromatograph in order to analyze quantitatively the very small amounts of gases produced during the radiolysis of organic compounds. It can also be used whenever it is necessary to measure and transfer small amounts of gas from a vacuum line to a gas chromatograph.

The essential part of the apparatus, which is shown in Fig. 1, consists of a combined Toepler pump (T), McLeod Gauge (M), gas burette (G) and sample injection device (I). This apparatus is connected by a Ward Still (W) to the part of the vacuum

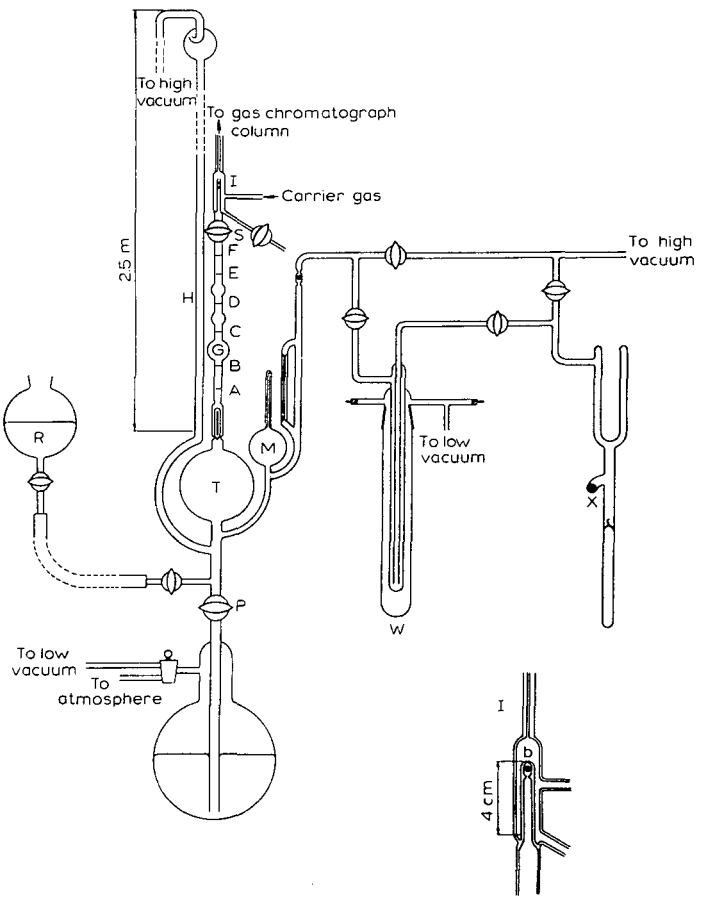

Fig. 1. Apparatus used for the separation, measurement and injection of gases into a gas chromatograph. The injection device is shown in the enlarged section. Calibrated volumes in the gas burette are S–A 5.89 ml, S–B 5.60 ml, S–C 2.29 ml, S–D 0.83 ml, S–E 0.18 ml, S–F 0.12 ml.

line which contains the gas to be analyzed. The gas sample injection device, which is shown in detail, is connected to the column of the gas chromatograph by about 1.5 m of 1 mm capillary tubing.

The gas chromatograph was calibrated by introducing known volumes of various gases from the vacuum line. A sample of gas was pumped into one of the calibrated bulbs of the gas burette by the Toepler pump. The difference in height between the mercury in the tube (H) and the appropriate calibration mark in the gas burette (G) was noted. The mercury reservoir (R) was then raised to such a height that the gas in (G) was compressed to a pressure greater than that of the carrier gas stream. In this particular case, the level of mercury in the tube (H) was some 1.5 m above the stopcock (S). The stopcock (P) was closed and the stopcocks connecting the Toepler pump to the mercury reservoir were opened. With a suitable carrier gas sweeping through the injection device, the stopcock (S) was then opened. The sample of gas, at high pressure, was thereby injected very rapidly into the stream of carrier gas, mercury being largely held back by the ball bearing (b). Any mercury which may escape past (b) collects in the side tube of the injection device and can be tapped off at a later stage.

The separation and resolution of the components of a mixture are greatly influenced by the technique of sample injection. The high rate at which the gas sample was injected into the carrier gas stream resulted in only a very small injection peak, even at the highest sensitivity used on the chromatograph. The small internal volume

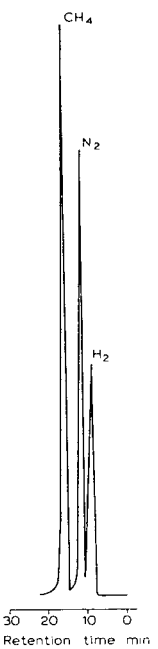

Fig. 2. Curve obtained from a sample of gas containing methane, nitrogen and hydrogen. Between the hydrogen peak and the nitrogen peak the sensitivity was reduced by a factor of 10. A six-foot silica gel column was used at room temperature with argon carrier gas flowing at a rate of 10 ml per minute. The chromatograph used was the Pye Gas Chromatograph with the katharometer detector.

of the injection device and the capillary connection with the column in the chromatograph successfully prevented any broadening of the peaks. A typical curve is shown in Fig. 2. Using samples of pure gases and also mixtures of gases which had been analyzed by other methods, calibration curves were prepared for each component present in the sample of gas which was to be analyzed. The reproducible way in which small samples of gas can be injected into the chromatograph is indicated by the straight lines obtained in the calibration curves; typical examples are shown in Fig. 3.

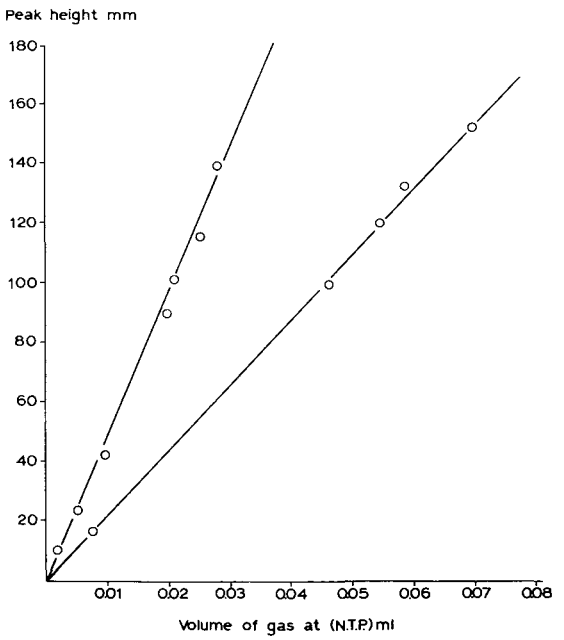

Fig. 3. Calibration curves obtained for pure hydrogen at two different sensitivities of the gas chromatograph. A six-foot silica gel column was used at room temperature with argon carrier gas flowing at a rate of 10 ml per minute.

The analysis of a complex mixture of gases may require the use of more than one column packing or carrier gas. It may be necessary, therefore, to fractionate the gas-mixture, holding back certain fractions until the column of carrier gas has been changed. The low temperature fractionation of small gas samples has been described by LE ROY[6] and this method, employing a Ward Still, has been used in the present work. The same technique was applied when analyzing the small amounts of gases produced when certain organic compounds were subjected to ionizing radiations. A known volume of an air-free organic liquid was enclosed in a sealed tube fitted with a break-seal and irradiated with X- or gamma-rays. After irradiation, the tube was sealed into the vacuum line at point (X), evacuated and the break-seal was broken. The temperature inside the Ward Still, which was immersed in liquid nitrogen, was adjusted by means of the built-in heating coil so that the irradiated organic liquid was just held back. The less condensible gases produced during the radiolysis were then pumped into the gas burette until the pressure in the vacuum line had fallen to 10^{-2}

to 10^{-3} mm as indicated by the McLeod gauge. The gas sample was then measured and injected into the gas chromatograph.

Using this apparatus, it was found that volumes of gases between 0.04 ml and 1.0 ml at N.T.P. could be analyzed. The usual size of gas sample produced by the radiolysis of organic compounds was, in the present work, of the order of 0.1 ml. It was found that this could be separated and that each component could be analyzed quantitatively, the accuracy of the analysis being dependent on the size and composition of the sample. In a typical experiment where methyl cyanide was irradiated with gamma-rays, the only gaseous radiolysis products were shown to be methane and hydrogen: each component in a 0.1-ml sample was analyzed with an accuracy of $\pm 5\%$.

The authors would like to express their appreciation of the contribution made by Mr. P. BOOTH and Mr. D. BRADLEY of the Royal College of Advanced Technology, Salford, in the development of this method of gas sample injection and analysis.

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Paper chromatography of some 2,4-dinitrophenyl S-alkyl-(L)-cysteines and corresponding sulfoxides

Recently we reported on the isolation of (+)S-methyl- and (+)S-*n*-propyl-(L)-cysteine sulfoxides from the onion (*Allium cepa*) as 2,4-dinitrophenyl derivatives[1]. In the course of this work, derivatives of some other S-alkyl-(L)-cysteines were also synthesized and studied. Before these compounds could be isolated in pure form by silicic acid chromatography, it was necessary to determine their chromatographic behavior and the feasibility of separating them from neutral amino acids obtained coincidentally from onion extracts.

This report describes the paper chromatography of the N-2,4-dinitrophenyl derivatives of these amino acids. Chromatography of N-2,4-dinitrophenylamino acids[2] has been widely used in end-group determinations of proteins and peptides and composition of protein hydrolysates[3]. The highly colored derivatives are easily detected on the chromatograms and can be eluted and measured colorimetrically[4,5].

However, little has been reported on its application to studies of complex mixtures of free amino acids in plant preparations.

We have found that N-2,4-dinitrophenyl-S-alkyl-(L)-cysteine sulfoxides crystallize readily and can be separated from corresponding dinitrophenyl-S-alkyl-(L)-cysteines and some other neutral amino acids. These findings have enabled us to separate the material on silicic acid columns and to use paper chromatograms to monitor the silicic acid chromatography.

Solvent systems reported in the literature[3–8] were, for the most part, unsatisfactory, because the spots migrated too quickly for effective separation or streaked too badly. Efforts to find a single solvent system that would resolve the mixtures satisfactorily were finally abandoned in favor of several solvent mixtures, which gave excellent results for the purposes to which they were applied.

Experimental

Preliminary paper chromatograms indicated that the dinitrophenyl-amino acid mixtures could be separated into a fast fraction containing the S-alkyl-(L)-cysteine derivatives (Table I) and a slow fraction containing the corresponding cysteine sulfoxides (Table II). The fast fraction could be resolved in a 16- to 24-h chromatogram. On the other hand, the slow fraction required a development time of 60 to 72 h with the fastest solvent for full resolution. Because of the long development time, the solvents were allowed to drip off the ends of the chromatograms, and the relative R_F values of the dinitrophenyl derivatives were calculated relative to that of N-2,4-dinitrophenyl-serine as an internal standard (Tables I and II).

Four solvent systems were used:

Ethyl acetate–phosphate buffer. Ethyl acetate saturated with pH 6.18 $M/4$ Sørensen buffer[11] was used in the preparative silicic acid column[1,9,10] as well as with

TABLE I

RELATIVE R_F'S OF DNP-S-ALKYL-(L)-CYSTEINES AND SOME INTERFERING NEUTRAL AMINO ACIDS (FAST FRACTION)[a]

Compound	*EtOAc–phosphate*[b]	*EtOAc–phthalate*[b]	*Heptane–propanol–phosphate*[c]	*Collidine–isoamyl alcohol–ammonia*[d]
S-Methyl-(L)-cysteine	4.8	4.7	4.3	2.0
S-Allyl-(L)-cysteine	5.8	6.8	7.4	2.6
S-*n*-Propyl-(L)-cysteine	5.7	7.9	9.3	2.7
Proline	5.4	8.0	6.3	1.6
Pipecolic acid	8.3	12.7	9.8	3.1
Methionine	4.5	6.7	6.7	2.4

[a] Calculated relative to R_F DNP-serine at 23°.
[b] S & S No. 589 Blue Ribbon paper; 8 h.
[c] Whatman No. 1 paper; 24 h.
[d] S & S Blue Ribbon paper; 24 h.

paper chromatograms. In the isolation of pure S-methyl- and S-*n*-propyl-cysteine sulfoxides, this solvent system consistently gave the best results. The fast fractions were eluted off rapidly and effectively, thus eliminating them as possible contaminants. Excessive amounts of asparagine and glutamine in the onion extracts interfered with

the separation of S-*n*-propyl-(L)-cysteine sulfoxide on the silicic acid column. However, the problem was not encountered on paper.

n-Heptane–n-propanol–phosphate. Heptane and propanol were mixed with the pH 6.18 *M*/4 Sørensen buffer (60:40:5) to give a single-phase system. Chromatograms developed with this mixture showed a slower migration rate with less distortion of spots. Whenever good resolution of fast-fraction components was desired, the mixture was very satisfactory if used with Whatman No. 1 paper.

Ethyl acetate–phthalate buffer. Ethyl acetate saturated with a pH 4.6 *M*/5 phthalate buffer[12] was compared with the phosphate buffered ethyl acetate. Results indicated a similarity in solvent behavior except that with the phthalate buffer the fast fraction moved faster and the slow fraction more slowly. There was less tendency toward streaking. Although the overall degree of resolution was not significantly improved, use of both ethyl acetate mixtures was rewarding in that one could readily resolve components that the other could not.

TABLE II

RELATIVE R_F'S OF DNP-S-ALKYL-(L)-CYSTEINE SULFOXIDES AND SOME INTERFERING NEUTRAL AMINO ACIDS (SLOW FRACTION)[a]

Compound	*EtOAc–phosphate*[b]	*EtOAc–phthalate*[c]	*Heptane–propanol–phosphate*[d]	*Collidine–isoamyl alcohol–ammonia*[e]
S-Methyl-cysteine sulfoxide	0.10	0.08	0.64	0.58
S-Allyl-cysteine sulfoxide	—	0.31	—	1.12
S-*n*-Propyl-cysteine sulfoxide	0.49	0.30	2.4	1.51
Asparagine	0.28	0.28	0.43	0.43
Glutamine	0.28	0.74	0.63	0.49

[a] R_F's calculated relative to R_F DNP-serine at 23°.
[b] S & S No. 589 Blue Ribbon paper; 16 h.
[c] S & S No. 589 Blue Ribbon paper; 24 h.
[d] Whatman No. 1 paper; 24 h.
[e] S & S No. 589 Blue Ribbon paper; 48 h.

Collidine–isoamyl alcohol–ammonia. This solvent mixture is adapted from one described by BISERTE AND OSTEUX[3]. The substitution of collidine for pyridine (without changing the 6:14:20 ratio), gave improved resolution. The migration rates were very slow, especially on Schleicher & Schüll, No. 589 Blue Ribbon paper. Chromatograms at 23° produced significant resolution of the fast fractions in 24 h, but 48 h were required for maximum resolution. For the slow fractions, even longer periods, up to 72 h, were required. However, these chromatograms were the most definitive and gave a resolution of the mixtures not obtainable with the other solvents.

For resolving the dinitrophenyl-S-alkyl-cysteine derivatives, the ethyl acetate–phthalate buffer solvent was particularly good for components of the fast fraction (Table I). The results, when viewed in conjunction with paper chromatograms of the slow fraction (Table II) developed with either the ethyl acetate–phosphate buffer or collidine solvent systems, presented a more complete picture of the behavior of these compounds. The collidine solvent system could probably be used to demonstrate the entire series of derivatives described, but the operation was much too slow. In

addition, there was only a partial resolution of dinitrophenyl S-allyl- and S-propyl-cysteines.

Actually, the mixture, *n*-heptane–*n*-propanol–phosphate buffer, approached closest to being the ideal single solvent. This was particularly true when the solvent was used to develop chromatograms on Whatman No. 1 paper, as the results show in Tables I and II. In spite of its apparent usefulness on paper, the *n*-heptane mixture was not satisfactory for silicic acid column work. When applied to the silicic acid column, development and elution of the various fractions were prohibitively slow. Furthermore, the dinitrophenyl-cysteine derivatives were only sparingly soluble in this medium. Since the primary objective was to prepare pure samples in sufficient amounts for further studies, the limitation on capacity negated use of the solvent.

The effect of paper was examined. Whatman No. 4 and No. 3MM[6] proved too fast, giving chromatograms with poor separation and bad streaking. Whatman No. 1 streaked badly with the phosphate-buffered ethyl acetate and somewhat with ethyl acetate–phthalate, but was very good when used with the heptane–propanol solvent. On the other hand, Schleicher & Schüll No. 589 Blue Ribbon paper (an acid washed paper) was particularly good with the buffered ethyl acetate solvents. Streaking was eliminated and the speed of the solvents tempered with resultant good resolution. This paper was used with the collidine mixture and, though very slow, gave the excellent results mentioned earlier. When a buffered solvent was used, the paper was dipped in a solution of the buffer and air dried before being spotted with the samples. Untreated papers were invariably unsatisfactory.

It is interesting to note that, in contrast to the behavior of methionine, the 2,4-dinitrophenyl-cysteines did not appear to undergo oxidation to corresponding sulfoxides during the time required to chromatograph them on the silicic acid columns.

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Gel electrophoresis of alkaloids*

Many papers have appeared in the literature describing the electrophoretic separation and identification of alkaloids on paper[1-5]. This paper describes briefly a method for separating alkaloidal mixtures via gel electrophoresis. The use of a solid gel media in electrophoresis has been described for proteins, amino acids, and other substances[6], but not for alkaloids. Although the method has no definite advantage over column chromatography as a preparative method, it may have utility for mixtures that do not lend themselves readily to chromatographic separation.

Experimental

The gel used in this laboratory was prepared via the method described by SMITHIES[7]. Eighteen grams of acetone washed Merck starch[6] were boiled under vacuum in an Erlenmeyer flask with the electrolyte, 1 *N* acetic acid (100 ml). The gel was poured into plastic trays (17 × 7 × 1.5 cm) and when the gel was nearly congealed (usually about 1 h) filter paper wicks were inserted on each end of the tray. The trays were then placed in the electrophoresis unit** and the wicks placed in contact with the electrolyte tanks (Fig. 1).

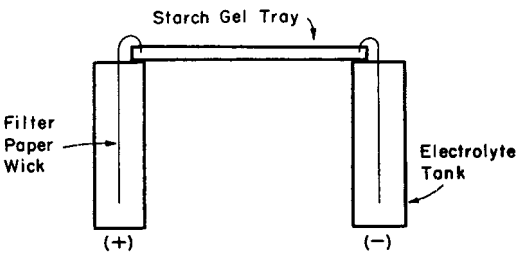

Fig. 1. Starch gel tray with filter paper wicks in contact with the electrolyte tanks.

A small cavity was cut into the congealed starch gel about 1 cm from the end with a razor blade or spatula insuring that it did not reach to either edge nor to the bottom of the starch gel tray (Fig. 2).

The "window" method described for paper chromatography and paper electrophoresis by POPOWICZ[8] works satisfactorily for applying the sample to the prepared gel tray. A piece of 17 MM Whatman paper was cut to the size 1 cm × 5 cm and the alka-

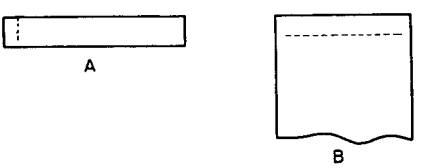

Fig. 2. (A) Side view of starch gel tray showing depth of cavity for insertion of "window" containing the alkaloid sample. (B) Partial top view of starch gel tray showing width of cavity for insertion of filter paper "window".

* This investigation was supported by Public Health Service Research Grant, RG-5640, National Institutes of Health.

** Spectrolator, supplied by Microchemical Specialties Co., Berkley, Calif.

loid mixture was adsorbed onto this strip and allowed to dry. This strip was then placed into the cavity.

To demonstrate the gel electrophoresis procedure two alkaloid mixtures were prepared that would contain alkaloids that fluoresced under ultraviolet light. Mixture No. 1 contained berberine HCl and quinine while mixture No. 2 contained berberine HCl and hydrastine.

Each mixture contained 10 mg of each alkaloid. The alkaloids were dissolved in methanol and adsorbed onto a Whatman strip as described and placed into the prepared starch gel tray.

The electrophoretic run was carried out at 50 mA and 400 V for 2 h. The migration distances for mixture No. 1 were 20 mm for berberine and 70 mm for quinine while mixture No. 2 had migration distances of 20 mm and 40 mm for berberine and hydrastine respectively. These distances were measured from the center of the fluorescent zones. The respective zones were cut out of the trays and placed in beakers. The gel was then mixed with water and solution effected on a steam bath. The final step of the procedure depends on the chemical nature of the alkaloid and this step actually represents a disadvantage over column chromatography and continuous curtain electrophoresis. The gel solutions of quinine and hydrastine were made alkaline with ammonium hydroxide and the alkaloidal bases extracted with chloroform. Berberine can be extracted from an acid solution with chloroform and so extraction of the gel solutions of berberine were effected in this way. The percent recovery of the alkaloids was not determined for this procedure.

For preparing larger quantities a pyrex dish (34 cm × 30 cm × 7 cm) was employed following the same procedure described and using 1000 ml of starch gel. Instead of the "window" method for applying the sample a "trough" is made in the gel by inserting a plate glass in the gel while still mobile and removing the glass plate when the gel is congealed. The sample is dissolved in the electrolyte (1 *N* acetic acid), starch added, and a gel prepared. When the gel is cool, but still mobile it is poured into the "trough" and allowed to congeal before applying the current.

This is a preliminary report and work with crude alkaloidal extracts is in progress.

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The detection of guanidine compounds on paper chromatograms

CONN AND DAVIS[1,2] found that ninhydrin (triketohydrindene hydrate) in alkaline media forms highly fluorescent products with guanidine, and with monosubstituted and N,N-disubstituted guanidines. The above authors developed a method for the fluorimetric estimation of guanidinium compounds.

It has now been found that the above method could be applied to the detection of certain guanidinium compounds on paper chromatograms. The method used was as follows:

The chromatogram was dried and sprayed with a solution of ninhydrin (0.25 % ethanolic solution). After drying the paper was sprayed with ethanolic sodium hydroxide (2 % ethanolic solution). On exposure to ultraviolet light, guanidinium compounds appeared as green fluorescent spots, which faded slowly.

The spray was tested successfully on the following compounds: guanidine, guanidine hydrochloride, guanidine nitrate, creatine, creatinine, glycocyamine, and arginine. Streptidine and diphenyl guanidine could not be detected by this method. Urea also did not give the fluorescent compound.

Although CONN AND DAVIS said that N,N'-disubstituted guanidines did not give the fluorescent compound, it was found that creatinine was detectable on paper chromatograms by this method.

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Colour photography of polycyclic compounds on alumina columns

Most polycyclic compounds are highly fluorescent in ultraviolet light hence their chromatographic development on alumina columns can be readily followed. However, the colour of various compounds and clarity of separation can be permanently recorded by modification of techniques recently developed for medical and biological photographic illustrations[1].

The equipment is illustrated in Fig. 1. A Witrona, 180 Joule Electronic Flash Unit at half power was used as the ultraviolet source. A Woods Glass Filter which

allowed passage of 3650 Å light was cut to the size of the flash holder and attached with a rubber clip ring, using a black felt gasket and opaque masking tape to completely seal off stray light.

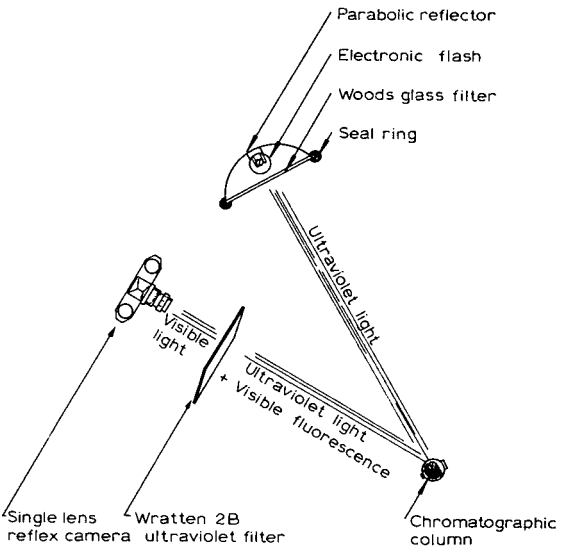

Fig. 1. Photographing chromatographic columns in ultraviolet light.

Ultraviolet energy irradiated the column emitting visible fluorescence plus reflected ultraviolet light. A Kodak Wratten 2B gelatin filter attached to the front of the camera lens absorbed the ultraviolet light and allowed only visible fluorescence to expose the film. Single lens reflex cameras, because of their freedom from parallax and facility for precise focussing proved the most satisfactory for this work. A Topcon 120 R camera with a f 1.8/58 mm lens was used.

The distance from light source to the column was 15 inches and the included angle to camera axis 20°.

Early difficulties with zone clarity and true colour production were overcome by using a fast film, High Speed Ektachrome, ASA 160.

For photographing the full column an exposure of f 5.6 proved most satisfactory while f 8 was used for close photographs of bright individual bands.

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Automatic spraying of paper chromatograms: An apparatus based on rotary motion suitable for quantitative analysis

An apparatus is described which permits reproducible, completely automatic spraying of paper chromatograms in such a manner that there is, experimentally, no evidence of excessive concentration of reagent over a wide area of paper. As such, the apparatus is especially suitable for studies involving quantitative paper chromatography, the spraying of dyes in order to observe the presence of acidic or basic compounds, or the spraying of chromatograms for illustrative purposes. The apparatus does not require the preparation of abnormally large amounts of spray reagent. Its dimensions, including base, are 36 in. (l) × 18 in. (w) × 15 in. (h). A chromatogram is sprayed in about 2 1/2 min.

A photograph of the apparatus is shown (Fig. 1). The apparatus includes a cylindrical stainless steel drum on which the chromatogram is mounted and secured*. The ends of the paper are easily slipped under bar clamps which are attached to the drum by removable screw-spring combinations.

The sprayer rides along a lead screw of eight threads per inch. The lead screw and drum are driven synchronously by an electric motor and pulley system.

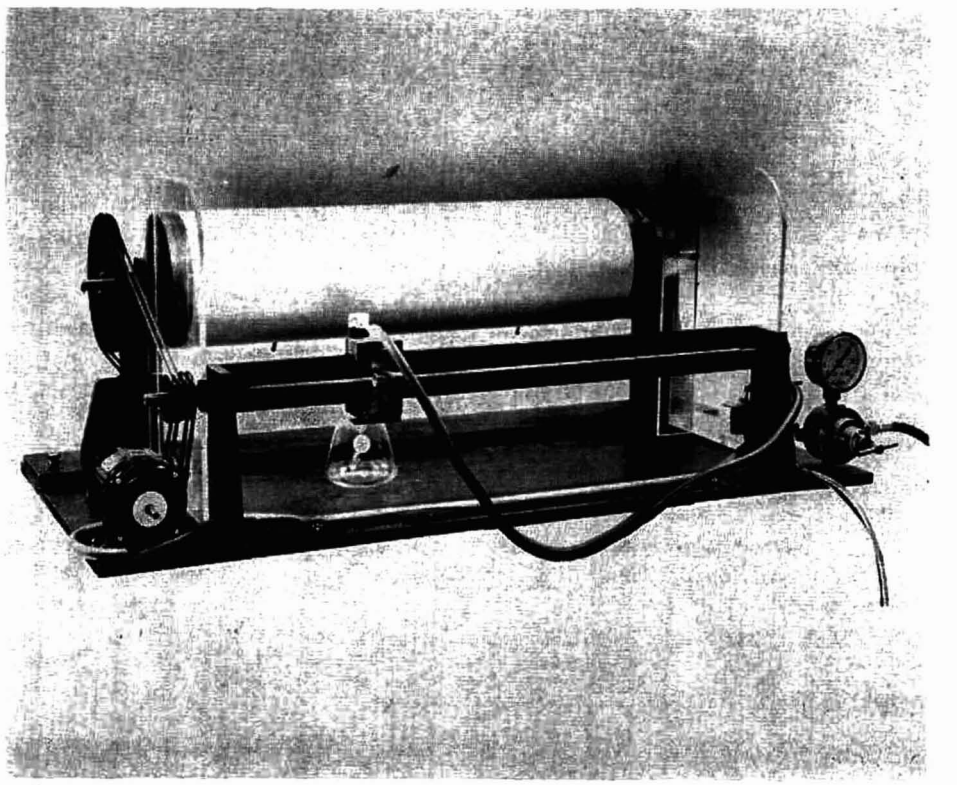

Fig. 1. An automatic sprayer for paper chromatograms.

* Non-metallic parts of the apparatus are Lucite and Benelex.

Pressure supplied from a tank of compressed gas is regulated to a fixed pressure (15–20 p.s.i.) by a secondary gauge attached to the apparatus. The position of the secondary gauge with respect to the on-off switch prevents the pressure from acting until the switch is thrown. The amount of spray applied can be regulated by the pressure at the secondary gauge.

Prior to "on" operation, the carriage is manually moved to the left side of the lead screw. When the on-off switch is turned on, the sprayer moves from left to right along the lead screw causing the chromatogram to be sprayed in an overlapping manner. When the carriage reaches the right end of the lead screw, it automatically switches off the current and the gas pressure.

Fig. 2 shows recordings obtained with a densitometer (Spinco model RB Analytrol). The upper graph shows that Whatman No. 3mm paper sprayed with methylene blue has an essentially even optical density in both directions. There was slightly

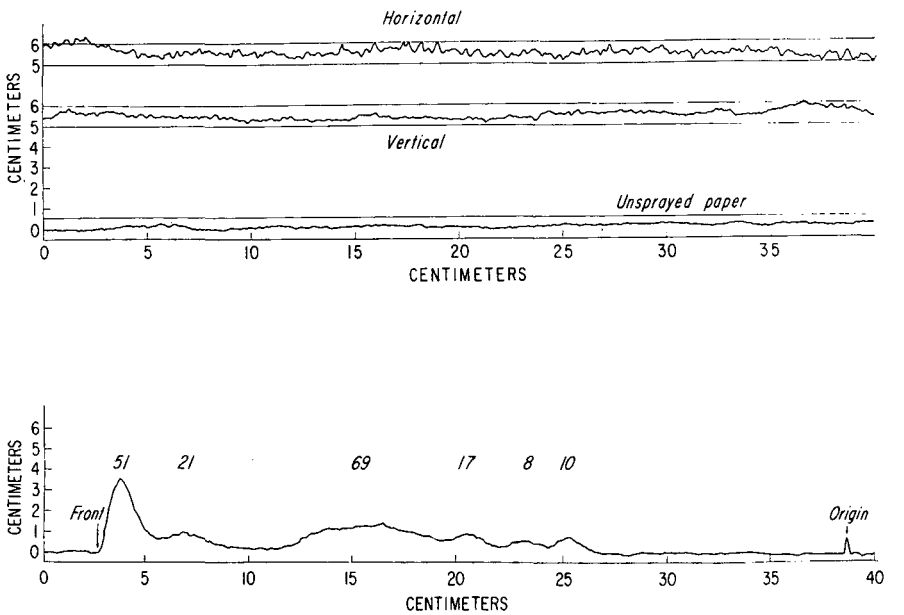

Fig. 2. The upper graph represents tracings obtained with a densitometer (Spinco model RB Analytrol) of Whatman No. 3mm paper sprayed with methylene blue and scanned in both directions. A scan of unsprayed paper is shown for comparison. The lower graph is that of a scan of a chromatogram of an extract obtained from the urine of a patient receiving chlorpromazine. It was sprayed with a reagent containing sulfuric acid in diluted ethanol. Numbers above each peak are the number of integration marks under the peak.

more variation at the ends of the strip and slightly more in the horizontal direction than in the vertical direction. A tracing obtained with unsprayed paper is shown for comparison.

The lower graph shows a tracing obtained with a chromatogram of an extract of urine from a patient receiving chlorpromazine. The chromatogram was sprayed with a sulfuric acid containing reagent which produced pink or purple colors against a colorless background. The base line shows little deviation from the zero. The success

of the analysis is, of course, partially dependent upon the degree of resolution obtained on chromatography. The numbers above each peak represent the number of integration marks obtained by dropping perpendiculars at the lowest point between peaks. The peak at the origin is the pencil line.

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BOOK REVIEWS

Cis–Trans Isomeric Carotenoids, Vitamins A and Arylpolyenes, par L. ZECHMEISTER, Springer-Verlag, Wien, 1962, 251 pages, price DM. 74.—, $ 18.50.

Cet ouvrage représente une mise au point des travaux de l'école de L. ZECHMEISTER parus au cours des deux dernières décades; les onze premiers chapitres (pp. 3–120) (généralités sur les caroténoïdes et l'isomérie *cis–trans*, propriétés des *cis*-caroténoïdes, isomérie *cis–trans* par réarrangement des formes tout-*trans*, ou par synthèse directe, *cis*-caroténoïdes naturels, rapports entre structure et propriétés physiques, étude détaillée de l'isomérie *cis–trans* des différents groupes de caroténoïdes naturels) ont déjà paru, il y a deux ans, dans le volume 18 des *Progrès de la Chimie des Substances Naturelles* bien connus. Ils sont présentés maintenant sous une forme légèrement modifiée et mise au point.

Vient ensuite un chapitre très important (pp. 121–148) consacré à la vitamine A et aux rétinènes.

Les quatre derniers chapitres (pp. 149–201) ont trait à des arylpolyènes synthétiques: mono-arylpolyènes, α,ω-diphénylpolyènes, cumulènes et polyène-azines.

Une bibliographie très détaillée et un index des auteurs et des matières complètent cet ouvrage, qui est imprimé de façon excellente et qui comporte 112 figures (le plus souvent des spectres ultraviolets et infrarouges).

L'importance de la configuration stérique des polyènes se manifeste de façon éclatante dans l'activité biologique des provitamines A et dans la stéréospécificité du rôle du néorétinal b dans la formation du pourpre rétinien.

Les problèmes traités dans ce livre sont donc d'un très grand intérêt physique, chimique et biologique et assureront à cet ouvrage le succès qu'il mérite.

E. LEDERER (Paris)

Some General Problems of Paper Chromatography. Relations between paper chromatographic behaviour and chemical structure. Attempts at systematic analysis. Edited by I. M. HAIS AND K. MACEK (Symposia ČSAV), Publishing House of the Czechoslovak Academy of Sciences, Prague, 1962, 220 pages, price Kčs 22.50.

The papers read at the Conference on Paper Chromatography in Prague and at the Symposium in Liblice, June 21st–23rd, 1961, have now appeared as a bound volume. Of the papers presented at the conference some are given in full and some as summaries, while all the symposium papers are published. Where necessary the papers were translated into English.

Paper chromatographers will find a number of new papers which have not been published elsewhere, others that have been published, as well as some reviews and introductory addresses. The discussions after each paper are also given. Altogether there are 31 articles. Both the editors and the translator (J. MICHL) are to be congratulated on an excellent production.

Announcement

LIPID DISTRIBUTION PROGRAM SPONSORED BY NATIONAL INSTITUTES OF HEALTH

As an extension of the cooperative program of the National Heart Institute and the Division of Research Grants, National Institutes of Health, ampoules of additional mixtures of saturated fatty acid methyl esters are now available to qualified investigators requiring such materials for their research programs. These samples include 200 mg of each of two mixtures, ranging from 8 to 16 and 14 to 24 carbons in chain length, respectively, thus extending the range beyond that previously available through other mixtures in this program.

Qualified investigators may request these samples as mixtures E and F, and should describe briefly the research purpose for which they will be used. Letters should be addressed in duplicate to Dr. WILLIAM H. GOLDWATER, Project Officer, Lipid Distribution Program, National Heart Institute, National Institutes of Health, Bethesda 14, Md., U.S.A.

Packages of four other mixtures, referred to previously as mixtures A through D, and of eight unsaturated fatty acid methyl esters, continue to be available as announced earlier. Requests for these samples should also be submitted as indicated above.

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PROCEEDINGS OF THE PRE-A.N.Z.A.A.S. CONFERENCE ON CHROMATOGRAPHY

INTRODUCTION

A number of discussions were recently held in Sydney before and in conjunction with the regular conference of the Australian and New Zealand Association for the Advancement of Science. One of these was devoted to Chromatography. This particular Conference was held on August 16th and 17th, 1962. Fifteen papers were presented, twelve of them being published in this Journal. The three remaining papers, by G. P. BRINER; D. G. BISHOP AND J. L. STILL; and J. H. RUSSEL, will be published elsewhere.

The time of the Conference was divided evenly between Paper Chromatography, including a review on Thin-Layer Chromatography by J. H. RUSSEL, and Gas Chromatography. Three films, the Whatman film on Chromatography, the Perkin Elmer film on Gas Chromatography, and that portion of the Czechoslovakian film on Organic Techniques which dealt with Chromatography, were shown. A Trade Exhibition of apparatus relevant to chromatography was given during the Conference.

Some 120 to 200 people attended the various sessions which were held in the Chemistry School and Theatrette in the University of New South Wales. The Conference was organized by Mr. E. C. MARTIN of the Chemistry School in the above University.

A PAPER CHROMATOGRAPHIC STUDY OF SOME PLATINUM (II) COMPOUNDS

II. THE SEPARATION OF *cis*- AND *trans*-DIHALOGENODIAMMINEPLATINUM (II) COMPLEXES

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INTRODUCTION

In a preceding paper[1] the separation of the *cis*- and *trans*-$[Pt(NH_3)_2Cl_2]^0$ complexes from each other and from other platinum (II) complexes was described using aqueous solvents as eluents on cellulose filter paper. This kind of adsorption cellulose chromatography appeared of interest since the adsorption mechanism of neutral complexes had not been previously investigated[2]. In addition preparative work (by F.B. and K.H.S.) also required a method for establishing the purity of the dibromo-, diiodo-, chloroiodo- and chlorobromo-diammineplatinum(II) complexes. Thus this paper reports the general study made on these halogeno complexes. The choice of methods is rather limited. Owing to the non-ionic character of all the compounds, ion exchange and paper electrophoretic methods are clearly unsuitable. During preliminary experiments it was also found that development for more than 1–2 h with partition solvents (such as butanol–water, butanol–1 *N* HCl etc.) produced long comets indicating transformations during the development. The presence of mineral acids even as dilute as 0.1 *N* led to substitution reactions at room temperature during a short development. Good separations were finally obtained by using neutral solvents under such conditions as to minimize hydrolysis during development.

TECHNIQUE

Ascending development in tightly closing jars (26 cm × 12 cm) was used throughout with Whatman No. 3 MM paper. Samples had to be dissolved in water only minutes before being placed on the paper to avoid hydrolysis. All spots were shown up on the developed chromatogram by holding the paper over iodine vapours which, depending on the compound, yielded yellow, brown or violet spots on a paler background. All R_F values given here are strictly relative. Considerable variations were observed from day to day and from one batch of paper to another. Thus the absolute R_F values for example in Tables I–IV are only indicative: however, the R_F differences are always readily reproducible. For identification purposes reference substances must be run on the same sheet of paper with the samples to be tested.

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EXPERIMENTAL

(i) *Studies on the adsorption of cis- and trans-*$[Pt(NH_3)_2Cl_2]^0$

The R_F values of *cis-* and *trans-*$[Pt(NH_3)_2Cl_2]^0$ in HCl, LiCl, NaCl and KCl of varying concentration are shown in Fig. 1. Unlike with chlorauric acid and other chloro-acids[3] there is no notable salting-out effect with these two complexes. However, it is interesting to note that the R_F values of the *cis-* and the *trans-*forms approach each other in higher concentrations of HCl. The adsorption seems to be related to the polarity

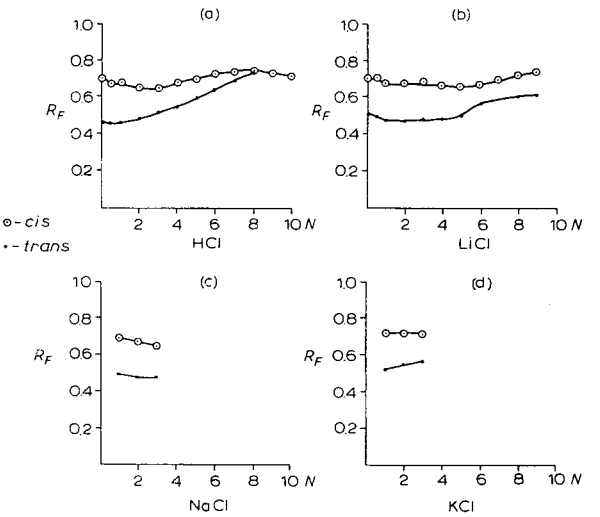

Fig. 1. The variation of the R_F values of *cis-* and *trans-*$[Pt(NH_3)_2Cl_2]^0$ on Whatman No. 3 MM paper at 20°. Eluent: (a) aqueous HCl, 0.1–10 *N*; (b) aqueous LiCl, 0.1–9 *N*; (c) aqueous NaCl, 1–3 *N*; (d) aqueous KCl, 1–3 *N*.

of the complexes and their environment. Thus whenever the ionic cloud around both complexes is very large the differences in hydration (and adsorption) disappear. This agrees with the results in Fig. 2 which show the changes in R_F values of the two isomers with increasing concentrations of ethanol in 0.5 *N* HCl. Again the separability decreases with a decrease in the polarity of the solvent system.

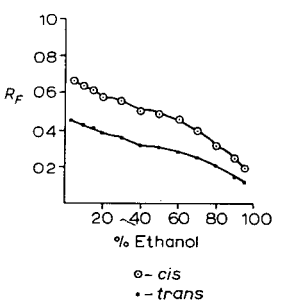

Fig. 2. The variation of the R_F values of *cis-* and *trans-*$[Pt(NH_3)_2Cl_2]^0$ on Whatman No. 3 MM paper at 20° with alcoholic 0.5 *N* HCl.

(ii) Separations of the dichloro- dibromo- and diiodo-diammineplatinum(II) complexes

The first attempts were to use butanol–water as partition solvent for these compounds. Long trails were formed which indicated that under the dynamic conditions of development, the hydrolysis reactions were too fast to yield separations. In order to repress the extent of hydrolysis, it was decided to acidify (0.1 *N* HCl) the aqueous and ethanolic solvents. At room temperature (18° ± 1°) the R_F values shown in Fig. 3a and 3b were obtained, thus it was possible to separate the *cis*-compounds from each other and from the *trans*-compounds, but not the *trans*-compounds from each other. The largest R_F differences were found to be in the region of very low or very high ethanol concentrations with an inversion of the sequence at about 40% ethanol.

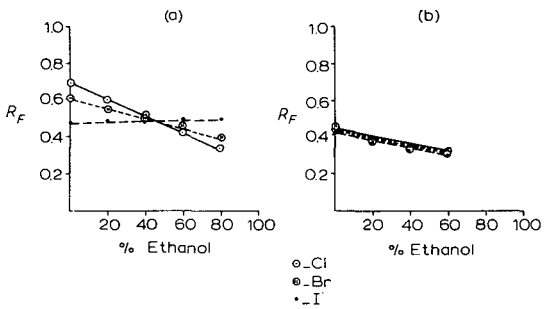

Fig. 3. The variation of the R_F values of $[Pt(NH_3)_2X_2]^0$ on Whatman No. 3 MM paper at 18° using mixtures of ethanol–water (all being 0.1 *N* with respect to HCl) as solvent. (a) *cis*-$[Pt(NH_3)_2X_2]^0$; (b) *trans*-$[Pt(NH_3)_2X_2]^0$.

It would appear that the adsorption differences are due to the hydration differences in aqueous solvents, the *cis*-diiodo being least hydrated and hence most adsorbed and the *cis*-dichloro being most hydrated and thus least adsorbed. This would be in accord with the greater electronegativity of chlorine *versus* iodine so that the dichloro system is expected to have the higher dipole moment and thus be more solvated. The *trans*-compounds having no dipole moment are not differentiated in aqueous solvents.

An attempt was made to apply these mixtures of ethanol–water (containing 0.1 *N* HCl) to the identification of the *cis*-chlorobromodiammineplatinum(II). However, it was noted that with a small increase in the room temperature (from 18° to 21°) the R_F values were no longer reproducible. The effect of temperature on the R_F values is shown in Table I. By carrying out chromatograms of solutions which were allowed to stand (in 0.1 *N* HCl) it could be shown that the change of R_F values was not due to temperature changes but to the conversion of the bromo complexes into the corresponding chloro complexes during development (see Table II). In 0.1 *N* HBr the reverse reaction could be observed (Table III).

Both hydrolysis and replacement reactions could be avoided by chromatographing in a refrigerator (+5°) with distilled water. Round spots and good separations were obtained with all of the *cis*-compounds as shown in Table IV.

After obtaining these results, a search was made for solvents capable of separating the *trans*-compounds. This search was limited to fast flowing solvents without the

TABLE I

R_F VALUES OF *cis*-DIHALODIAMMINEPLATINUM (II) COMPLEXES AT VARIOUS TEMPERATURES

(Solvent: aqueous 0.1 *N* HCl; paper: Whatman No. 3 MM)

Complex	R_F		
	5°	*18°*	*21°*
$Pt(NH_3)_2Cl_2$	0.63	0.70	0.70
$Pt(NH_3)_2ClBr$	0.60	—	0.68
$Pt(NH_3)_2Br_2$	0.54	0.62	0.66

TABLE II

EFFECT OF AGEING ON SOLUTIONS OF *cis*-DIHALODIAMMINEPLATINUM (II) IN 0.1 *N* HCl

(Paper: Whatman No. 3 MM; temperature for ageing and development: 18°)

Complex	R_F *after ageing for:*		
	0 h	*7 h*	*24 h*
$Pt(NH_3)_2Cl_2$	0.71	0.70	0.71
$Pt(NH_3)_2ClBr$	0.68	0.69	0.71
$Pt(NH_3)_2Br_2$	0.64	0.67	0.68

TABLE III

EFFECT OF AGEING ON SOLUTIONS OF *cis*-DIHALODIAMMINEPLATINUM (II) IN 0.1 *N* HBr

(Paper: Whatman No. 3 MM; temperature for ageing and development: 18°)

Complex	R_F *after ageing for:*		
	0 h	*12 h*	*24 h*
$Pt(NH_3)_2Cl_2$	0.69	0.67	0.66
$Pt(NH_3)_2ClBr$	0.66	0.66	0.66
$Pt(NH_3)_2Br_2$	0.60	0.62	0.64

TABLE IV

R_F VALUES OF *cis*-DIHALODIAMMINEPLATINUM (II) COMPLEXES

(Solvent: water; paper: Whatman No. 3 MM; temperature: 5°)

Complex	R_F
$Pt(NH_3)_2Cl_2$	0.65, 0.65, 0.65
$Pt(NH_3)_2ClBr$	0.58, 0.58, 0.58
$Pt(NH_3)_2Br_2$	0.52, 0.53, 0.53
$Pt(NH_3)_2ClI$	0.56
$Pt(NH_3)_2I_2$	0.44, 0.44, 0.43

addition of mineral acids. Neither pure methanol nor ethanol yielded good separations; however mixtures of acetone and water at 18° gave excellent separations of both the *cis*- and the *trans*-compounds.

The R_F values are shown in Fig. 4a and 4b.

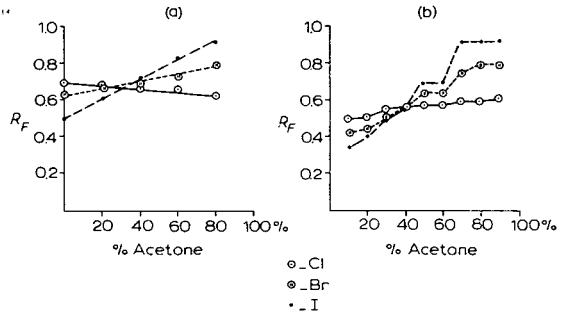

Fig. 4. (a) R_F values of *cis*-$[Pt(NH_3)_2X_2]^0$ with acetone–water mixtures. Temperature: 20°; paper: Whatman No. 3 MM. (b) R_F values of *trans*-$[Pt(NH_3)_2X_2]^0$ with acetone–water mixtures. Temperature: 20°; paper: Whatman No. 3 MM.

RESULTS

The *cis*- and *trans*-dibromo and diiodo complexes were all prepared by reacting the dichloro complex with two equivalents of $AgNO_3$, filtering off the precipitated AgCl and then adding two equivalents of either NaBr or KI. This resulted in the separation from solution of the desired compounds. Paper chromatography showed all these reactions to yield only a single spot corresponding to the dihalogeno complex of the

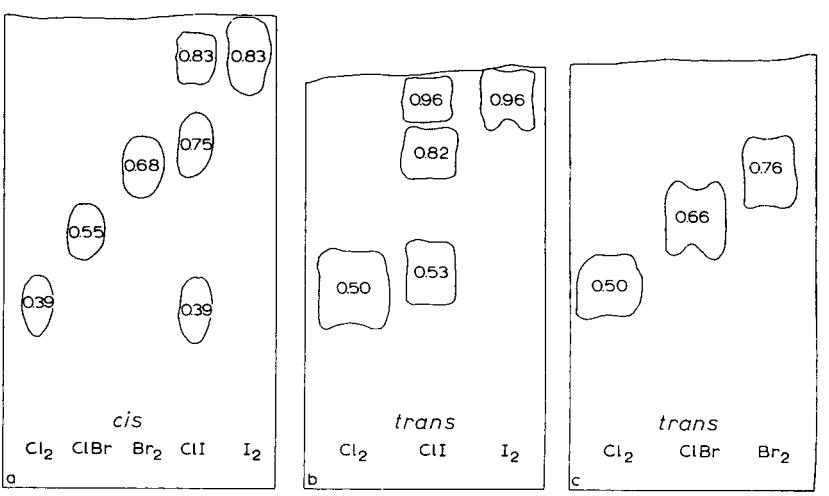

Fig. 5. Some typical chromatograms obtained with acetone–water (9:1) at 20° on Whatman No. 3 MM paper. Bottom line: line of application; top line: liquid front. (a) Samples of *cis*-$[Pt(NH_3)_2X_2]^0$ run side by side on the same sheet. Note that *cis*-ClI is impure containing also *cis*-Cl_2 and I_2 complexes. (b) Samples of *trans*-$[Pt(NH_3)_2X_2]^0$ run side by side on the same sheet. Note that *trans*-ClI contains also *trans*-Cl_2 and I_2 complexes. (c) Samples of *trans*-$[Pt(NH_3)_2X_2]^0$ run side by side on the same sheet.

same geometric structure. It may thus be concluded that chromatographically pure compounds are formed by this reaction.

The mixed dihalogeno complexes were prepared by reaction of the required dichloro complex with one equivalent of $AgNO_3$ after which the precipitate of AgCl was removed on a filter and one equivalent of either NaBr or KI was added. The chlorobromo compounds so prepared were also found to be pure within the limits of detectability on a paper chromatogram. On the other hand the presumed chloroiodo complexes yielded usually three spots: the dichloro, the chloroiodo and the diiodo complexes (Fig. 5). The reaction of *trans*-dichloro with equivalent amounts of *trans*-diiodo in boiling water was also tried and yielded only a weak spot of the chloroiodo complex in presence of preponderant quantitites of the dichloro and the diiodo complexes.

The details of these syntheses as well as further preparative work will be described elsewhere.

SUMMARY

The separation of *cis*- and *trans*-dihalogenodiammineplatinum (II) complexes was effected by paper chromatography using neutral fast flowing solvents such as water at 5° or acetone–water (9:1). Practically all mixtures of *cis*- and *trans*- as well as of dichloro-, dibromo-, diiodo as well as chloroiodo- and chlorobromo-complexes could be separated.

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M. LEDERER, *J. Chromatog.*, 6 (1961) 518.

THE CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONES ON ACETYLATED PAPER

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INTRODUCTION

Aliphatic carbonyl compounds play a major role in the odours of plants, insects and oxidized foods. Since these compounds often occur in amounts below one part per million, special techniques are necessary for their identification[1]. One of the most widely used techniques is the study of their 2,4-dinitrophenylhydrazones, especially by paper chromatography. The relative rates of movement of the 2,4-dinitrophenylhydrazones and the light absorption maxima of the spots, which may be measured directly on the paper after chromatography[2], are important qualitative criteria. Moreover, the pure compounds may be extracted from the paper for further examination such as determination of infrared spectra[3] or melting points.

There is a considerable variety of methods for the paper chromatography of 2,4-dinitrophenylhydrazones in which either treated or untreated paper is used[4]. MEIGH[5], HUELIN[6] and SCHEPARTZ[7], all using untreated paper with the same solvent system, report good separation for the lower homologues but decreasing separation for the higher homologues of the hydrazones of *n*-alkanals and *n*-alkan-2-ones. Better separation throughout the range is obtained by treating the paper with an involatile stationary phase such as phenoxyethanol[8], paraffin oil[9] or vaseline[10]. However, RITTER AND HARTEL[11], discussing these methods, point to the disadvantages associated with lack of reproducibility of the amount and distribution of the stationary phase, possible effects on the stationary phase during spotting, and interference by the non-volatile stationary phase during subsequent measurements. Acetylated paper has been found useful in the chromatography of the 2,4-dinitrophenylhydrazones of carbonyl fission products of sugars[12], polycyclic aromatic hydrocarbons[13], steroids[11], indole derivatives[14], polonium and other ions[15], 3,5-dinitrobenzoates of aliphatic alcohols[16] and the 2,4-dinitrophenylhydrazones of lower aliphatic carbonyl compounds[17]. Although the use of acetylated paper obviates the need for the impregnation with its attendant disadvantages, the acetylation procedures are tedious and not particularly reproducible. Little use has been made of commercial papers, possibly due to their high price.

EXPERIMENTAL

In this study a commercially available acetylated paper (Schleicher & Schüll 2043b/21ac, 20–25 % acetylated; 54 × 57 cm) was used for the paper chromatography of 2,4-dinitrophenylhydrazones of the C_{3-13} *n*-alkan-2-ones, the C_{1-14} *n*-alkanals, the $C_{3-11,16}$ *n*-alk-2-enals and the $C_{5-12,14,16,18}$ *n*-alka-2,4-dienals. The first batch re-

ceived in 1959 had a softer and more open texture than five batches received in 1962.

Using the first batch, excellent chromatograms were obtained with the solvent system described by MEIGH[6]. The 2,4-dinitrophenylhydrazones were dissolved in methanol (except the hydrazones of the C_{11-14} *n*-alkanals which were dissolved in ether) and spots 0.5 cm in diameter and 2.5 cm apart were placed on the baseline 10 cm from one end of the paper. The chromatogram was run parallel to the 57 cm edge of the paper which is apparently at right angles to the fibre direction. The paper was equilibrated overnight in a Shandon 12-in. Universal Strip Chromatank (57 cm high) with methanol saturated with "heptane" (light petroleum, b.p. 100–120°) and then developed by the descending method with "heptane" saturated with methanol. Small circular spots were obtained and the separation can be seen from the data presented in Fig. 1.

With the five later batches of paper good separation was obtained but the faster moving compounds gave "streaked" spots. This streaking was unaffected by the size

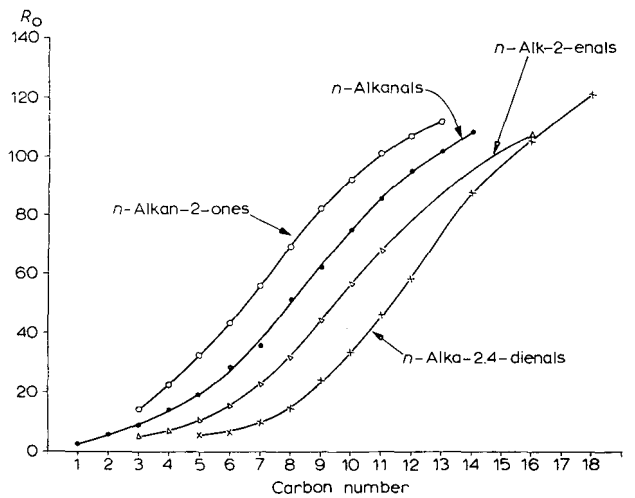

Fig. 1. Mobility of 2,4-dinitrophenylhydrazones. $R_o = \frac{\text{Distance moved by a hydrazone}}{\text{Distance moved by } n\text{-octanal hydrazone}} \times 50$.
O—O *n*-Alkan-2-ones; ●—● *n*-Alkanals; △—△ *n*-Alk-2-enals; ×—× *n*-Alka-2,4-dienals.

of the initial spot or by the distance of the baseline from the end of the paper but was increased by running the compounds in the direction parallel to the 54 cm edge. Several other solvent systems, see for example refs. [13,14], were tried but none were better than the methanol–"heptane". Drying the methanol–"heptane" with calcium sulphate increased the streaking while the addition of 5 % water had no effect.

The acetylated papers were compared by macroscopic and microscopic examination, staining and washing tests, solubility in chlorinated solvents, resistance to air flow and measurement of thickness. While the papers varied considerably, it was not possible to determine the reason for the superiority of the paper received in 1959. This aspect was not pursued as good chromatograms could be obtained by the simple techniques described below.

Two other grades of paper were tested on a limited scale (Schleicher & Schüll

2045b/21ac, 20–25 % acetylated; and 2043a/45ac, 40–45 % acetylated). The former was a harder paper and the latter a more highly acetylated but neither was markedly superior to the 2043b/21ac.

Two modifications with the methanol–"heptane" system each eliminated the streaking. In one, the paper was equilibrated with methanol alone instead of methanol saturated with "heptane". Alternatively, as suggested by RITTER AND HARTEL[11] and SPOTSWOOD[13], the mobile phase was run through about 12 cm of a "retardation strip" of slow paper (Whatman No. 2 or No. 20) before the acetylated paper. Both modifications increased the development time to 4–7 h with two papers in the tank. Experiments were carried out at 15°, 20°, 30° and 40°. The best chromatograms were obtained at 30° with a "retardation strip".

As was noticed by BURTON[18], the spots obtained by acetylated paper chromatography of 2,4-dinitrophenylhydrazones were smaller than those obtained with untreated Whatman paper particularly for the lower homologues. Individual members of the homologous series were separated from each other. The R_F values of the 2,4-dinitrophenylhydrazones of the C_x alkan-2-one, C_{x+1} alkanal, C_{x+3} alk-2-enal and C_{x+5} alka-2,4-dienal were roughly equivalent (*cf.* refs. [2,4]). However, each series could be characterized by light absorption measurements of the spot[2,19]. In this way, a combination of R_F values and λ_{max} enables any spot belonging to the above series to be identified (see also ref. [20] for a comparison with C_{4-10} *n*-alk-1-en-3-one hydrazones).

CONCLUSIONS

The advantages of using acetylated paper for chromatography are the speed and simplicity of preparing the chromatograms, the excellent separation obtained, and the ease with which subsequent measurements may be effected. With the current availability of reagents for the formation of colored derivatives from amines, alcohols, carbonyl compounds, mercaptans and other compounds, it is likely that acetylated paper may be used with advantage in their chromatography.

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SUMMARY

The paper chromatography of the 2,4-dinitrophenylhydrazones of the C_{3-13} *n*-alkan-2-ones, the C_{1-14} *n*-alkanals the $C_{3-11,16}$ *n*-alk-2-enals and the $C_{5-12,14,16,18}$ *n*-alka-2,4-dienals on six batches of acetylated paper (Schleicher & Schüll 2043b/21ac) is described. With a batch obtained in 1959 excellent chromatograms were obtained by equilibration overnight with methanol saturated with "heptane" followed by descending development with "heptane" saturated with methanol. With the other five batches obtained in 1962, the separation of all compounds was also excellent but the faster moving spots tended to streak. This was prevented by equilibration with

methanol alone or preferably by running the mobile solvent through a "retardation strip" of about 12 cm of a slow paper (Whatman No. 2 or No. 20) before the acetylated paper. The optimum temperature for the latter chromatograms was 30°.

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ASPECTS OF THE DIRECT PHOTOMETRY OF SUBSTANCES DISTRIBUTED ON TRANSLUCENT SHEETS

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INTRODUCTION

Provided that reasonably complete resolutions of mixtures of substances can be effected on sheet material (zone-electrophoretically, chromatographically), that the individual substances can be located and identified, and that either they themselves or something with which they have reacted or the two together can be eluted satisfactorily from excised portions of the sheet, then circumstances exist for the quantitative estimation of the individual substances. And the circumstances suffice whether the resolution can or cannot be conducted in a closely reproducible way.

The current biochemical literature furnishes various examples of the use of methods of this type. However, a desire to reduce the time and equipment involved by eliminating the elution step has led to the development of many methods involving direct photometry (transmission, reflection) of the substance bands on the sheets, only a limited number of which have proved satisfactory, however.

It is the purpose of this communication to recount an analysis of the difficulties which appear, hitherto, to have militated against the achievement of success in work of this type, and of means which might be adopted to surmount these difficulties and to place the photometry on a footing more nearly comparable with that prevailing in work with solutions (LUGG AND MCEVOY-BOWE[1]). Although transmission methods will be considered primarily here the conclusions have implications for reflection methods also, and these will be indicated later.

In "one-dimensional" resolutions, with the mixture to be resolved streaked uniformly on a starting line, the bands will normally move with but little lateral spreading, a small defect which can be eliminated by carrying the starting line along the full width of a strip of sheet. Provided that the sheet material is of uniform thickness and of fine texture, and that the absorption of light by the substance is in effect in accordance with Beer's law in respect of "concentration" (density in the sheet), then any such clearly separated band may readily be submitted to scanning with a slit photometer of suitable design, with integration for the number of slit-frames required to cover the band. The resolution does not need to be conducted in a closely reproducible way if the photometry can be conducted in an ideal way. Any overlapping of bands, however, must obviously lead to some uncertainty about values.

With "two-dimensional" techniques the resolution is much higher and can usually be made much more complete. However, with sheets of uniform thickness and fine texture, and the most favourable conditions for reproducible chromatography

prevailing, the distribution of substance in a band about the position of maximum density will yet not, in general, be radially symmetrical nor will it be of the same form for all bands, even if the initial spot has been applied uniformly as a disc of definite diameter to the sheet. It can readily be shown on optical grounds that, even if the absorption of light by the substance in different parts of the band conforms in effect with Beer's law in respect of density and the photometry can be conducted in an otherwise ideal way, yet the integrated value obtained by slit-scanning such a band will vary with the orientation of the band relative to the direction of traverse of the slit.

And so, even if the resolution can be made reproducible, there remains the problem of ensuring reproducible orientation in the slit-scanning photometry, and in any event the abandonment of any hope of establishing other than an empirical relationship between the integrated photometer results and the quantity (Q) of that substance in the initial spot, irrespective of the possible existence of a formal relationship between Q and the density of substance in a specified part of its band. For these reasons the slit-scanning of such bands cannot be favoured: the so-called "maximum density" type of photometry holds far more promise.

The photometer (densitometer) must, however, have a circular aperture and the response of the photocell to light must be uniform across the exposed portion of its face, if the reading is to be independent of the orientation of the band in the instrument. We (LUGG AND MCEVOY-BOWE[1-3]) have exclusively employed instruments of this type, in which also the response has been closely proportional to light flux.

CHOICE OF CHROMATOGRAPHIC PROCEDURE: IDEALISED CONDITIONS

However regular may be the construction of an actual sheet the distribution of centres of specific attraction (Van der Waals and electrostatic type) is unlikely to exhibit the degree of regularity desirable for the purpose under consideration. That is to say, liquid–liquid partition rather than adsorption or ion-exchange should determine the R_F value. Again, it is with this type of partitioning that the distribution coefficient of the substance will usually vary least with concentration. Correspondingly, if the cross-sectional thicknesses of stationary and mobile phase do not vary over the sheet, the more likely is the relationship between Q and the maximum density of substance in its band to be linear.

The plain fact is that every substance is distributed in a two-dimensional chromatogram over the entire sheet beyond the position of first contact with each of the two mobile phases in turn, up to the limit of excursion of these phases. The confines of a band, however sharply defined they may appear to be, are merely the limits of detectability under the detection criteria in use. The maximum density, however, has a position and a value, related indeed to the sheet-wide overall distribution, but instrumentally determined by a single reading and requiring no precise information about the sheet-wide distribution

For ideal liquid–liquid partition chromatograms on sheets of fine structure and uniform gross thickness the overall distribution might be computed from MARTIN AND SYNGE'S[4] theoretical treatment of the column partition chromatogram for substances having constant coefficients of distribution between the two liquid phases, if the cross-sectional thicknesses (areas, per unit width) were known for stationary

phase and mobile phase. The computation requires that due allowance be made for diffusion (diffusion coefficients in both phases being assumed constant) which, in the direction of the "run" has the effect of increasing the "height-equivalent of the theoretical plate" and which, normal thereto, leads to the lateral spreading of the bands. The effect can be computed by analogy from the well-established equation for the loss of heat by conduction from a source (see also BRIMLEY[5]). There is also the effect of micro-scale angle-channelling of mobile phase between the textural elements of the sheet upon lateral spreading, but the channelling could reasonably be assumed independent of the (low) concentration of substance.

Under these conditions the quantity of substance through which the light is regarded as passing is directly proportional to Q, neglecting the minute contributions from the attenuated parts of other bands and the (usually) far more important contribution of the "blank" (the total being the "background"). Reasonable allowance for the "background" can be made by subjecting a portion of the sheet remote from visible limits of bands to the photometry.

IDEALISED TRANSMISSION PHOTOMETRY OF BAND AND BACKGROUND

Consider the transmission photometry of a substance in solution contained in a cuvette. Of the monochromatic incident light flux (IL) some (RL) is reflected back from the interfaces and irretrievably lost. Of the balance, which we may call the "entering" light (EL), a portion (AL) is absorbed and the rest, the transmitted light (TL), reaches the photocell and is measured:

$$EL = IL - RL = AL + TL \tag{1}$$

If, as is usually the case, the absorption of entering light by materials in the light path is governed by Lambert's law in respect of thickness, if the absorption by the substance is governed by Beer's law in respect of density, if optical densities are additive, and if due correction can be made for the blank, then the concentration of the substance can be estimated accurately if the entering light can be maintained constant. But, whereas the constancy of IL can be assured readily enough, EL must be presumed to vary with substance concentration to some extent if IL is constant. This variation is customarily ignored, but it is of interest that, such as it may be, the variation is in the direction which would account, at least in part, for the commonly observed departure from Beer's law.

In both the strip-scanning and maximum density types of transmission photometry of bands on a sheet, RL is usually a large, not small, fraction of IL, so that even a relatively small variation with substance density can seriously affect EL when IL is constant. Any attempt, therefore, to employ the classically derived relationship.

$$Q = kD = k \log (1/T) \tag{2}$$

where D is the optical density of the band material, T being the transmittance relative to the sheet, and k a constant, requires that the reflected-back light be restored. This can be done conveniently in one or both of two ways which will be discussed later.

WORK WITH ACTUAL SHEETS

Our work has been confined to so-called "single-phase" two-dimensional chromatography of amino acids[1-3] and of sugars[6] on Whatman No. 1 filter paper sheets, colour development being conducted under conditions designed to yield colour substances in quantities proportional to those of the parent substances. With amino acids, ninhydrin and moist chlorine gas were colour and bleaching reagents, respectively. The evidence is that these single-phase solvents behave as conventional solvent pairs in association with the fibre (see MARTIN[7]), and the preliminary assumption is, then, that the chromatograms are ideal within the limitations imposed by the deficiencies of the sheets.

These sheets are of fibrous cellulose and their structure is depicted in Figs. 1–3. This evidence and information obtained by weighing various areas of sheet indicate that individual sheets are of appreciable uniformity in respect of gross physical thickness, but that there is some variation between sheets of a batch, and that the textural irregularities are not notably fine in relation to the magnitude of the instrument aperture (3 mm diam.). Thus, photometry of a remote part of the sheet could not be assumed to yield an adequate estimate of the optical density of sheet plus background in the region of minimum transmission of the band.

A solution of this problem was sought by measuring the transmissions in the two places on excised portions of the sheet, bleaching the portions and returning them in precisely the same positions and orientation for re-measurement. A microscope mechanical stage attached to the instrument is used for the purpose. From these values the transmittance of the band material relative to the sheet could be computed much more reliably than was otherwise possible, the variance of the mean with replicate chromatograms being reduced to about one quarter of what was otherwise attainable.

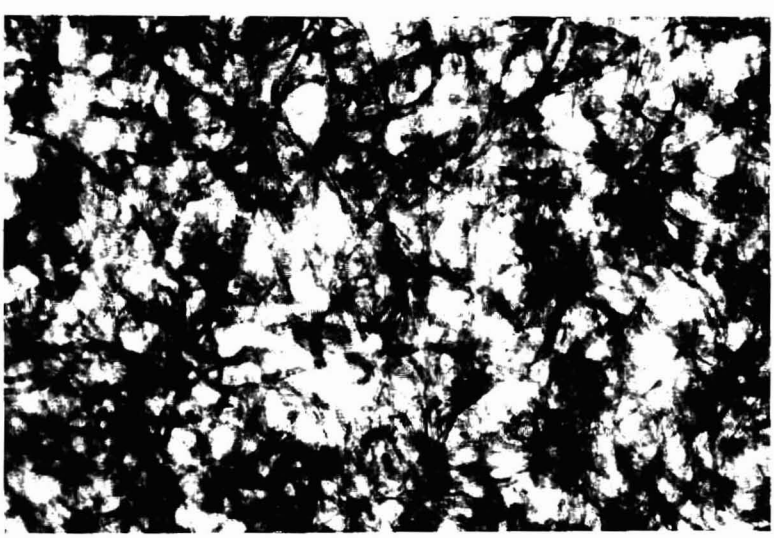

Fig. 1. View into Whatman No. 1 filter paper, with light source below. Focus half-way through the paper. Mag. (paper to photo) × 40.

Fig. 2. View into Whatman No. 1 filter paper, with light source below. Focus almost superficial. Mag. (paper to photo) × 200.

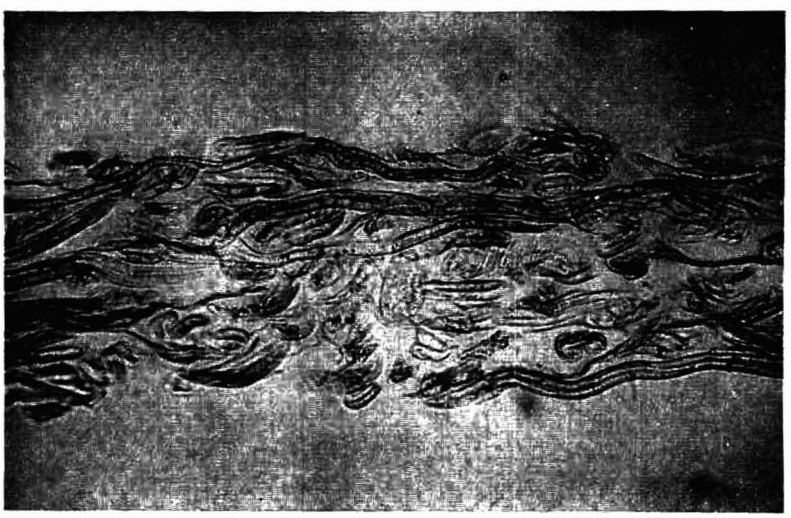

Fig. 3. Cross-section (10 μ thick, wax-embedded, wax removed) of Whatman No. 1 filter paper. Note some shrinkage of fibre due to embedding. Mag. (section to photo) × 200.

As the transmittance so computed is still in respect of constant incident light instead of constant entering light it is under-estimated to a degree which increases with substance density, and the correction for background is likewise somewhat defective. Equation (2) could therefore not be expected to hold, even if monochromatic light were used in place of the near-white light actually employed in our work. The value, however, could be used with a calibration curve and, within fairly wide limits, was found to be reasonably satisfactory also for use in a semi-empirical equation:

$$Q = k_1 [(1/T) - 1] \tag{3}$$

where k_1 is a constant.

Close control of the chromatography and of the colour development, coupled with this photometry refinement, yielded means of triplicate assays of amino acids which were as reliable[3] as the means of about one hundred replicates in the procedure recommended by BLOCK, DURRUM AND ZWEIG[8].

FURTHER CONSIDERATIONS

With the advances related above we have thus far been satisfied in applied studies, despite the appreciable empiricism still plaguing the photometry and the fact that eqn. (3) could be far less valid with other types of sheet. The challenge has been taken up, however, in further study and in the designing of instrument accessories.

A presumed (in effect) validity of Lambert's law for "thickness of sheet" in respect of entering light can be tested by measuring the transmitted light with increasing numbers of sheets in contact, incident light being kept constant and the reflected-back light being absorbed by the black enclosure. For sheets of Whatman No. 1 paper beyond the third (and so, for part but not all of the third itself) accordance with the law has been found excellent, near-white and green-filtered light being used in this work; and we conclude that the flux of reflected-back light has by then become virtually constant. In passing it should be mentioned that the term "thickness of sheet" in the above context must signify "mean density of sheet material in the region for unit thickness, times the number (including fractional number) of thicknesses considered".

The observation furnishes a simple solution of the problem of ensuring constancy of the entering light—simply interpose several thicknesses (or their optical equivalent in other material) as backing sheets between the source and the test sheet and as close to it as practicable.

An alternative solution is to collimate the incident beam and use a large almost hemi-spherical reflector with its centre of curvature at the site of incidence of the beam on the sheet—nearly all the light could be restored in summation of an infinite series of successive reflections from and restorations to the sheet. Such a reflector would make the instrument cumbersome. A smaller reflector is less efficient but, used with only a few backing sheets, achieves a satisfactory result. Interposition of the backing sheets reduces the sensitivity of the instrument, but compensation for this can be made by inserting a Perspex light conductor in the collimator tube.

The instrument and its accessories have been described fully elsewhere[1] and need not be detailed here. Figs. 4 and 5 show the main body of the densitometer with the lamp-house detached and folded back to reveal the position of the collimator-reflector

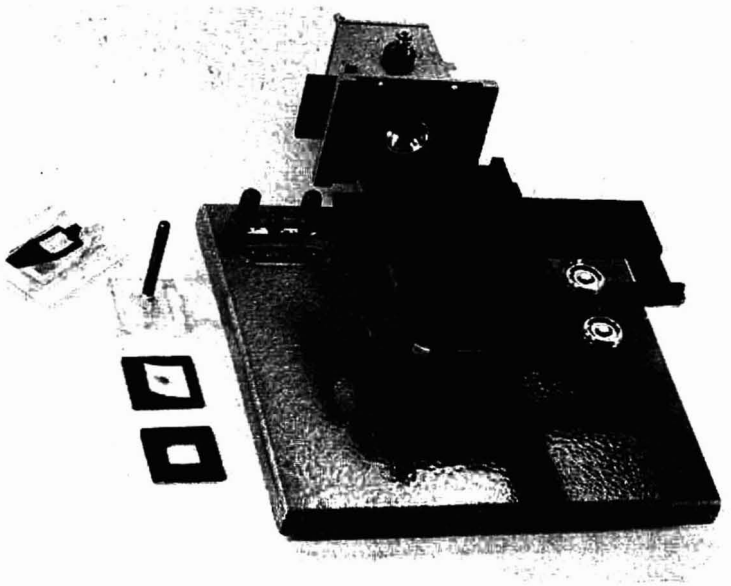

Fig. 4. The densitometer, with the lamp-house folded back to show the collimator-reflector assembly. A sheet-portion is shown on one (inverted) of the sheet-holders at the left.

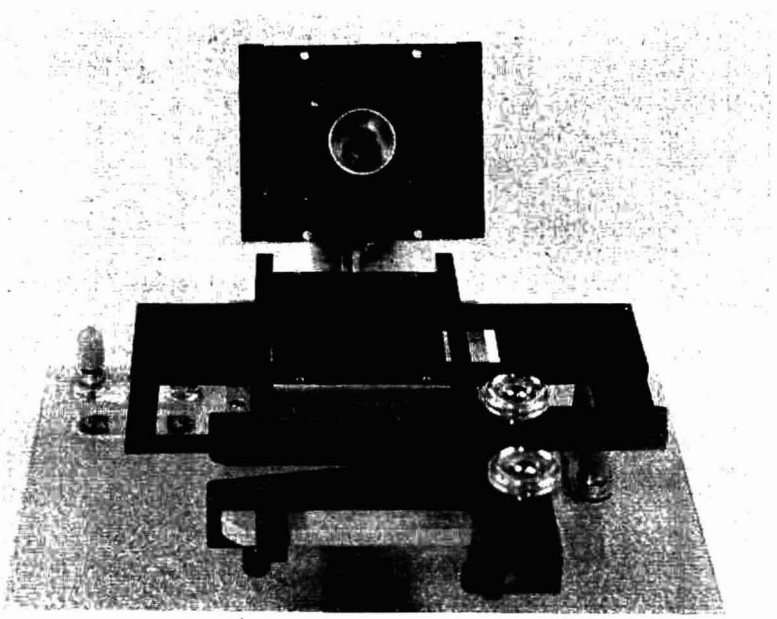

Fig. 5. Densitometer, showing microscope mechanical stage fitted to carrier, and with the lamp-house folded back to reveal the collimator-reflector assembly in its base.

assembly in the base. At the left hand side of Fig. 4 are shown the creaser and stamper (the crease ensures positional replacement of a sheet portion after bleaching), an inverted sheet-holder with sheet portion attached, and an unloaded sheet-holder with its securing wedge at its side.

This model differs from the earlier one[1] in that the slide to carry a diffuser and/or colour filter has been raised to just beneath the lamp and just above the top of the collimator, thereby obviating the need to insert diffuser and/or filter in a collimator well.

To some extent the nature of the textural irregularities might affect the density of chromatographed substance in and upon the fibre there. If, for example, the irregularities were in respect of fibre density for uniform physical thickness of sheet the cross-sectional thicknesses of mobile and stationary phase would not be in as constant ratio minutely over the entire sheet as would be expected if the fibre density were minutely uniform, the irregularities being only in local physical thickness of sheet.

In the latter of these extreme cases the amount of substance in the light path would be deemed proportional not only to Q but to the amount of fibre there. Equation (2) might be expected to apply more closely then, if the term on the right were multiplied by the ratio of the mean thickness over all the sheet to the mean thickness at the photometer aperture. An approximation to this factor could be computed from the photometer readings for sundry bleached portions of the sheet. The prospect is of potential importance in that the variability of replicates might thereby be substantially reduced.

In the former of these two extreme cases the amount of substance in an irregularity would not be proportional to the amount of fibre there. A possibility is that its distribution at the aperture might be rather uniform, with eqn. (2) applying fairly closely.

The photomicrographs of the paper we used suggest, perhaps, that the irregularities are somewhat of a mixture of the two extreme types considered above. It can readily be shown that for sheets of grossly uniform texture and physical thickness the constant, k, in eqn. (2) or its variant should be constant for small variations in physical thickness between individual sheets of a batch, if the assumptions concerning the chromatography are valid. The soundness of this important principle is not dependent upon the nature of the (small) irregularities[1].

The data with which eqn. (3) was found to accord fairly closely for a ten-fold range of Q were, in fact, fairly well represented by eqn. (2) for much smaller ranges of Q. Although systematic studies of the same type have not been made with provision for restoration of reflected-back light, existing data[1] make it plain that such provision would greatly increase the range of Q for which eqn. (2) might apply fairly closely. Indeed, with monochromatic (if not even with appropriately filtered) light, eqn. (2) could, in the author's opinion, be used to summarize data with many different types of sheet material.

Just how it comes to pass that Beer's law should show such promise of being valid (in effect at least) is a matter of conjecture The "in effect" qualification might merely be an aspect of real validity if the substance is wholly dissolved in sorbed moisture on the sheet material. Indeed, we have recently found[9] that in drier environments eqn. (3) is reasonably applicable only up to somewhat lower levels of Q.

With the development of newer types of sheet of fine and uniform structure the

irregularity problem may be so reduced as to make the bleaching and repositioning of sheet portions unnecessary in some work. Indeed, much has been claimed for the slit-scanning of bands on granular-textured cellulose acetate strips[10] after the state of the sheet material has been changed from translucent to virtually transparent with the aid of organic solvents. However well-justified the claim may be, it is questionable that successful "maximum density" work could be done with sheets so treated: if the sheet thickness and with it the condition and/or state of aggregation of the substance were to vary capriciously, more could be lost than gained in so simple a solution of the reflection-back problem.

The problem posed by "reflection-back" in the transmission photometry of substances on translucent sheets has as its logically obvious counterpart a "transmission-forward" problem in reflection photometry. Analogous solutions are, of course, to be found in the use of backing sheets and/or reflector on the side of the test sheet distal to the light source, but a plane reflector against the test sheet (or backing sheets, if used) is required. Such reflection photometry may have advantages over the transmission type if the sheet transmission itself is very low.

ACKNOWLEDGEMENT

The help of Dr. W. F. C. BLUMER of the Anatomy Department, University of Western Australia, for the preparation of the section and of the photomicrographs, is gratefully acknowledged.

SUMMARY

In the direct photometry of substances resolved on translucent sheet electrophoretograms and chromatograms textural irregularities in the sheet may be responsible for pronounced variability of replicates, and loss of reflected-back light in transmission work or of transmitted-forward light in reflection work can result in serious departure from otherwise valid, formally derived relationships between the quantity or density of a substance and the photometer results.

These difficulties and means which have been devised to reduce their significance are discussed, primarily in relation to "maximum density" type transmission photometry of liquid–liquid partition chromatograms. A photometer and procedure suitable for such work are briefly discussed. However, the conclusions reached are considered to be of widely general validity, and the broad design of a corresponding reflection photometer is indicated.

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SOME FACTORS INFLUENCING THE CONSTANCY OF R_F VALUES IN PAPER CHROMATOGRAPHY UNDER AUSTRALIAN FIELD CONDITIONS

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INTRODUCTION

The application of paper chromatographic techniques to geological problems required that, on some occasions, analyses had to be carried out in the field or under base-camp conditions. The methods of WARD AND MARRANZINO[1], NEVILL AND LEVER[2] and RITCHIE[3] certainly were designed for use under these conditions. Published data on R_F values generally relate to laboratory studies made under controlled conditions of temperature and humidity. The realization that the extremes of temperature and humidity which were likely under Australian field conditions, would influence seriously the R_F values prompted the investigations, the results of which are now reported.

As far as possible the data presented have been compiled from field tests. The technique used was by ascending development, using rectangles of Whatman No. 1 paper (25 cm × 30 cm) standing in the solvents in screw-top 6-l polythene jars. Sometimes the "runs" were done in a small tent but more often in a box trailer fitted with a canvas hood. In some cases the field conditions were simulated in laboratory tests with good correlation.

RESULTS

The main results are shown in Table I.

Apart from the variations in the R_F values, the significance of which will be discussed below, extreme humidity presents some special problems. One important difficulty is the maintenance of the paper in a workable condition over periods of weeks in the field. This is a difficulty encountered in Africa by BURSILL[4] as well as in Australia. The ascent of the solvents by capillarity was almost stopped when the paper became moist. The vapour phase seemed to react with the water in the paper and on occasions the metal ions moved downwards under the influence of gravity.

Even on the one chromatogram, inconsistent results were obtained when the vessels were exposed to wind or direct sunlight. The problem was overcome by placing boxes or cardboard cartons over the vessels. Apart from this effect, very consistent results were obtained on any one chromatogram.

The variations which are revealed in Table I may be generalized as follows:

1. Hot-dry conditions are conducive to higher R_F values. Notable exceptions are Co^{2+} and Mn^{2+} in solvent 1.

TABLE

R_F VALUES OF METAL IONS UNDER

Metal ion	Solvent 1				Solvent 2			
	Published	*40° Humid 10% Hot-dry*	*35° Humid 80% Hot-wet*	*12° Humid 80% Cold-wet*	*Published*	*40° Humid 10% Hot-dry*	*35° Humid 80% Hot-wet*	*12° Humid 80% Cold-wet*
Ag^{+}	0.77 + C	0.90 + C	0.88 + C	0.80 + C	1.0 + C	0.90 + C	0.88 + C	0.92
Pb^{2+}	0.44 + C	0.62 + C	0.57 + C	0.48 + C	0.64 + C		0.64 + C	0.62 + C
Cd^{2+}	0.92	1.0	1.0 + C	0.97 + C	1.0			0.94
Bi^{3+}	0.75	0.97	0.93	0.74	0.85			0.97 + C
Hg^{2+}	1.0	1.0	0.93	1.0	1.0			0.93
Sb^{3+}	0.88	0.95	0.96	0.89	1.0	0.95	0.93	0.83 + C
As^{3+}	0.92	0.92	0.88	0.80	0.92	0.91	0.90	0.59
Fe^{3+}	Up to 0.93	0.89	0.80	0.50	Up to 0.75	0.49	0.40	0.35
Cu^{2+}	0.53	0.34–0.92	0.34–0.67	0.45	0.54C–1.0	C–0.92	C–0.58	0.32
Co^{2+}	0.45	0.28	0.40–0.80	0.42	0.30–0.38	0.28	0.20	0.24
MnO_4^{2-}	0.73	0.78	0.72	0.80	0.53 + C			0.55
Ni^{2+}	0.52	0.27	0.61	0.40	0.32	0.27	0.24	0.28
Mn^{2+}	0.48	0.28	0.33	0.32	0.32	0.28	0.33	0.30
Zn^{2+}	0.94	0.97	0.90	0.90	1.0			0.87
Al^{3+}	0.46	0.52	0.46	0.44	0.36			0.28
Be^{2+}	0.60	0.66	0.62	0.64	0.60			0.46
Mg^{2+}	0.47	0.50	0.47	0.46	0.37			0.28
Au^{3+}	1.0	0.77	0.83	0.93	0.8–1.0			0.94
Pt^{4+}	1.0 + C	0.92 + C	0.88 + C	1.0 + C	1.0 + C			0.85 + C
Cr^{3+}	0.38	0.46	0.40	0.44	0.23			0.28
Ti^{4+}	0.64			0.67	0.43			0.36
UO_2^{2+}	0.46			0.48	0.37–0.43			0.41
V^{5+}	0.47			0.75 + C	0.43			0.33
WO_4^{2-}	0.70	0.70 + C		0.80 + C	0			0

Solvent 1 = Butanol–conc. HCl–conc. HF–water (100:50:2:48).
Solvent 2 = Butanol fraction of butanol–conc. HBr–water (100:10:90) + conc. HBr (40).

2. Hot-wet conditions produce values slightly lower than the hot-dry.

3. Cold-wet conditions produce lower R_F values than the hot conditions but not always lower than the published results which were obtained near 20°.

Some disadvantage is revealed in that, within the range of conditions experienced, identification of ions cannot be made by reference to the published R_F values obtained under controlled laboratory conditions. The disadvantage may be overcome by running parallel chromatograms in such a way that known and unknown ions may be compared[3].

Advantageous features appear to be a better separation at temperatures from 35° to 40° with less variation of R_F over small temperature ranges. Further results obtained at more critical temperatures might reveal that, for the solvents used, 20° ± 1° is an unfavourable temperature for R_F determinations.

CONCLUSIONS

R_F values of common metal ions vary considerably under the influence of:

1. temperature,

I

AUSTRALIAN FIELD CONDITIONS

Solvent 3				*Solvent 4*			
Published	*40° Humid 10% Hot-dry*	*35° Humid 80% Hot-wet*	*12° Humid 80% Cold-wet*	*Published*	*40° Humid 10% Hot-dry*	*35° Humid 80% Hot-wet*	*12° Humid 80% Cold-wet*
0.13 + C		0.12 + C	0.13 + C	0.59 + C	0.76 + C	0.70 + C	0.55
0.45 + C	0.81 + C	0.72 + C	0.45 + C	0.48 + C	0.81 + C	0.76 + C	0.54 + C
0.96	0.93	0.90	0.77	0.77–0.83	0.94		0.58
0.88	0.89	0.88	0.76	0.85	0.88	0.82 + C	0.86 + C
1.0	1.0	0.95	0.90	1.0	0.95	0.92 + C	0.94 + C
0.80 + C	0.95 + C	0.88 + C	0.67	0.82	0.90	0.85	0.90 + C
0.92	0.88	0.86	0.71 + C	0.65	0.94	0.92	0.80 + C
0.73	0.86	0.85	0.62	0.86	0.97	0.92	0.74
0.65	0.77	0.72	0.73 + C	0.56	0.87	0.80	0.45
0.70	0.74	0.72	0.66	0.45–0.55	0.58	0.42	0.26
0.60	0.55	0.50		0.83–0.92	0.90 + C	0.90 + C	1.0 + C
Up to 0.67	0.77	0.65	0.58	0.03	0.02	0.02 + C	0.25 + C
0.70	0.72	0.70	0.72	0.25	0.27	0.15	0.15
0.94		0.95		0.90	0.65	0.75	
0.75	0.84	0.80		0.02	0	0	0
0.86	0.88	0.86		0.22	0.28	0.26	0.28
1.0	0.78	0.82	0.78	0.03	0.46	0.26	0.24
0.91	0.95	0.92		0.97	0.95	0.90	1.0
0.90 + C	0.86 + C	0.86 + C	0.87	0.93 + C	0.90 + C	0.91 + C	0.97 + C
0.73		0.73	0.73	0.06	0.10	0.10	0.08
0.76	0.82	0.78	0.69	0.64	0.84	0.76	
0.76		0.78		0.58–0.64	0.52	0.48	0.62
0.74	0.80	0.75	0.61	0.64	0.65	0.62	0.92
1.0	1.0	1.0	1.0	1.0 + C	1.0 + C	1.0 + C	0.97 + C

Solvent 3 = Ethanol–methanol–2 *N* HCl (60:60:80).
Solvent 4 = Acetone–conc. HCl–conc. HF–water (180:10:2:8).

2. humidity (which affects the moisture content of the paper),
3. temperature variations from one side of the vessel to the other.

The difficulties thus created can be overcome to some extent by using a comparative method in which known metal ions are chromatographed alongside the unknown ions. An air-tight container kept dry by silica gel should overcome the moisture problem.

SUMMARY

R_F values of metal ions obtained under Australian field conditions are reported. The effects of temperature and humidity are shown. Precautions leading to effective use of the technique in the field are mentioned.

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THE ROLE OF THE STATIONARY PHASE IN GAS CHROMATOGRAPHY

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INTRODUCTION

The large number of stationary-phase liquids described in the literature and offered for sale by suppliers is bewildering to the gas chromatographer. The choice of a suitable stationary phase for a given separation of volatile compounds can be facilitated by a study of the balance of intermolecular forces between the solute and solvent. A qualitative prediction of the retention behaviour of a solute on a given phase can be made using the vapour pressure of the solute and the classification of liquids first proposed by EWELL, HARRISON AND BERG[1]. This has been discussed in some detail by KEULEMANS[2], AMBROSE AND AMBROSE[3] and HARDY AND POLLARD[4].

In gas chromatography, apart from the vapour pressure of the solute, the most important single factor governing the magnitude of the solute retention on a stationary phase is the nett electron donor–acceptor interaction between solute and solvent. The most common example of this is hydrogen-bond formation. The electron donor–acceptor properties of both solute and stationary-phase liquid can be used as a basis for a quantitative classification of phases and as a basis for a method of identification of unknown volatile compounds by gas chromatography.

MOLECULAR STRUCTURE AND INTERMOLECULAR INTERACTIONS

First we must consider briefly the structure of the molecules, their intermolecular interactions and the factors which govern the specific retention volume of a solute. Non-polar molecules in gas chromatography are confined mainly to gases and saturated hydrocarbons. Polar molecules contain one or more polar atoms or groups together with a neutral or non-polar part, usually saturated hydrocarbon chains. The polar part may be the strongly electronegative atoms F, O or N or electron attracting groups, *e.g.* $-NO_2$, $-C\equiv N$, $-CF_3$ or electron repelling groups such as $-NMe_2$, $-CH_3$, $-CMe_3$, $-OMe$. It is the type and number of polar groups in a molecule relative to the size of the neutral part which determines the polarity or distribution of electrons on the different parts of the molecule. If the molecule contains double bonds with their mobile π-electrons this allows electron attracting or repelling groups to transmit their effects further through the hydrocarbon part of the molecule than is possible with saturated hydrocarbon chains. Most polar molecules can act as electron donors or acceptors or both, given the required environment, but usually one effect is predominant. It is the concentration of electrons or the electron-cloud density which governs the potential donor or acceptor property of the molecule.

In compounds which form hydrogen bonds with electron donors the proton, because of its small size, can present a concentrated area of effective positive charge (low electron density) close to a donor molecule which has an area of high electron density. For example: chloroform has a low concentration of excess electrons spread over the three chlorine atoms leaving a concentrated deficit of electrons on the proton. The compound 1-nitropropane has an excess of electrons on the nitro group and a deficit of electrons on the α-methylene group while the remaining part of the alkyl chain is substantially neutral. The amino hydrogen atoms of primary and secondary amines act as acceptor sites while the nitrogen of tertiary amines act as donors. In aromatic compounds there can be an excess or deficit of π-electrons on each side of the ring depending on whether the substituent groups are electron repelling or attracting. In aromatic compounds both the π-electron atmosphere of the ring and the substituent groups can act as donor or acceptor sites. Steric hindrance may also play an important part in the availability of donor or acceptor sites. For example: the work of FITZGERALD[5,6] shows that 2,6-dimethylphenol has a larger retention volume than the close boiling 3-methylphenol on the donor-type phase diaminodiphenyl sulphone and 2,6-dimethylpyridine has a larger retention volume than the close boiling 3-methylpyridine on the acceptor-type phase tris-(2-cyanoethyl)-nitromethane.

THE FACTORS GOVERNING THE RETENTION VOLUME

Now let us consider the factors governing the retention volume. If ideal behaviour is assumed in the gas phase the partial pressure of a solute 1 above a solution in solvent 2 at temperature $T°$ K is given by:

$$p_1 = Py_1 = x_1\gamma_1 p°_1$$

where x_1 and y_1 are the mole fractions of solute 1 in the liquid and vapour respectively, P is the total pressure, $p°_1$ is the vapour pressure of solute 1 at T °K and γ_1 is the activity coefficient of component 1 (referred to the standard state of pure liquid solute at the same temperature and pressure). The partition coefficient K has been defined[3] as:

$$K = \frac{x_1}{y_1} \cdot \frac{n_s}{n_m} = \frac{P}{\gamma^\infty_1 p°_1} \cdot \frac{n_s}{n_m} = \frac{n_s RT}{\gamma^\infty_1 p°_1}$$

where n_s and n_m are the moles per ml in the stationary phase and mobile phase respectively and γ^∞ refers to infinite dilution. The specific retention volume per gram of stationary phase V_g is given by[3]:

$$V_g = \frac{K\ 273}{\rho_L\ T}$$

where ρ_L is the density of the stationary phase at the column temperature T. Then:

$$V_g = \frac{273\ R}{M_L} \cdot \frac{1}{\gamma^\infty_1 p°_1}$$

where M_L is the molecular weight of the stationary-phase liquid. The absolute retention volume V is given by:

$$V = \frac{NRT}{\gamma^{\infty}{}_1 p^{\circ}{}_1}$$

where N is the number of moles of stationary phase on the column and T is the temperature at which the volumetric flowrate is expressed.

For ideal solutions $\gamma^{\infty} = 1$ and V_g is inversely proportional to the vapour pressure of the compound. However, in gas chromatography there is usually a large molecular size difference between the volatile component and the stationary phase so, even when these are both normal paraffins, there is a small negative deviation from Raoult's law due to entropy effects. This is of the form[7]:

$$\log \gamma^{\infty} = D(n_1 - n_2)^2$$

where n_1 and n_2 are the number of carbon atoms in the solute and solvent and D is a constant having a negative value.

Many volatile compounds and stationary phases form non-ideal solutions so that V_g is controlled by both p° and γ^{∞} and for a given p°, V_g is controlled by the value of γ^{∞}.

The value of γ^{∞} is determined by the balance of intermolecular forces between the molecules of solute 1 and stationary-phase liquid 2. For solutions where excess entropy effects are small we have:

$$\log \gamma^{\infty} = k\,(E_{11} + E_{22} - 2E_{12})$$

where E is the energy of interaction between the molecules. High values of E may be due to dipole, induced dipole and electron donor–acceptor interactions. Let us consider three circumstances.

(1) E_{11} *predominant.* Here the solute has a high positive value of $\log \gamma$ and therefore a small retention volume relative to a compound of the same boiling point which forms an ideal solution. For example: associated compounds such as nitromethane and methanol on the non-polar phase Apiezon L. (This is equivalent to saying that at the high temperature and low concentration in the liquid phase these compounds are much less associated than in the pure liquid state and would therefore have a much higher "effective" p°.)

(2) E_{22} *predominant.* The solute has a high positive value of $\log \gamma$ and therefore a smaller retention volume than a compound of the same boiling point which forms an ideal solution, *e.g.* saturated hydrocarbons on a glycerol phase.

(3) E_{12} *predominant.* Here electron donor–acceptor interaction between the solute and solvent leads to a negative value of $\log \gamma$ and thus to long retention times, *e.g.* 1,1,2-trichloroethane on Reoplex 400 or primary amines on polyethylene glycol.

PIEROTTI *et al.*[8] have studied and correlated the value of γ^{∞} for a wide range of solutes and solvents.

The effects of these intermolecular interactions are often larger at lower temperatures.

CLASSIFICATION OF STATIONARY PHASES

An approximate measure of the electron donor or acceptor properties of a stationary phase can be obtained from the ratio of the retention volumes of two compounds of about the same boiling point, one an acceptor and one a donor. Table I shows examples of this method based on retention data measured at 125° C on a number of

TABLE I

ACCEPTOR/DONOR RETENTION RATIOS ON PHASES

*Phase**	*Ratio of retention volumes (125°C)*				
	$\frac{CHCl_3}{CCl_4}$	$\frac{CHCl_2\text{-}CH_2Cl}{Dioxane}$	$\frac{Cyclohexanol}{Cyclohexanone}$	$\frac{Pyrrole}{Pyridine}$	$\frac{Aniline}{NMe_2\text{-}aniline}$
REO	1.66	1.32	1.17	—	2.52
XF 1150	1.65	1.64	—	2.14	1.79
DGS	1.10	1.26	—	1.73	2.88
APL	0.665	1.74	0.981	0.676	0.48
m-Bis	0.890	1.32	—	0.862	0.70
ZONYL	0.925	0.595	—	0.435	0.91
FCP	0.874	0.572	0.622	0.700	0.705
QF 1	0.840	0.986	—	0.714	0.706
AROCLOR	0.636	0.720	—	0.705	0.507
TNB	1.25	0.568	0.490	—	1.01

* The phases are identified under Fig. 1.

phases using the apparatus and methods described by BROWN[9]. A high value of the ratio indicates a donor-type phase while a low value indicates an acceptor-type. The selectivity of a given phase for a particular separation has been determined by BAYER[10] who uses the ratio of retentions of two homologous series corrected to identical boiling point as a selectivity coefficient. A similar approach has been used by HUEBNER[11] who used the retention ratio of methanol to alkane to determine the "polarity index" of surface active agents.

The use of the V_g of only two compounds, donor and acceptor, to determine the properties of a stationary phases does not give much information about the relative polarity of the phase and does not permit one to distinguish readily between a non-polar phase and one which has both donor and acceptor properties. If, however, three test compounds are used, one non-polar, one an acceptor and one a donor it is possible to determine the relative polarity of the phase as well as its donor or acceptor potential. If V_n, V_a and V_d are the retention volumes of the non-polar, the acceptor and donor compounds on the phase we can calculate the "retention fractions".

$$F_n = V_n/(V_n + V_a + V_d)$$

and similarly F_a and F_d and these can be plotted on a triangular graph. The retention volumes of *n*-decane, 1,1,2-trichloroethane and dioxane were measured on a number of phases at 125° and the retention fractions calculated to give the classification of phases shown in Fig. 1. The non-polar phases are close to the *n*-decane apex and have high values of F_n and the polar phases have low values of F_n. The acceptor-type

phases are towards the dioxane apex and have a high value of F_d and a low value of F_a, while the donor-type phases are towards the trichloroethane apex and have a high value of F_a and a low value of F_d.

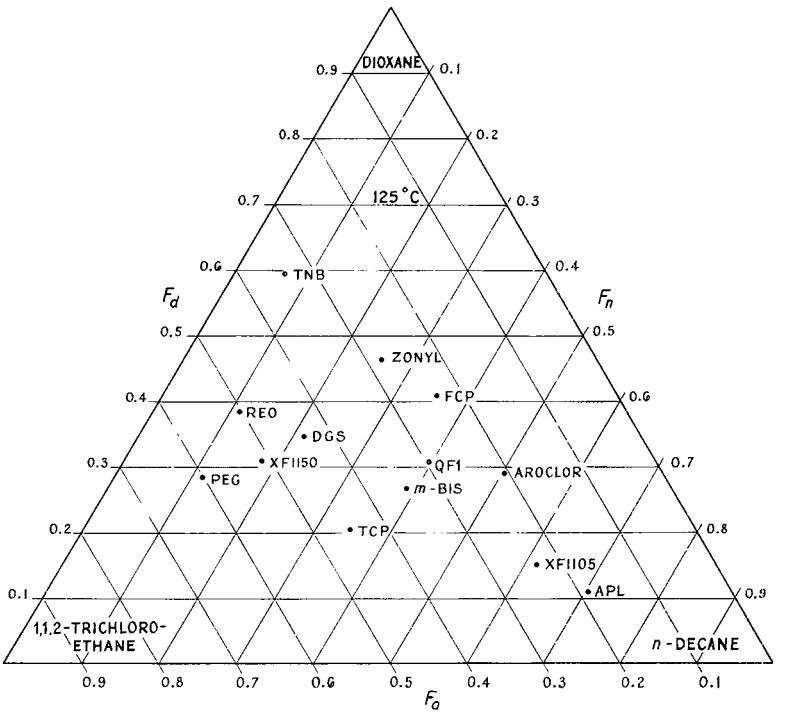

Fig. 1. Classification of stationary phases. F_n, F_a, F_d are retention fractions (see text) calculated from retention data measured at 125° by the author. The phases are: APL = Apiezon L (Metropolitan, Vickers); XF 1105, XF 1150 = cyanoethylated silicones (General Electric) with 5% and 50% cyanoethyl groups; AROCLOR = Aroclor 1262 (Monsanto) chlorinated diphenyl; QF 1 = fluorinated silicone (Applied Science Labs.); *m*-Bis = *m*-bis-(*m*-phenoxyphenoxy)-benzene (Eastman); TCP = tricresyl phosphate (Albright and Wilson); Zonyl = Zonyl E 7 (Du Pont) pyromellitic perfluoro ester; DGS = diethylene glycol succinate (Research Specialities Co.); PEG = polyethylene glycol 1500 (Carbide and Carbon); REO = Reoplex 400 (Geigy); TNB = 1,3,5-trinitrobenzene; FCP = diester of tetrachlorophthalic acid and 1-H,1-H,5-H-octafluoro-1-pentanol.

Similarly, using the retention data of McNAIR[12], TENNEY[13] and BROWN[9] for *n*-hexane, ethanol and 2-butanone at 100° C we have the classification of the phases shown in Fig. 2. The position on the triangular graph for a given phase is determined by the choice of the three test compounds and these can be varied to suit a particular problem. The "functional group retention ratio" to be described below can be used with advantage instead of the retention volume of a single compound in the classification of phases by this method.

The number of donor-type phases available to the gas chromatographer is adequate, but there are few strong acceptor-type phases suitable for use over a range of temperature. Many strong acceptor compounds have a rigid molecular structure which gives them melting points close to a temperature where they have an appreciable vapour pressure. The compound di-*n*-butyl tetrachlorophthalate is a useful

weak acceptor-type phase (Fig. 2) but it has sufficient alkyl content to give appreciable retention values for aliphatic hydrocarbons. If this compound could be modified by replacing the butyl groups by more strongly electron withdrawing non-hydrocarbon

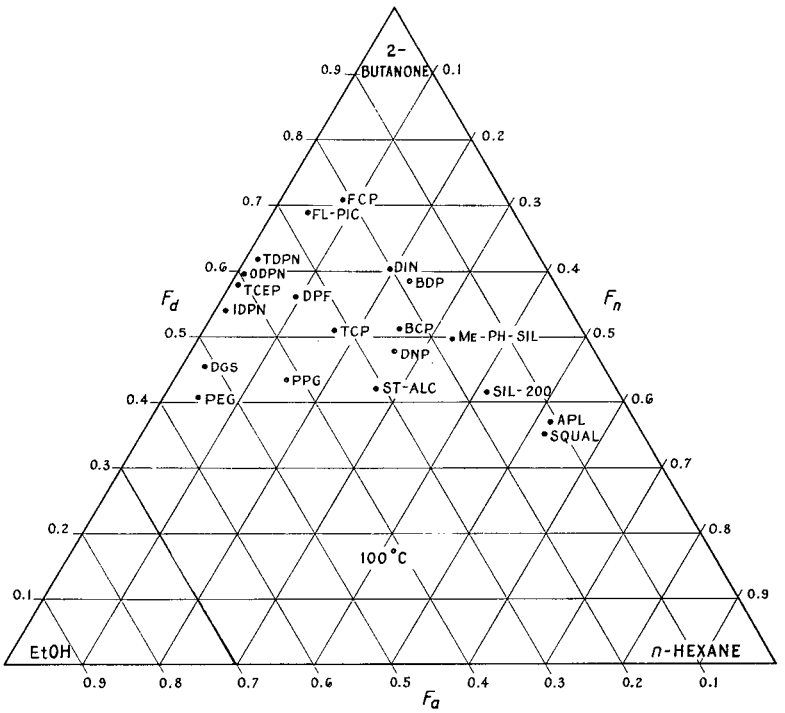

Fig. 2. Classification of stationary phases. F_n, F_a, F_d are retention fractions (see text) calculated from the retention data at 100°C and 67°C of BROWN[9], MCNAIR[12], TENNEY[13], and RAUPP[14]. The phases are: APL = Apiezon L; SQUAL = squalane; Sil-200 = Silicone DC-200; Me-Ph-Sil = methylphenyl silicone (General Electric 81705); St-alc = stearyl alcohol; DNP = dinonyl phthalate; BCP = di-*n*-butyl tetrachlorophthalate; BDP = benzyldiphenyl, DIN = di-*n*-octyl ester of 4,4-dinitrodiphenic acid; TCP = tricresyl phosphate; PEG = polyethylene glycol; PPG = polypropylene glycol; DGS = diethylene glycol succinate; DPF = diphenyl formamide; IDPN = iminodipropionitrile; ODPN = oxydipropionitrile; TDPN = thiodipropionitrile; TCEP = 1,2,3-tris-(2-cyanoethyl)-propane; FL-PIC = fluorene picrate; FCP = diester of tetrachlorophthalic acid and 1-H, 1-H, 5-H-octafluoro-1-pentanol.

groups a more polar and more strongly acceptor-type phase should be obtained. The diester of tetrachlorophthalic acid with 1-H,1-H,5-H-octafluoro-1-pentanol was made and on testing proved to be a much stronger acceptor than the butyl ester as can be seen from Fig. 2. This stable fluoroester is a liquid at room temperature and has a boiling point of 180° C/0.6 mm Hg.

IDENTIFICATION OF ORGANIC COMPOUNDS BY GAS CHROMATOGRAPHY

If we reverse the procedure just described for the classification of phases and run a number of compounds on three selected phases, one neutral, one an acceptor and one a donor and plot the three "affinity fractions" analogous to the "retention fractions"

on a triangular diagram we have a method for studying the donor–acceptor properties of the volatile compounds. In an earlier communication[15] the author proposed this method for the identification of compounds by gas chromatography. From Fig. 1 of the earlier communication and from Fig. 3 of this paper which is based on data of HORNING *et al.*[16] it can be seen that the position on the graph is governed by the relative strength of molecular interaction of the donor, acceptor and neutral parts of the

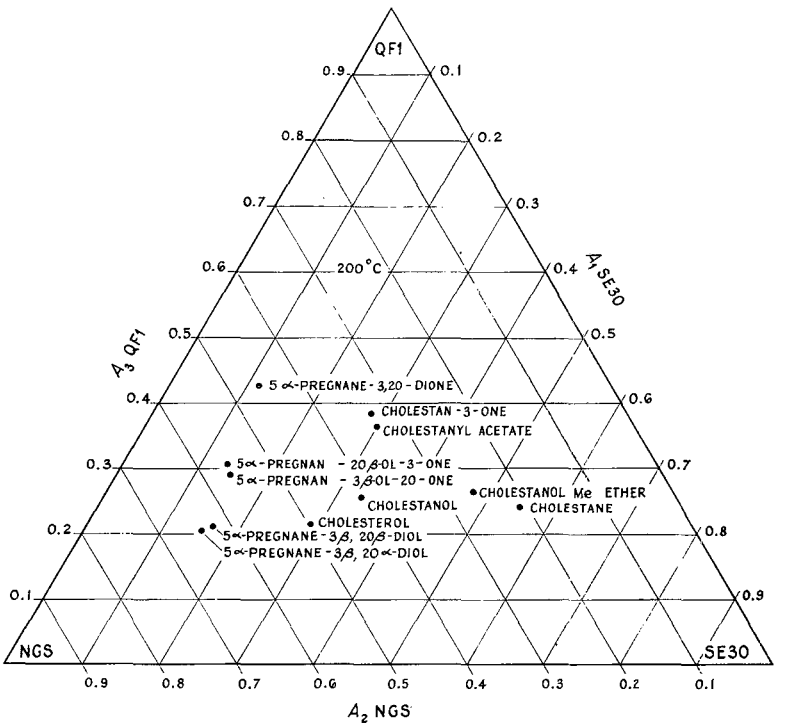

Fig. 3. Identification of steroids. A_1, A_2, A_3 affinity fractions (see BROWN[15]) from the data of HORNING *et al.*[16]. Phases: SE 30 = silicone; NGS = neopentyl glycol succinate; QF 1 = fluorinated silicone.

molecules with the stationary-phase liquids, the position of a given compound is fixed for 3 given phases and the position of a member of a homologous series depends on the length of the hydrocarbon chain.

The value of this method of identifying volatile compounds by gas chromatography would be improved considerably if the effect of hydrocarbon chain length on the retention volume could be eliminated and we could study the behaviour of the functional groups alone. This can be done by taking the ratio or log of the ratio of V_g of a compound RX containing the functional group X to the V_g of a suitable homomorph hydrocarbon, *e.g.* V_g *n*-hexanol/V_g *n*-hexane or nitrobenzene/benzene. For a homologous series of compounds containing the same functional group we should expect this to be a constant at a given temperature as the graphs of log V_g against carbon number for two homologous series are often almost parallel straight lines. Values of the "functional group retention ratio", V_g RX/V_g RH for a number of

different homologous series on various phases are shown in Table II. These were calculated from the data of BROWN[9], MCNAIR[12] and RAUPP[14] using extrapolation of the plot of V_g RH against carbon number to obtain values for V_g of the two lower hydrocarbon homomorphs. It can be seen that the functional group retention ratios are constant to within the accuracy of the experimental data. The choice of homomorph hydrocarbon can be made to suit the problem; for example, the homomorph

TABLE II

FUNCTIONAL GROUP RETENTION RATIOS

*Phase**	*DIN*	*APL*	*FL-PIC*	*DNP*	*SQUAL*	*ADOL-40*		
Temp.(C°)	*100*	*100*	*100*	*100*	*100*	*153*	*153*	*153*
Group	*> C = O*	*> C = O*	*–OH*	*–OH*	*–OH*	*–OH*	*–Br*	*–Cl*
Carbon No. 3	—	—	87	8.9	3.4	15.0	11.0	6.25
4	—	—	81	9.8	3.8	15.6	11.0	6.32
5	11.3	2.97	84	9.5	3.8	15.6	10.8	6.28
6	11.3	3.04	86	8.9	3.9	15.5	10.4	6.15
7	11.1	2.92	—	8.6	4.0	14.9	—	—
8	10.7	2.98	—	—	3.9	15.0	—	—

* These data were calculated from the data of BROWN[9], MCNAIR[12] and RAUPP[14]. The phases are identified under Fig. 2. Adol-40 is 9,10-octadecen-1,12-diol.

for 2-pentanone could be *n*-pentane or *n*-propane if the functional group is taken as $-CO-CH_3$, or for the study of the acceptor interactions of chloroform with donor phases the homomorph could be taken as fluorotrichloromethane. The best choice is probably the hydrocarbon having the same carbon skeleton or the same number of carbon atoms as the compound containing the functional group. In measuring these ratios for compounds containing functional groups which interact strongly with the stationary phase the retention volumes of the homomorph hydrocarbons may be too small to be measured with sufficient accuracy and it is recommended that these be determined from a log V_g against carbon number graph for the higher members of the series.

The functional group retention ratio for a few aromatic compounds has been determined by BORER[17] using benzene as the homomorph. A similar approach has been employed by CLAYTON[18] with steroids. EVANS AND SMITH[19] have used *n*-nonane as a universal homomorph and KOVATS[20] a more complicated function of retention volumes which he named the Retention Index and which EVANS AND SMITH[19] state is, "100 times the carbon number of a hypothetical hydrocarbon having the same retention as the unknown". SWOBODA[21] goes one step further and uses the difference between KOVATS' Index for a compound and for its homomorph. Another method for the identification of unknown compounds from retention data has been proposed recently by MERRITT AND WALSH[22] using the ratio of retention volumes of the unknown on two carefully chosen phases.

Let us return to the functional group retention ratio. The value of this ratio for a given homologous series is a measure of the interaction of the functional group with the stationary phase. In fact $-RT \ln RX/RH$ is the nett free energy change ΔG on the transfer of one mole of a compound RX from the vapour at one atmosphere

in the mobile phase to solution at a low given concentration in the stationary phase plus that for removal of one mole of the homomorph from the stationary phase to the mobile phase under the same conditions. Values of the functional group retention ratio or ΔG, measured on one or more stationary phases, are very useful for the identification of unknown compounds. This easily measured thermodynamic quantity which is a quantitative measure of functional group to phase interaction can be divided into its enthalpy and entropy terms by measuring its temperature dependence.

By using the values of the functional group retention ratio on three carefully selected phases it is possible to plot the group retention fractions derived from them on a triangular graph which shows only one point for each class of compounds. Fig. 4 is such a graph based on the data of BROWN[9], MCNAIR[12] and RAUPP[14]. This method can be employed for the identification of unknown compounds provided that a method is available for transforming the unknown compound into its unknown

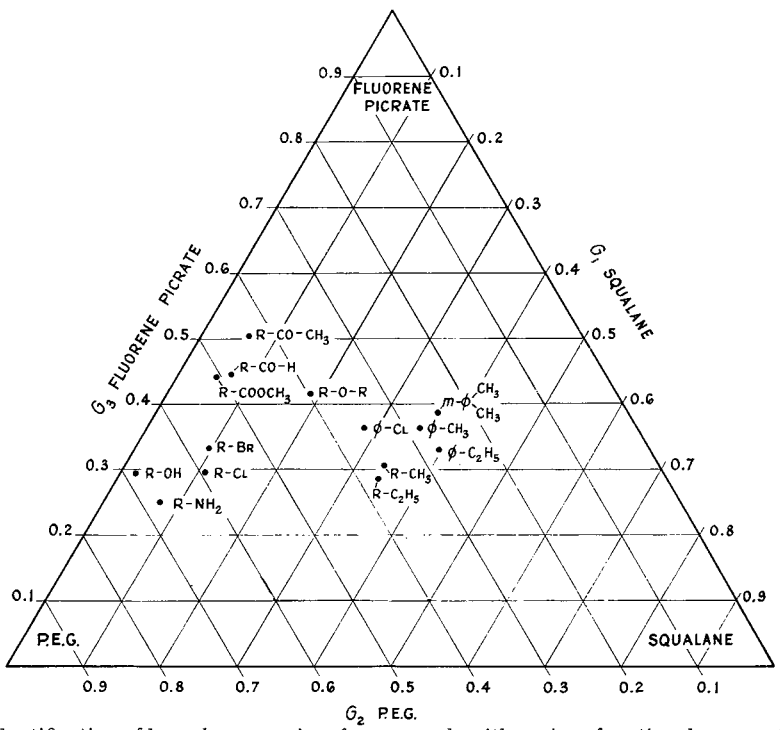

Fig. 4. Identification of homologous series of compounds with various functional groups. G_1, G_2, G_3 group retention fractions (see text) calculated from the data at 100° C of BROWN[9], MCNAIR[12] and RAUPP[14]; R = alkyl group; φ = phenyl group; PEG = polyethylene glycol.

homomorph so that this can also be gas chromatographed on the same three phases. This degradation has been done by micro vapour-phase hydrogenation for a number of oxygen, nitrogen and sulphur compounds by workers at the Bartlesville, U.S. Bureau of Mines Research Laboratories[23, 24].

When the donor–acceptor interaction between a polar solute and a polar phase is not too high we find that the free energy change $\Delta G = -RT \ln \mathrm{RX/RH}$ is a constant

for a given homologous series on a given phase over a range of column temperatures. However, if the specific donor–acceptor interaction is large there is a difference in orientational freedom on the phase between the polar compound and its homomorph leading to a change in entropy and this gives a variation of ΔG with temperature. For very strong interactions this may lead to variation in entropy and thus in ΔG with the length of the hydrocarbon part of the polar molecule.

SUMMARY

The molecular structure of volatile solutes and stationary-phase solvents, their molecular interactions, and the factors governing the magnitude of the retention volumes are briefly discussed.

A method is described for the classification of stationary phases into types according to their polarity and behaviour as electron donors or acceptors by measuring the retention volumes of three selected compounds on each phase.

A similar method is described for the identification of unknown compounds from their retention data on three carefully selected phases. This method uses the "functional group retention ratio", a quantitative measure of the interaction of the functional group in an homologous series of polar compounds with the stationary-phase liquids, to identify the functional group of the unknown compound.

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FLAME IONIZATION DETECTORS

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INTRODUCTION

One method of investigating food flavours involves the analysis of minute amounts of volatile materials in the air surrounding the food at its normal consumption temperature. Gas–liquid chromatography using flame ionization detection is a well-known method for this type of problem. This paper presents a discussion of the theoretical limits to the ultimate sensitivity and linearity of the flame ionization detector and describes methods for approaching these limits.

THE DIODE DETECTOR

(*a*) *Basis*

The flame ionization detector of McWILLIAM AND DEWAR[1] may be treated as a diode (Fig. 1a) with the collector as anode and the jet as cathode. To obtain the maximum linearity of the detector the current meter A should introduce negligible voltage drop in the circuit and have high sensitivity, negligible time constant and negligible drift. The usual method of measuring the ionization current is shown in Fig. 1b where the

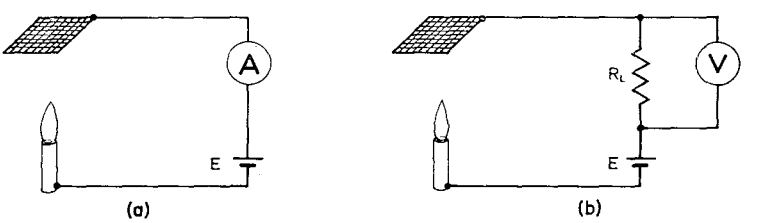

Fig. 1. Basic diode measuring systems.

voltage drop across the load resistor R_L is measured with a high impedance voltmeter V. Electrometer amplifiers are commonly used for this purpose. Resistor R_L introduces an appreciable voltage drop with consequent non-linearity.

(*b*) *Voltage drop and non-linearity*

The manner in which this non-linearity arises may be seen from Fig. 2 which shows the ionization current obtained for different flow rates of ethylene as a function of the

positive voltage applied to the anode of the detector. The sloping portion of the curves represents the region where the ion collection is not complete, while the horizontal plateau represents maximum collection efficiency. Load lines for $R_L = 0\,\Omega$, $10^9\,\Omega$, $10^{10}\,\Omega$ and $10^{11}\,\Omega$, are shown for an applied voltage E of 200 V. For $R_L = 10^{10}\,\Omega$ and an ethylene flow rate of 10^{-7} g/sec the voltage drop (RI) is 23 V and the voltage remaining across the detector ($E - RI$) is 177 V. Similarly, RI is 93 V and $E - RI$ is

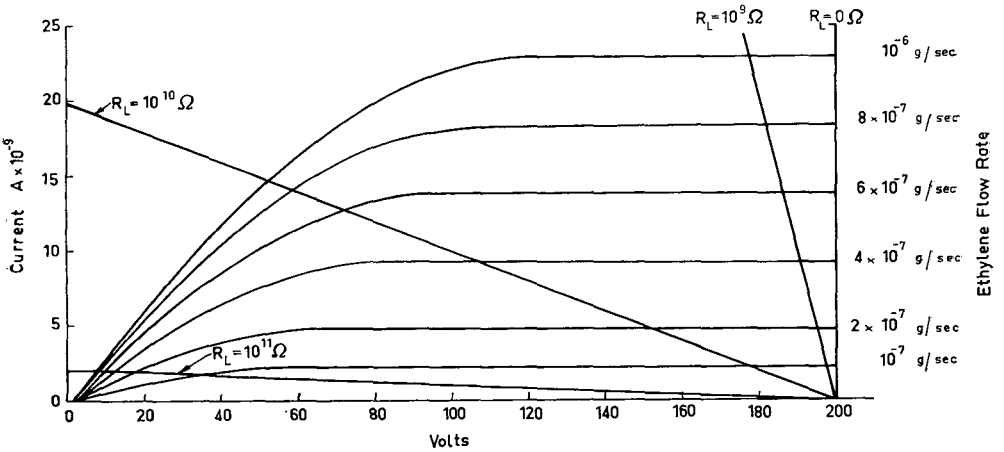

Fig. 2. Diode current curves.

107 V for an ethylene flow rate of $4\cdot10^{-7}$ g/sec. From Fig. 2 it can be seen that the voltage drop across the load resistor is proportional to the ethylene flow rate only if the load line intercepts the horizontal portion of the appropriate curve. As soon as the voltage across the detector falls below that required for complete collection of ions the output of the detector becomes non-linear. The linear region extends up to $5\cdot10^{-8}$ g/sec with $R_L = 10^{11}\,\Omega$, $5\cdot10^{-7}$ g/sec with $R_L = 10^{10}\,\Omega$ and to about $3\cdot10^{-6}$ g/sec with $R_L = 10^9\,\Omega$. At $R_L = 0\,\Omega$ the linearity extends to about 10^{-5} g/sec which is the limit of the inherent linearity of the detector for an applied voltage of 200 V.

In selecting a load resistor, a compromise must be made between the conflicting requirements of output voltage, which increases with increasing resistance, and linearity which decreases with increasing resistance. This difficulty is avoided if the applied voltage E is arranged to vary in such a manner that it always cancels the effect of the voltage drop in R_L. This is shown in Fig. 3 where an additional voltage E_1 is continuously adjusted to be equal to the voltage drop RI. Under these conditions the potential across the diode remains equal to E, independent of the ionization current. The effective load line then becomes $R_L = 0\,\Omega$, independent of the actual value of R_L. This permits a large output voltage to be obtained without this voltage being subtracted from the potential being applied across the diode.

Before discussing how this voltage E_1 is obtained in practice, it is necessary to consider the properties of a perfect unity gain amplifier (P.U.G.A.). Fig. 4a shows a diagrammatic representation of a P.U.G.A. Its three principal properties are (i) the ratio of output voltage to input voltage is very close to, but less than, unity (about 0.999), (ii) the input resistance is so high that the input current is negligible, (iii) the

output impedance is negligible. If a P.U.G.A. is connected as in Fig. 4b, the effect produced is the same as shown in Fig. 3. The output voltage V of the P.U.G.A. is equal to the input voltage $V = RI$ and is connected so as to oppose it; consequently the output voltage is equivalent to E_1 of Fig. 3 and the voltage across the diode will remain at E volts. Note that any point on this circuit may be earthed. A typical

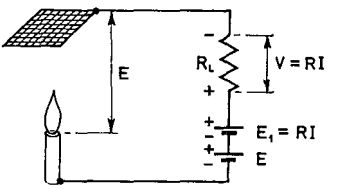

Fig. 3. Constant potential diode.

practical circuit employing this principle is shown in Fig. 4c. The earth point chosen is at one side of the P.U.G.A., so that with no ionization current the anode is at earth potential; as the current increases, the anode and cathode simultaneously move away from earth potential by the same amount *viz.* RI.

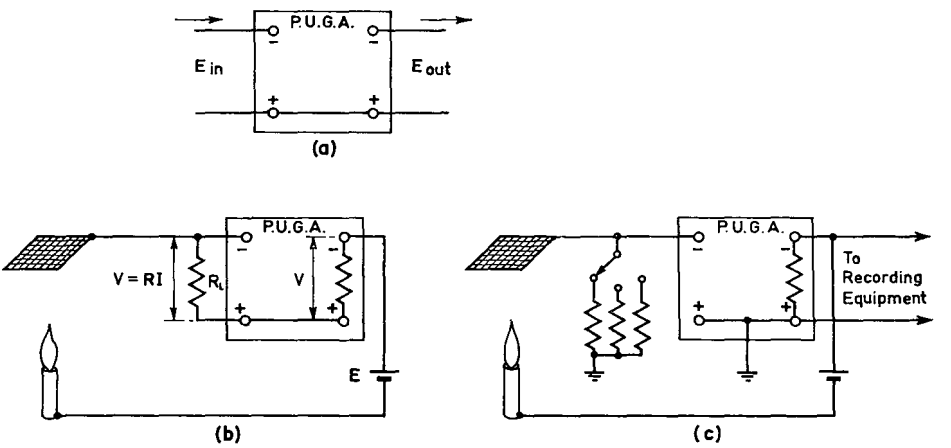

Fig. 4. Constant potential diode.

(*c*) *Time constant*

The connection from the anode of the detector to the input of the P.U.G.A. (or in fact, any amplifier or electrometer) has a capacitance C to earth. It is usual to use a coaxial cable for this connection and a typical value of C for 60 cm of cable is 40 pF ($40 \cdot 10^{-12}$ F). If there is an increase in the ionization current this capacitor charges up. The time constant τ, which is the time required to charge this capacitor to 63% of its final value, is given by $\tau = R_L C$. If $R_L = 10^{10}\,\Omega$ and $C = 40 \cdot 10^{-12}$ F then $\tau = 0.4$ sec; for $R_L = 10^{11}\,\Omega$, $\tau = 4$ sec; and for $R_L = 10^{12}\,\Omega$, $\tau = 40$ sec. The time required to charge to 99% of final value is about 5 τ so that the use of high value resistors, even with low capacitance cable, leads to inadmissibly long time constants. The time

constant of the input circuit can be reduced by a large factor if the shield of the coaxial cable is connected not to earth, but to the output of the P.U.G.A., as in Fig. 5. Now, the effective capacitance $C_{eff} = C$ (1 — gain) and for a gain of 0.9996*, $C_{eff} = 0.0004\,C$. Hence $\tau = 0.2$ msec for $R_L = 10^{10}\,\Omega$, $\tau = 2$ msec for $R_L = 10^{11}\,\Omega$ and $\tau = 20$ msec for $R_L = 10^{12}\,\Omega$. Since the cathode of the diode is also connected to the output through battery E the anode–cathode capacitance is also reduced by a factor of 0.0004.

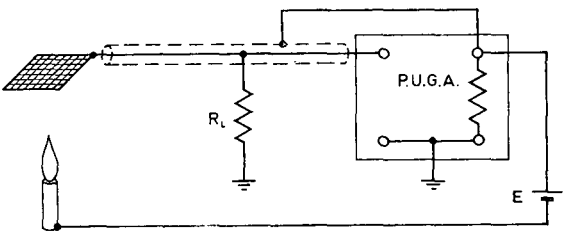

Fig. 5. Reduction of time constant; cancellation of cable capacitance.

If the self capacitance of resistor R_L is not negligible, it is also reduced by a factor of 0.0004 by returning it to the output of the P.U.G.A. However, the effective resistance is now given by $R_{eff} = R_L\,(1 - \text{gain})^{-1} = 2500\,R_L$, so to obtain an effective input resistance of $10^{12}\,\Omega$ an actual resistor of $4 \cdot 10^8\,\Omega$ is used.

(d) Drift

The design of the P.U.G.A. employs a considerable amount of negative feedback, which means high stability can be achieved and drift rates can be as low as 2 % of full scale per hour, which is adequate for many gas chromatographic applications.

(e) Limit of detection of diode

The sources of noise which set the final limit of detection of the diode are:

(1) *Chemical noise in the flame.* This arises from stationary phase eluting from the column and from impurities present in the carrier gas, hydrogen and air.

(2) *Physical noise in the flame.* The ionization current consists of discrete charged particles which arrive at the anode in a random fashion, producing what is known as "shot" noise. The root mean square value of the shot noise current is given by $i_{RMS} = \sqrt{3.2 \cdot 10^{-19}\,I\,\tau^{-1}}$ A where I = D.C. current in A and τ = time constant in sec. The noise voltage produced across the load resistor is $E_{RMS} = R_L \sqrt{3.2 \cdot 10^{-19}\,I\,\tau^{-1}}$ V. For $R_L = 10^{12}\,\Omega$, $\tau = 1$ sec and $I = 10^{-11}$ A (typical value of the ionization current produced by commercially pure gases), $E_{RMS} = 1800\ \mu$V approximately.

(3) *Physical noise in the load resistor.* At room temperature the Johnson noise in the load resistor is given by $e_{RMS} = 1.29 \cdot 10^{-10} \sqrt{R_L\,\tau^{-1}}$ V. For $R_L = 10^{12}\,\Omega$ and $\tau = 1$ sec, $e_{RMS} = 129\ \mu$V.

(4) *Amplifier noise.* Noise is generated in the first valve of the P.U.G.A. (or any amplifier) due to grid and plate currents. With careful design this noise can be kept to about 300 μV (R.M.S.).

If chemical noise is disregarded, shot noise sets the limit of detection. For a

* See "Measurements", section (*f*).

background current of 10^{-11} A the corresponding shot noise current is $1.8 \cdot 10^{-15}$ A (R.M.S.) which corresponds to an ethylene flow rate of 10^{-13} g/sec approx. If a signal to noise ratio of unity is selected the limit of detection would be 10^{-13} g/sec of ethylene.

In practice, using commercially pure hydrogen and nitrogen, it is possible to attain such a background current. If further purification of these gases reduces this background current to 10^{-12} A the shot noise only decreases by $\sqrt{10}$ and the limit of detection is then $3 \cdot 10^{-14}$ g/sec of ethylene. It seems unlikely that the ionization current can be reduced much below 10^{-13} A, which indicates a theoretical limit of detection of 10^{-14} g/sec of ethylene.

(f) Measurements on the diode

To determine the optimum detector operating conditions, systematic changes in each of the dimensions and flow rates have been made using a Keithley Model 150A Micromicroammeter connected to a high speed X-Y recorder. This method eliminates the drifts in these variables which can occur between manual observations. A stainless steel tube 0.5 m long × 4.5 mm bore packed with 40–60 acid washed celite is used as a flow resistance between the sample introduction and the detector. Nitrogen (50 ml/min) is passed through the column and ethylene is metered into it as required. Hydrogen (50 ml/min) is added to the column effluent and the mixture burns at a tapered platinum–20 % rhodium jet having an 0.325 mm orifice (see Fig. 8). The anode consists of a disc of 1 cm dia. platinum gauze (48 mesh) situated 1 cm above the jet. An airflow of 1 l/min is maintained through the detector. A load resistor of 10^{12} Ω is used and the detector output signal is connected via a guarded coaxial connector to a Halex 302E Electrosensor* used as a P.U.G.A. This P.U.G.A. has a gain of 0.9996, input impedance of 10^{17} Ω and output impedance of 0.3 Ω. The output of the P.U.G.A. is attenuated and then recorded with a Speedomax Model G, one second recorder**. Using commercially pure gases a background ionization current of 10^{-11} A is obtained, the associated noise being 10^{-14} A (R.M.S.). Since the shot noise for 10^{-11} A is only $1.8 \cdot 10^{-15}$ A (R.M.S.) it appears that the chemical noise in the flame is the limiting factor setting the present practical limit of detection at $5 \cdot 10^{-13}$ g/sec of ethylene. The drift rate under these conditions is the equivalent of 10^{-11} g/sec ethylene/h.

THE FLAME IONIZATION TRIODE

(a) Basis

To reduce the drift rate of the D.C. amplifying system associated with the diode, an A.C. system and the flame ionization triode have been developed[2].

Fig. 6a shows the basic triode in the grounded cathode configuration. An alternating voltage is applied between the grid and the cathode and the resultant alternating plate current flows through load resistor R_L producing an alternating voltage at the output; R_L has values similar to those in the D.C. system. The time constant of the input circuit must be small compared with the time for one cycle of the A.C. used, so capacitance effects are of great importance. Fig. 6a shows the capacitances associated with the components of the detector. Since the grid has a large A.C. signal on it, some A.C. will be fed directly to the anode via the large capacitance C_2. To

* Halex Inc., California, U.S.A.
** Leeds and Northrup Co., Philadelphia, Pa., U.S.A.

avoid this the grounded grid configuration of Fig. 6b is used, in which the A.C. is applied to the cathode and the feedthrough to the plate is via C_1. The screening effect of the earthed grid makes C_1 several orders less than C_2. Since the capacitance C_3 is directly across the transformer, it has no effect on the system. However, as C_1 is between anode and earth this adds to the stray capacitances which, in association with R_L, set the time constant of the system. For convenience 50 c/s is chosen as the

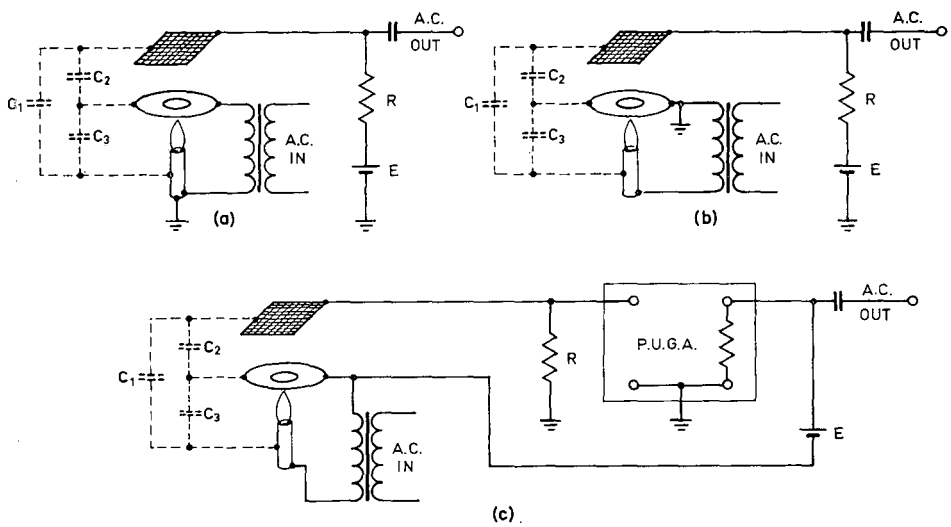

Fig. 6. Flame ionization triode.

operating frequency, so the time constant must be less than 10 msec, consequently capacitance reduction must be employed. Fig. 6c shows how the P.U.G.A. may be used to keep the grid-anode voltage constant and hence reduce the effective value of C_2 to about 10^{-2} pF. C_1 is already reduced to 10^{-3} pF by the presence of the grid.

The A.C. output of the P.U.G.A. is fed through an attenuator to a combined A.C. amplifier and phase-sensitive detector (Fig. 7). The amplifier follows normal practice; 0.05 μF capacitors tune the signal and reference transformers to 50 c/s. The neon Ne2 conducts as soon as the voltage across the signal transformer exceeds 60 V. By this means the D.C. output to the 10 mV recorder is linear to about 14 mV but is sharply limited at 15 mV. A Bristol Syncroverter used as a phase-sensitive detector has negligible drift compared with valve and transistor systems. Any residual feedthrough via the cathode-plate capacitance is removed by applying an out-of-phase voltage to C_9; however, this capacitance is kept small since its use increases the input time constant.

(*b*) *Linearity*

The considerations of the inherent linearity of the diode detector apply also to the triode and Fig. 6c may be considered as the A.C. equivalent of Fig. 4c. The effective load resistor R_L is zero, independent of the actual value of R_L.

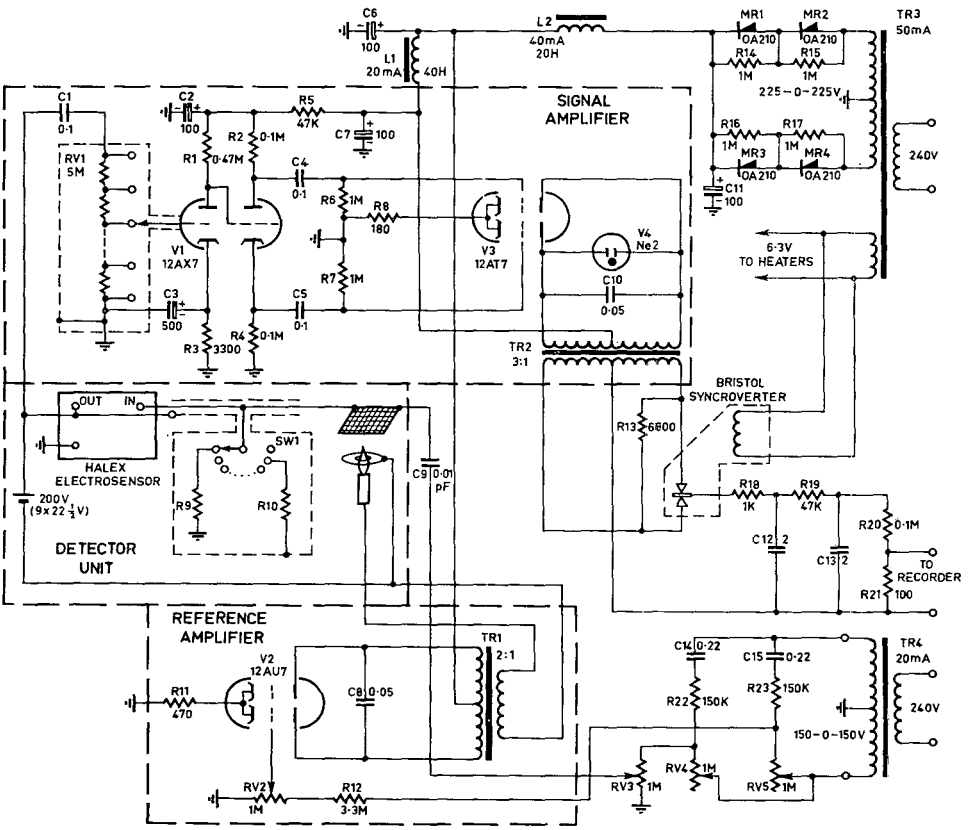

Fig. 7. Amplifier for flame ionization triode.

(c) Time constant

The time constant associated with the input to the P.U.G.A. sets a limit to the maximum frequency at which the system can be operated. For a total effective input capacitance of 10^{-2} pF and a load resistor of $10^{12}\,\Omega$ the input time constant is 10^{-2} sec which permits the use of frequencies up to 50 c/s. The time constant of the overall system is set by the integration time of the R.C. network following the phase-sensitive detector (Fig. 7). To provide satisfactory integration of the output of the phase-sensitive detector this time constant is somewhat greater than 0.2 sec.

(d) Drift

As the D.C. output voltage is independent of the D.C. conditions of the P.U.G.A., the major source of drift is eliminated. Some long-term drifts are caused by changes in the feedthrough capacitances due to changes in dimensions of the detector; variations in the cancelling voltage can also occur.

(e) Limit of detection of triode

The sources of noise in the triode are the same as those in the diode and similar considerations apply to their magnitudes. For an overall time constant of 1 sec, the

shot noise is 1800 μV (R.M.S.) for a background current of 10^{-11} A. The magnitude of this noise can be reduced by increasing the integration time of the network after the phase-sensitive detector but only at the cost of increased time constant of the whole system. For equivalent time constants the diode and triode detectors have the same limit of detection.

(f) Measurements on the triode

Fig. 8 is a diagram of the flame ionization triode used in these measurements. It consists of the basic diode with the addition of an annular grid. An analysis of vari-

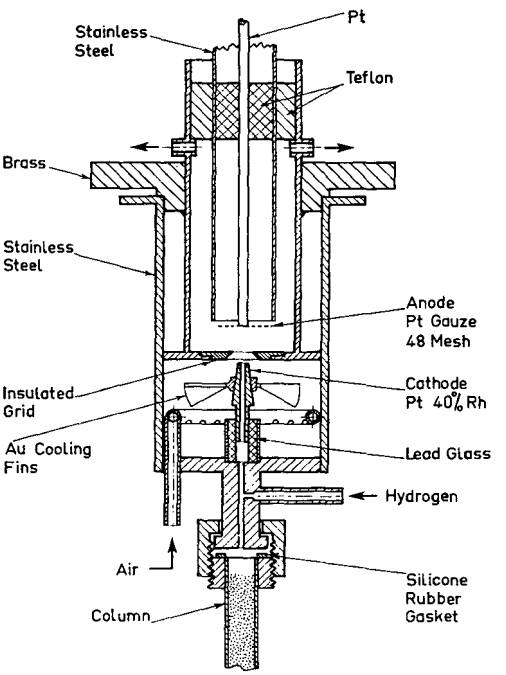

Fig. 8. Flame ionization triode.

ables, similar to that undertaken for the diode, has shown that optimum conditions for the diode and triode are similar. The size and position of the grid are shown in Fig. 8.

The drift rate (with a $10^{12}\,\Omega$ load resistor) is less than 10 μV/h, which is equivalent to 10^{-15} g/sec ethylene/h and is an improvement of four orders of magnitude over the diode. The electronic noise is again about 300 μV (R.M.S.) which is negligible as compared with the shot noise (1800 μV) in the background ionization current. The overall electronic noise is negligible compared with chemical noise in the flame.

CONCLUSION

Using the methods and circuits which have been described it is possible to approach the inherent limits of linearity and detection of both the diode and triode flame ionization detectors.

Linearity over a range of 10^8:1 is possible. The major theoretical noise contribution is due to the shot effect in the background ionization current of the hydrogen flame. For a background current of 10^{-11} A, obtained from commercially pure gases, the theoretical limit of detection is 10^{-13} g/sec of ethylene using a bandwidth of 1 c/s. Chemical noise due to impurities in the gases used sets the present limit of detection at about $5 \cdot 10^{-13}$ g/sec of ethylene.

The diode and the triode detectors have the same noise limitations but the drift of the diode D.C. system is approximately 100 times its theoretical noise level (peak to peak) per hour, whereas the drift of the triode is negligible. The drift rate produced by stationary phase elution from some types of columns can be greater than that due to the diode electronic system and, under these circumstances, there is little to be gained by using the triode system.

SUMMARY

Methods are described for improving the performance of the amplifiers associated with the diode and triode flame ionization detectors. The effect of these amplifiers on linearity, time constant and drift rate is discussed together with the theoretical and practical limitations of detection.

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GAS CHROMATOGRAPHY OF VINYL KETONES

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INTRODUCTION

During the characterization of vinyl *n*-amyl ketone[1], a compound responsible for metallic flavour in dairy products, it was noticed that its retention behaviour in gas chromatography was anomalous. It had been expected that differences in retention between vinyl *n*-amyl ketone and ethyl *n*-amyl ketone would parallel those between *n*-oct-2-enal and *n*-octanal as the two compounds of each pair have corresponding differences in structure. This was not observed. Consequently, a study of the retention behaviour of vinyl ketones and alcohols was undertaken using stationary phases of various polarities. The investigation was extended to include some other classes of carbonyl compounds and alcohols.

Several catalogues of retention data have been published[2–5] and various methods of presentation of data have been suggested including retention indices[3], functional retention indices[6], retention relative to theoretical nonane[7] and group retention factors[8,9]. All these concepts are inter-related and each may be expressed in terms of any other. The data obtained in this work are expressed as group retention factors. The group retention factor (K) for a functional group (X) is defined by:

$$K_X = R_A/R_B \qquad (1)$$

where R_A, R_B are retention times of A and B and A and B differ only in that B contains group X in place of the appropriate hydrogen atoms.

EXPERIMENTAL

Apparatus and procedure

Apiezon M, silicone oil (May & Baker, Embaphase), Carbowax 4000, diethylene glycol succinate (Applied Science Laboratories, Inc., State College, Pa.) and fluorosilicone QF1 (Applied Science Laboratories) were used as stationary phases. The support was 100–140 mesh Gas Chrom A (Applied Science Laboratories). Stationary phase (20 parts by weight), dissolved in just sufficient solvent to wet the support, was mixed with support (80 parts by weight) and the solvent removed at 20° and 15 mm pressure using a rotary evaporator. The dry packing was then evacuated at 50° and 0.05 mm pressure. Glass columns (185 cm long by 0.4 cm i.d.) were vibrated while portions of packing were added. Chromatograms were obtained at 70°, 100° and 130° using a Perkin-Elmer 154D Fractometer with a thermistor detector and helium as carrier gas. A carrier gas pressure of 10 p.s.i. was used to give flow-rates of 35–75 ml/min. Samples (less than 100 μg per component) were introduced with a Hamilton

TABLE

GROUP RETENTION FACTORS

	Apiezon M						*Embaphase silicone*					
	70°		100°		130°		70°		100°		130°	
	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}
Alkanal	3.85	3.90	3.23	3.20	2.66	2.72	3.82	4.14	3.17	3.22	2.78	2.70
Alkan-2-one	3.48	3.22	2.86	2.84	2.35	2.42	3.54	3.56	2.98	2.99	2.60	2.54
Alkan-3-one	3.42	3.20	2.86	2.84	2.35	2.42	3.51	3.50	2.87	2.86	2.49	2.48
sym-Alkanone	3.00	2.78	2.57	2.52	2.23	2.30	3.24	3.14	2.74	2.66	2.40	2.32
Alk-1-en-3-one	3.22	2.94	2.73	2.64	2.33	2.40	3.41	3.35	2.74	2.94	2.38	2.43
Alk-1-en-3-ol	3.22	2.94	2.73	2.63	2.33	2.40	3.43	3.65	2.78	3.05	2.38	2.43
Alk-2-enal	6.10	—*	4.65	4.77	3.54	4.04	5.66	5.90	4.29	4.64	3.66	3.80
Alkan-1-ol	6.38	7.10	4.88	5.00	3.61	3.96	6.40	7.15	4.90	5.26	3.82	3.97
V_g (ml/g)	20.9	3560	11.6	903	6.85	251	20.6	1810	10.8	480	6.07	147

* The large retention of these compounds could not be determined with the small samples available.

1-μl syringe. Mixtures of homologous series of C_{4-10} *n*-alk-1-en-3-ones (vinyl ketones), C_{6-10} *n*-alk-1-en-3-ols (vinyl alcohols), C_{3-12} *n*-alkanals, C_{3-11} *n*-alkan-2-ones, C_{4-12} *n*-alkan-3-ones, C_{7-11} *sym-n*-alkanones, C_{6-9} *n*-alk-2-enals, C_{2-9} *n*-alkan-1-ols and C_{5-12} *n*-alkanes were examined together with *n*-hexa-2,4-dienal, *n*-nona-2,6-dienal, *n*-hex-3-en-1-ol, *n*-hept-3-en-1-ol, *n*-hex-1-en-5-one and a number of diones and cyclic ketones. Different chromatograms were related by using an internal standard.

Calculation of group retention factors (K)

Retention distances were measured from the air peak to the peak maximum and log retention distance was plotted against carbon number. The best straight line was drawn through the experimental points for an homologous series and retention distances read off at C_5 and C_{11}. Division by the corresponding values for the *n*-alkanes gave the group retention factors.

RESULTS

The C_5 and lower ketones were retained by the stationary phases too long for their retention distances to fit the log plot. This fact, also noticed by KOVATS[3], suggests that the carbonyl group is less shielded by the hydrocarbon chain and interacts more strongly with the stationary phase. Thus, the values of K at C_5 and C_{11} (Table I)* represent the effective range of carbon number which has been studied. Variation between the C_5 and C_{11} values for a particular series arises from a slight difference in the slopes of the log plots for that series and the *n*-alkanes.

Table III shows the group retention factors for an isolated double bond (by comparison of the pairs hex-3-enol/hexanol, hept-3-enol/heptanol, nona-2,6-dienal/non-2-enal, hex-1-en-5-one/hexan-2-one), a vinyl double bond (by comparison of alk-1-en-3-one/alkan-3-one pairs) and a double bond conjugated to a carbonyl group (by comparison of alk-2-enal/alkanal pairs).

Insufficient diones and cyclic ketones were examined to allow their representation in Table I.

* The complete data, from which Table I is constructed, are available from the author.

I

RELATIVE TO ALKANES

Diethylene glycol succinate						*QF 1*						*Carbowax 4000*					
70°		100°		130°		70°		100°		130°		70°		100°		130°	
C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}	C_5	C_{11}
32.4	36.2	28.5	25.2	17.3	17.0	14.9	20.8	12.9	11.9	7.66	8.50	27.2	28.8	16.0	18.6	10.5	11.6
36.1	38.6	30.4	26.1	17.9	17.5	18.4	22.3	13.6	12.6	8.50	9.21	26.2	27.0	15.5	17.5	10.1	10.9
29.8	30.1	24.4	21.1	15.9	14.5	17.9	18.4	11.5	10.8	7.19	7.95	21.6	22.1	13.7	14.5	9.26	9.76
24.8	25.2	20.6	17.7	14.2	12.3	14.2	16.0	10.5	9.67	3.82	4.21	18.4	18.3	11.5	11.6	8.29	8.06
41.0	43.3	34.3	29.8	20.9	18.4	14.2	16.0	11.5	10.8	6.73	7.59	29.4	30.4	17.3	19.5	11.0	12.1
74.5	89.5	57.5	50.5	30.2	27.2	6.80	8.04	6.35	5.87	3.82	4.21	76.4	93.4	35.5	40.5	19.0	21.0
90.5	101	70.0	63.2	37.4	35.7	34.4	42.5	25.6	23.8	13.7	15.2	64.0	—*	35.5	—*	20.0	22.9
127	158	83.5	80.6	40.7	42.8	13.3	21.4	10.5	10.1	6.37	7.25	141	196	63.7	86.0	30.0	38.4
2.59	65.9	1.32	24.4	105	11.1	7.30	267	4.11	97.6	2.79	34.3	4.63	234	3.03	80.4	2.13	33.7

DISCUSSION

Utility of group retention factors

Table I shows that the variation of K from C_5 to C_{11} is about 10 %. However, values of K for any particular carbon number may be reproduced with an accuracy of ± 1 %. Table II shows values of K calculated from published retention data for Apiezon L. It can be seen that there is good agreement with the present results obtained with Apiezon M except for *n*-alkan-3-ones. Thus K can readily be used for the prediction

TABLE II

GROUP RETENTION FACTORS CALCULATED FROM THE LITERATURE[3,4]

(Apiezon L at 130°)

Series	K_{C_5}	$K_{C_{11}}$*
Alkanal	2.66	2.72
Alkan-2-one	2.37	2.41
Alkan-3-one	2.47	2.43
sym.-Alkanone	2.22	2.19
Alkan-1-ol	3.76	3.80
Alk-1-ene	0.88	0.90

* Values at C_{11} or the nearest carbon numbers.

of retention times or as an aid in identification of an unknown compound if its carbon skeleton is known. In many cases the carbon skeleton of a compound, isolated by gas chromatography, may be determined by microhydrogenation to the parent hydrocarbon[6,10]. Provided there is no interaction between two functional groups in the same molecule, their effect on its retention may be predicted by successive applications of eqn. (1). For the substitution of groups X and Y in A to give C, the retention time of C (R_C) is given by:

$$R_C = K_X K_Y R_A \qquad (2)$$

Both CLAYTON[9] and EVANS AND SMITH[11] (using an equivalent expression) have been able to predict retention times in this manner.

TABLE III

RETENTION FACTORS FOR DOUBLE BONDS

		Apiezon M			*Embaphase silicone*			*Diethylene glycol succinate*			*QF 1*			*Carbowax 4000*		
		70°	*100°*	*130°*	*70°*	*100°*	*130°*	*70°*	*100°*	*130°*	*70°*	*100°*	*130°*	*70°*	*100°*	*130°*
Hex-3-en-1-ol/hexan-1-ol	*trans*	0.84	0.85	0.89	0.93	0.94	0.88	1.21	1.21	1.19	0.90	0.88	0.96	—	1.15	1.10
	cis							1.37	1.38	1.32				—	1.21	1.18
Hept-3-en-1-ol/heptan-1-ol	*trans*	0.79	0.81	0.86	0.89	0.90	0.87	1.15	1.15	1.11	0.83	0.87	0.94	—	1.09	1.02
	cis							1.36	1.35	1.29				—	1.23	1.17
Nona-2,6-dienal/non-2-enal	*trans-trans*	—	0.88	0.96	0.92	0.93	0.91	1.14	1.15	1.25	—	0.87	0.94	—	—	1.16
	trans-cis							1.48	1.49	1.38	—	0.93	1.00	—	—	1.25
Hex-1-en-5-one/hexan-2-one		0.90	0.92	0.98	0.90	0.89	0.78	1.56	1.48	1.40	0.90	0.97	0.92	—	1.24	1.26
Alk-1-en-3-one/alkan-3-one	C 5	0.94	0.95	0.99	0.97	0.96	0.96	1.38	1.41	1.32	0.79	1.00	0.94	1.36	1.26	1.20
	C 11	0.92	0.93	0.99	0.96	1.03	0.98	1.44	1.41	1.27	0.87	1.00	0.95	1.37	1.35	1.24
Alk-2-enal/alkanal	C 5	1.58	1.44	1.33	1.48	1.36	1.32	2.80	2.46	2.16	2.30	1.98	1.80	2.35	2.22	1.99
	C 11	—	1.49	1.48	1.43	1.44	1.47	2.79	2.50	2.10	2.04	2.00	1.79	—	—	1.97

Retention of vinyl ketones

Table III shows that the vinyl double bond, although conjugated, contributes to the overall retention of an alk-1-en-3-one as if it were an isolated rather than a conjugated double bond. The values of K for a double bond in the carbonyl compounds studied with Apiezon M agree well with the values for the *n*-alk-1-enes shown in Table II. Thus, in solution in a gas-chromatographic stationary phase, there is little interaction between the vinyl double bond and the carbonyl group and retention times may be predicted by use of eqn. (2).

A similar comparison was not made for the vinyl alcohols as samples of the alkan-3-ol series were not available. Nevertheless, it is interesting to note that, although the alkanal and alkan-1-ol series are completely separated on all five phases, the vinyl ketones and alcohols can only be separated with the three polar stationary phases.

Choice of stationary phase

Gas chromatography is often used as a qualitative analytical technique for the separation of mixtures following which the individual components may be identified by a combination of retention data with the results of other physical and chemical measurements. Thus, the choice of stationary phase will be dictated by the types of compounds likely to be present, the separation required and the need to avoid contamination which may complicate the interpretation of further measurements, particularly spectral examination.

(*a*) *Separation of geometrical isomers.* Retention times of the *cis*- and *trans*-isomers of the 3-enols or 2-*trans*, 6-*cis*- and 2-*trans*, 6-*trans*-nonadienals are slightly different on all five stationary phases. The *trans*-isomer is eluted before the *cis*-isomer on polar phases whereas the reverse is true for the non-polar phases. However, a mixture of *cis*- and *trans*-isomers gives separate peaks only with the more polar diethylene glycol succinate and Carbowax 4000. (QF 1 gives separate peaks for the nona-2,6-dienals but not the alk-3-enols.)

In practice, conclusive differentiation of *cis*- and *trans*-isomers is obtained by infra-red spectroscopy. For this, Carbowax 4000 is the less suitable stationary phase as it frequently gives off low molecular weight "bleed". Furthermore, retention times on Carbowax 4000 are generally longer than on diethylene glycol succinate or QF 1. For separation in a fixed time, higher flow-rates or higher temperatures are necessary with Carbowax 4000. The former causes difficulties in trapping the eluted material and the latter increases contamination and decreases resolution. Thus on all counts the preferred stationary phase for separation of geometric isomers is diethylene glycol succinate.

(*b*) *Separation of positional isomers.* Table I shows that the order of elution on all five stationary phases is alkane, *sym.*-alkanone, alkan-3-one, and alkan-2-one. This is to be expected as the shielding effect of the hydrocarbon chain on the carbonyl group decreases through this series allowing more interaction with the stationary phase and so increasing the retention. On Apiezon M, Embaphase silicone oil, and Carbowax 4000, alkanals elute after the alkan-2-ones whereas on diethylene glycol succinate and QF 1 they lie between alkan-3-ones and alkan-2-ones. An optimum separation may be obtained for any pair of these classes by careful choice of stationary phase.

SUMMARY

The apparently anomalous behaviour of vinyl ketones in gas chromatography was investigated. Retention data for homologous series of *n*-alkanes, *n*-alkanals, *n*-alkan-2-ones, *n*-alkan-3-ones, *sym.*-alkanones, *n*-alk-2-enals, *n*-alk-1-en-3-ones, *n*-alk-1-en-3-ols and *n*-alkan-1-ols were determined and considered in terms of group retention factors. Five stationary phases were evaluated and some criteria for the choice of a stationary phase for qualitative analysis are considered.

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DETERMINATION OF NEUTRON-PRODUCED GASES IN BERYLLIUM BY GAS CHROMATOGRAPHY

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INTRODUCTION

The low atomic weight of beryllium and its small cross section for thermal neutrons has led to considerable interest in this material and its oxide as moderators for fast neutrons in nuclear reactors. However, beryllium has an appreciable cross section for the following reactions with fast neutrons:

$$^{9}_{4}\mathrm{Be}\ (\mathrm{n},2\mathrm{n})\ ^{8}_{4}\mathrm{Be} \rightarrow 2\,^{4}_{2}\mathrm{He}$$

and:

$$^{9}_{4}\mathrm{Be}\ (\mathrm{n},\alpha)\ ^{6}_{2}\mathrm{He}\ (\beta)\ ^{6}_{3}\mathrm{Li}\ (\mathrm{n},\alpha)\ ^{3}_{1}\mathrm{H}\ (\beta)\ ^{3}_{2}\mathrm{He}\ (\mathrm{n},\mathrm{p})\ ^{3}_{1}\mathrm{H}$$

Since the final products are gases, which can under certain circumstances concentrate as bubbles at grain boundaries, some concern has been felt that the physical properties of the moderator may be adversely affected as these reactions proceed[1-4]. A method is therefore required for determining isotopes of helium and hydrogen in beryllium-containing materials after various periods and conditions of irradiation.

ELLS[5] showed that helium and hydrogen may be quantitatively removed from beryllium by heating the metal under vacuum. The gases are rapidly removed if the beryllium is melted and this method has been adopted at the A.A.E.C. Research Establishment. Several methods of analysing the extracted gases have been compared.

EXPERIMENTAL

The vacuum extraction apparatus is shown in Fig. 1. Samples of beryllium weighing about 0.3 g are loaded into the system in such a manner that they can be dropped by means of a magnetically operated release mechanism into a silica crucible. Samples are melted by means of a radio frequency heater at approximately 1300°. The evolved gases are pumped rapidly from the furnace by means of a mercury diffusion pump. The gas is then Toepler pumped into a calibrated volume and the pressure measured. Fractions of the collected gas are set aside for analysis by mass spectrometry and gas chromatography and a fraction for the determination of tritium by gas counting.

(a) *Tritium counting*

Tritium is counted with a coaxial type counting tube in the proportional region using 10 % methane in argon at a counting gas pressure of 10 cm of mercury. These tubes

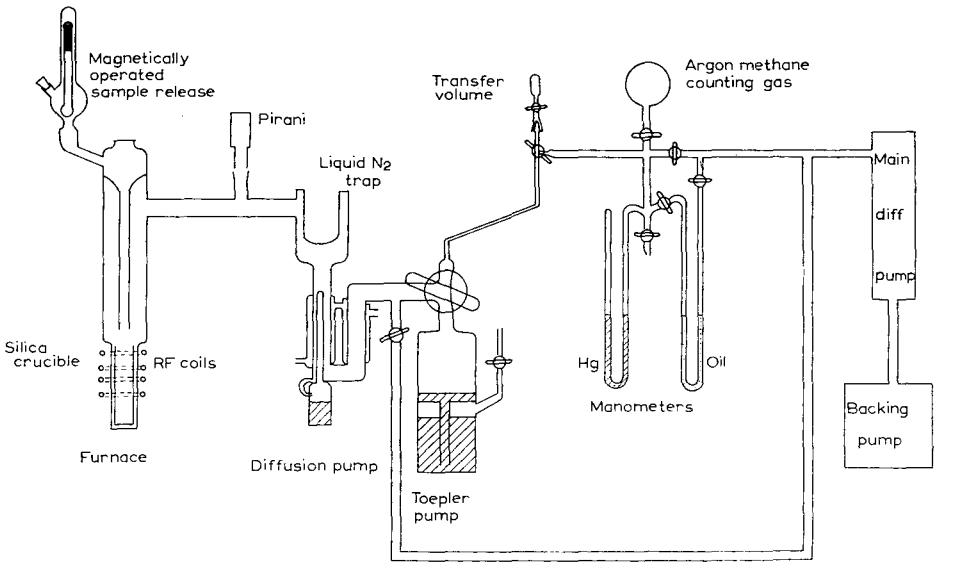

Fig. 1. Vacuum extraction apparatus.

have previously been calibrated against a pair of stainless steel compensating tubes. Owing to the high specific activity of tritium, gas dilutions of 10^4–10^6 times are required to obtain suitably low count rates.

(b) Mass spectrometry

A Metropolitan Vickers MS3 gas mass spectrometer is used for this purpose. In the lower mass range, the main contributors to various mass peaks are as given in Table I. The origin of deuterium is not known but by lowering the ionization voltage to about 20 V (when helium does not ionize) it has been shown that the mass 3 peak is very largely due to HD^+ and not $^3He^+$. This is to be expected in samples which have

TABLE I

Mass No.	*Species*
1	H^+
2	H_2^+
3	HD^+ $^3He^+$ (H_3^+)
4	HT^+ $^4He^+$ (D_2^+)
5	Nil
6	T_2^+

recently been removed from the reactor since ^3He has a very large cross section for thermal neutrons and is burnt up as rapidly as it is produced. Changing the ionization voltage also permits HT^+ to be distinguished from $^4He^+$. The large quantities of protium probably result from moisture present during processes of metal fabrication although a little is produced by the n,p reaction of ^3He.

(c) *Gas chromatography*

Molecular sieves were first used for the separation of the permanent gases by KYRYACOS AND BOORD[6] in 1957. This was followed by the work of TIMMS *et al.*[7] for the separation of impurities in carbon dioxide. The separation of hydrogen and helium has been reported by TOTH AND GRAF[8] and JANAK *et al.*[9,10] who first used palladium uniformly distributed on celite and later molecular sieves. More recently, MEYER *et al.*[11] reported the use of molecular sieves for the analysis of neutron-produced gases extracted from beryllium oxide.

The operating conditions for results reported in this paper were:

Detector. Perkin Elmer thermistor type maintained at 45°.

Column. Coiled stainless steel 7 ft. 6 in. long, 22 gauge, 1/4 in. O.D. packed with Linde 5A molecular sieve —30 to + 44 mesh B.S.S.

Column temperature 0°.

Carrier gas. Argon; flow rate of 65 c.c./min controlled by Negretti and Zambra precision pressure regulator.

Sampling system. Perkin Elmer gas sampling valve connected in line to a vacuum system and both oil and mercury manometers.

Column preparation. The molecular sieves were crushed and sieved to obtain the —30 to + 44 fraction. The crushed sieves were then dried by heating in an oven at 400° for 3 h. The column was packed and then coiled at a red heat. Regeneration of the column was necessary initially and at intervals of about 3 months. This was accomplished by heating to 350° in a stream of argon.

Calibration. The column was calibrated by injecting standard mixtures of hydrogen in argon and helium in argon at various pressures.

A graph of peak height against volume of hydrogen or helium was linear up to 25 μl of both gases. Full scale deflection of the 1 mV recorder at maximum sensitivity corresponded to 2 μl of hydrogen and 2.5 μl of helium.

The uncorrected retention volumes for helium and hydrogen, at a carrier gas flow of 65 c.c./min. were 54.2 c.c. and 71.5 c.c. respectively.

Resolving power. At equimolar concentrations and so long as the quantity of either hydrogen or helium does not exceed 25 μl (*i.e.* column is not overloaded), the hydrogen is completely separated from helium. With concentrations of hydrogen larger than 25 μl, the hydrogen peak becomes asymmetrical with a sharp tail and a sloping front which overlaps the helium peak. Thus, helium in hydrogen may be detected down to approximately 0.1 % v/v, while in the reverse case (*i.e.* small concentrations of hydrogen in helium), no overlapping occurs and hydrogen may be detected down to 40 p.p.m. in 0.25 c.c. samples of helium.

RESULTS

Table II gives a comparison of results obtained by gas chromatography and mass spectrometry on samples taken from the helium gas blanket above the reactor HIFAR. The analyses were for total hydrogen ($H_2 + D_2 + T_2$) formed by radiolysis of the moderator.

A similar comparison showing results for total hydrogen and total helium extracted from several irradiated beryllium samples is shown in Table III.

The coefficient of variation of all the results is about 10 %. By the use of larger

TABLE II

ANALYSIS OF HELIUM GAS BLANKET ABOVE THE REACTOR HIFAR FOR HYDROGEN (p.p.m., v/v)

Sample No.	*Gas chromatography*	*Mass spectrometry*
1	200	180
2	500	580
3	200	190

TABLE III

COMPARISON OF THE COMPOSITION OF GAS FROM IRRADIATED BERYLLIUM BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

Sample No.	*Gas chromatography*		*Mass spectrometry*	
	H_2%	*He*%	H_2%	*He*%
1	64.5	31.1	64.4	32.6
2	51.4	48.6	48.3	49.0
3	73.5	23.5	70.4	23.0
4	54.3	45.7	51.0	45.3
5	83.3	10.4	83.7	10.7

TABLE IV

ANALYSIS OF RADIOGENIC GASES FROM BERYLLIUM METAL

	Sample No. 1			*Sample No. 2*		
Volume gas extracted per g of beryllium (c.c.)		*0.609*			*0.432*	
	Mass spectr. (%)	*Gas chrom. (%)*	*Counting (%)*	*Mass spectr. (%)*	*Gas chrom. (%)*	*Counting (%)*
H_2	64.0	64.5	—	48.0	51.4	—
D_2	0.02		—	Not detected		—
T_2	0.3		0.67	0.3		0.71
^3He	Not detected	31.1	—	Not detected	48.6	—
^4He	32.6		—	49.0		—
$(CO + N_2)$	0.9	—	—	0.73	—	—
O_2	0.1	—	—	0.1	—	—
A	1.4	—	—	0.3	—	—
CH_4	0.3	—	—	1.5	—	—
CO_2	0.1	—	—	0.1	—	—

samples even smaller concentrations of hydrogen could be detected by gas chromatography.

The chief source of error in the results obtained by gas chromatography, is in the measurement of the sample pressure. The gas sampling valve has a measureable leak rate at sample pressures in the region of 1 cm of mercury which are used and this involves a small correction.

Table IV is the complete gas analysis of two typical samples by all three methods.

The quantity of tritium is usually too low to be determined with any precision by the mass spectrometer.

Protium, methane and carbon monoxide originate from impurities present in the metal before irradiation.

DISCUSSION

These preliminary results show that gas chromatography is a feasible method for determining hydrogen and helium extracted from irradiated beryllium-containing materials. In the near future we shall try to separate isotopes of hydrogen on columns maintained at low temperatures as described by several previous workers[12–14]. This will help to confirm the presence of the deuterium observed by the mass spectrometer since it is interesting to decide whether this is produced during irradiation or arises from contamination from the reactor moderator.

The gas chromatographic results can be obtained more cheaply and rapidly than those using the mass spectrometer and are less liable to error. When using gas chromatography alone, the entire sample can be diluted with argon to a pressure which makes the effect of the sampling valve leak negligible. An ionisation detector in the column exit in addition to the thermistor detector should also allow an independent gas chromatographic estimation of tritium in the same sample.

SUMMARY

Gases present in beryllium after neutron irradiation have been extracted by vacuum melting. The helium and hydrogen content of the extracted gas has been determined by gas chromatography and the results compared with those obtained by mass spectrometry.

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THE EFFECT OF PRESSURE DIFFERENTIALS ON GAS CHROMATOGRAPHY SEPARATION

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INTRODUCTION

Difficulties may be experienced in the separation and collection of the components of volatile mixtures by gas chromatography, which neither increase in column length nor change of stationary phase can overcome.

Little attention appears to have been paid to the effect of pressure changes in the carrier gas at a point in the gas chromatography column intermediate between inlet and outlet, the pressure at the latter being held constant.

An investigation of the effects of such intermediate pressure changes on column efficiency has now been carried out, using a modified analytical gas chromatography apparatus. This study has led to applications which can facilitate the collection of pure components.

APPARATUS

The instrument used throughout was a Bodenseewerk Perkin-Elmer Fraktometer Model 154C in conjunction with a Hartmann and Braun Linecomp recorder with a 5 mV full scale deflection. 8,000 Ω thermistors were used in the detector block. The columns were packed with Celite 545 impregnated with 20 % Silicone D.C. 550 fluid by the conventional slurry method and dried at 100°. Helium was used as the carrier gas, and a soap bubble flowmeter was used in conjunction with the rotameter in the instrument. The samples were injected with Hamilton syringes of 10 μl and 50 μl capacities. The following modifications were made to the instrument.

The monitoring system (see Fig. 1)

The normal gas inlet was fed directly into the sample injection block (F), without the bypass into the reference thermistor chamber, by unscrewing the T-piece and sealing the bypass outlet. A hole was drilled from the front face of the detector block (T) to the original reference thermistor chamber (H). This hole was tapped and a brass elbow (S), with a column connection at one end, was screwed into it. A three-way brass elbow was screwed into the original reference thermistor inlet. This arrangement allows the gas stream to pass directly over the original reference thermistor now called the monitoring thermistor.

The multichannel stopcock (see Fig. 2)

The stopcock was constructed from two brass discs held together on a brass spindle (U). One disc (V) was faced and the disc (X) had a Teflon insert (W), which was also faced.

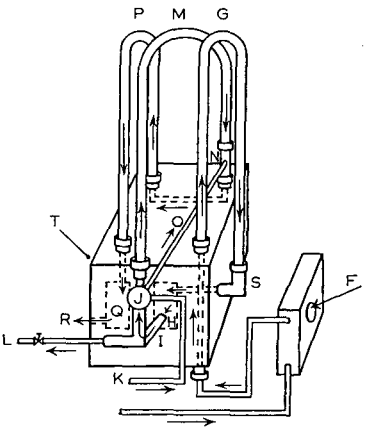

Fig. 1. Detector block assembly.

Disc (V) had four holes drilled into its edge which coincided with four holes drilled into its face. Copper tubes (I), (O), (M) and (K) were soldered into the holes in the edge of the disc. Two grooves were cut into the Teflon face (W). Face (V) was fixed and face (W) revolved, its movement being controlled by a switch (Z) connected by a castle system (Y) to the spindle (U). The multichannel stopcock will now be referred to as m.c. stopcock.

The gas inlets (see Figs. 1 and 4)

A gas regulator (B_1), connected to the gas cylinder (A), controlled the supply to both the fine gas regulator in the instrument (D) and the gas regulator (B_2) for the second-

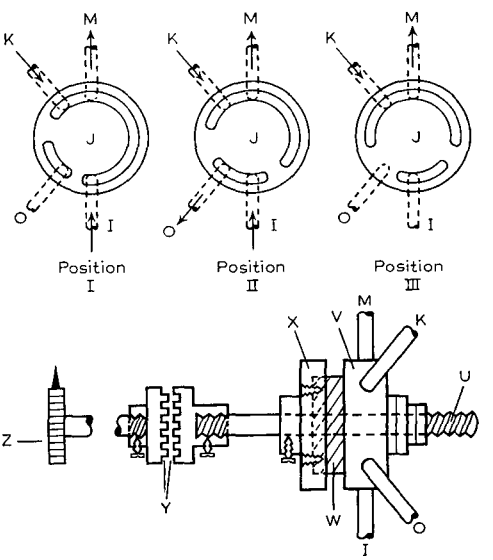

Fig. 2. Multichannel stopcock.

ary gas inlet (K). A copper tube (O.D. ¼ in.) with a two-way brass stopcock led from the secondary gas inlet regulator through the instrument's gas sampling port to tube (K) in the m.c. stopcock (J).

The bypass system (see Figs. 1 and 2)

One of the tubes (O) on the m.c. stopcock was connected by a copper tube (O.D. 1/8 in.) to a brass column connection (N) which was fixed to a port on the detector block.

The secondary gas outlet stopcock (see Figs. 1 and 2)

The secondary outlet stopcock was manufactured in a manner similar to that of the m.c. stopcock except that here only one groove was made in the Teflon face to permit connection of the inlet and outlet in the open-to-atmosphere position. The outlet tube on the stopcock was constricted by crimping to prevent too abrupt pressure adjustments when the stopcock was opened. The inlet of the stopcock was connected by a copper tube (O.D. 1/8 in.) to tube (I) (Fig. 2) on the m.c. stopcock (J).

The column arrangement (see Fig. 1)

Three packed aluminium columns (I.D. 4 mm) were used in the modified fractometer. The columns were connected as follows: Column (G) led from the inlet port on the detector block to elbow (S) leading to chamber (H). Column (M) led from the m.c. stopcock (J) to the bypass connection (N) attached to the detector block. Column (P) joined the remaining two ports on the detector block, one being the outlet port leading to the original detecting thermistor.

The monitoring system allowed the operator to check on the behaviour of a sample and enabled precise cutting just before the sample reached the m.c. stopcock. A typical trace so obtained on the recorder is shown for a mixture of α-pinene and β-pinene (Fig. 3). Here the base line was run in the middle of the chart and the response of the monitoring thermistor to the sample was shown as a negative signal, (dashed curve), whereas the response of the original detecting thermistor was shown as a positive signal (full curve). A range of intermediate pressures can be obtained by adjusting the secondary gas inlet regulator. Tube (O) permitted column (M) to be bypassed if so desired. The monitoring detector, m.c. stopcock and bypass were placed in the column line at a distance of approximately one third of the total column length from the normal inlet. A shorter distance was found to be unsatisfactory in view of the poor resolution of some multicomponent mixtures at the monitor. A greater distance could give rise to overlapping of monitor and final detector responses on the chart, especially with regard to a mixture with a wide range of retention times. The m.c. stopcock was to provide a choice of several paths to a sample (see Fig. 4).

Gas flow paths

When a sample is injected into block (F) it is carried by the gas stream through column (G), monitor thermistor chamber (H) and either by tube (I) to m.c. stopcock (J) or through the secondary outlet (L). If the latter is closed to the atmosphere, the following paths are possible:

Position I (see Fig. 2). With or without a gas stream from the secondary gas inlet (K), the sample passes through columns (M) and (P), thermistor chamber (Q),

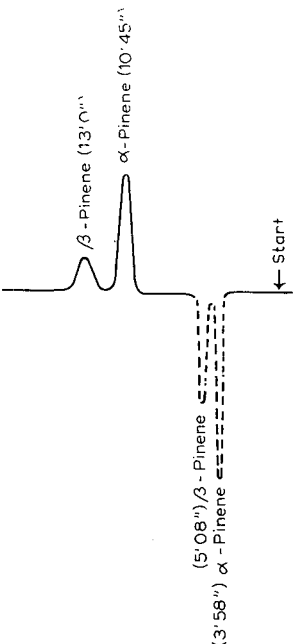

Fig. 3. Chromatogram of a mixture of α- and β-pinene. Temperature: 138°; normal inlet pressure: 2.5 kg/cm²; column length: 2.8 m; attenuation: 32; chart speed: 300 mm/h; sample volume: 7 μl.

and normal outlet (R), except in the case where the intermediate pressure is equal to the normal inlet pressure.

Position II (see Fig. 2). The sample passes through bypass tube (O), connection (N), column (P), chamber (Q) and outlet (R). Simultaneously a separate gas stream from inlet (K) can be made to pass through columns (M) and (P).

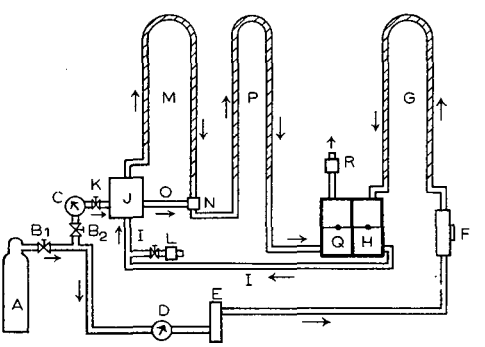

Fig. 4. Gas flow diagram.

Position III (see Fig. 2). Sample cannot enter (J), hence may be eluted from the secondary outlet (L). Simultaneously a stream of gas can be passed from inlet (K) through columns (M) and (P).

EXPERIMENTAL

The m.c. stopcock was held in Position I affording use of the columns according to normal gas chromatography procedure except that the intermediate pressure could be adjusted. The intermediate pressure was brought to the desired level by adjusting the secondary pressure regulator (B_2) (Fig. 4) with stopcock (K) closed, after which it was opened. The fine pressure regulator in the apparatus and the regulator on the gas cylinder were at all times adjusted to the same pressure; thus the intermediate pressure could not be made to exceed the normal inlet pressure. The rotameter (E) was calibrated against the soap bubble flowmeter for flow rate measurements.

RESULTS

At maximum sensitivity (attenuation 1) a sudden pressure change caused an abrupt recorder response followed by a gradual base line adjustment to its original position. At lower sensitivities (*e.g.* attenuation 32) with a sudden pressure change, the base line remained constant except for an instantaneous negative and positive deflection of the recorder pen. Sudden pressure changes caused no deterioration of the thermistor beads, which was also observed by BORFITZ[1]. When the intermediate pressure was raised in the column, while outlet and normal inlet pressures were held constant, a decreased flow in the column before the secondary inlet, and an increased flow in the column after the secondary inlet was observed. When the intermediate pressure was made equal to the normal inlet pressure, gas flow ceased in the column before the secondary inlet.

Raising the intermediate pressure prior to the injection of a compound had the effect of increasing its retention time, as well as broadening its elution curve. Raising the intermediate pressure after the compound had passed the secondary gas inlet, a slight drop in retention time accompanied by a slight sharpening of the elution curve was noted.

Raising the intermediate pressure, whilst the compound was retained in column (G), caused much the same effects as in the case where the intermediate pressure was raised prior to sample injection. Column efficiency dropped markedly when the pressure gradient in column (G) was small.

When the intermediate pressure was raised just after the first component of a binary mixture, almost completely resolved at the monitor, had passed the secondary inlet, no appreciable change was observed in the retention times and shapes of the elution curves, except when the pressure gradient in column (G) was small. Under the latter condition the difference in retention times of the two components was considerably increased, but column efficiency for the second component dropped below a useful limit. When the pressure gradient was zero a compound could be held stationary in column (G). However, when it was stationed just before the secondary inlet, diffusion effects caused it to leak into column (M). On reducing the secondary inlet pressure the compound, which was held up, resumed its passage. This effect was studied on single compounds, *e.g.* ethyl alcohol, benzene and *n*-butyl acetate, and the results are shown in Fig. 5. The period for which the compound was held stationary was called the hold-up time, H_t. The total residence time R_t, less the hold-up time, H_t, was called the travelling time, T_t, *i.e.*, $R_t - H_t = T_t$. To obtain some measure of the effect of

the hold-up time, the concept of column efficiency was used. For this purpose the column efficiency was measured by the number of theoretical plates (KEULEMANS[2]) which was adapted by replacing retention time by travelling time in the equation, column efficiency $= (4\ T_t/W)^2$, where W (in mm) is the base of the triangle on the base line obtained by drawing the tangents to the inflection points of the elution curve.

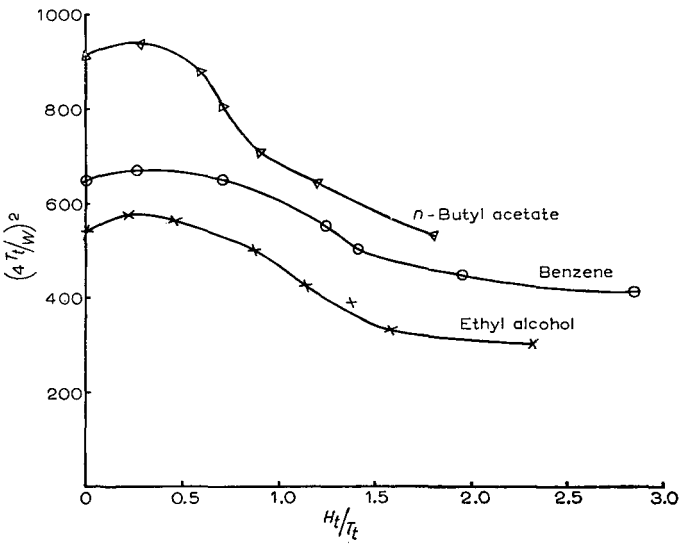

Fig. 5. Effect of hold-up time on column efficiency. Normal inlet pressure: 2.5 kg/cm²; column length: 3 m; temperature: 139°, except for *n*-butyl acetate: 130°.

Travelling time, T_t, is converted to length units by multiplying by the chart speed. Column efficiency was then plotted against the ratio of hold-up time to travelling time. It was noted that column efficiency did not drop appreciably until the hold-up time equalled approximately three quarters of the travelling time. Beyond this value the column efficiency decreased considerably and was probably due to the fact that diffusion of the zone became more marked during the hold-up period.

Therefore the effect of intermediate pressure changes on the separation of components is of little use unless a hold-up technique can be used.

APPLICATIONS

By using the gas chromatography apparatus as outlined in the Experimental section, only one application, which has little practical value, is possible, namely further separating components which are already resolved at the secondary gas inlet. This application may be repeated by the following technique. The zero pressure gradient is obtained in column (G) (Fig. 4), by switching m.c. stopcock to Position III and simultaneously using the secondary gas inlet, instead of using Position I. However, both suffer from the disadvantage that the monitor and final thermistor responses interfere with each other. By using the above gas chromatography apparatus with the secondary outlet and bypass for samples in which the total range of retention times lies within certain limits, the above disadvantage may be eliminated.

In view of the observation that a component can be held stationary under pressure for a limited period without great loss of column efficiency, the modified apparatus may be used for the following applications.

Using the secondary outlet any component of a mixture can be flushed out to atmosphere during a run. In the case of a binary mixture (*e.g.* α- and β-pinene), where the second component with the longer retention time *i.e.* β-pinene, was flushed out, bleeding back of α-pinene was prevented by switching the m.c. stopcock from Position I to Position III, prior to the flush-out period. This example is shown in Fig. 6. A reduction of approximately 20 % in run-time is gained in this way. A further reduction in time can be obtained by the use of the secondary gas inlet.

The bypass allows any component of a mixture to be eluted from the column before any preceding components. For example in the case of α- and β-pinene, (Fig. 7), the α-pinene is allowed to pass through column (M), with m.c. stopcock in Position I, then β-pinene is channelled through the bypass by switching to Position II and is eluted before the α-pinene. Switching back to Position I now allows α-pinene to be eluted. The α-pinene can be made to travel along column (M) by using the secondary gas inlet with m.c. stopcock in Position II and this again leads to a reduction in total run-time.

The secondary outlet and the bypass become particularly useful with regard to partially resolved and multicomponent mixtures.

An example of the former is a mixture of α-pinene and camphene, (Fig. 8a) which was partially resolved after passing through the total column length of 3 m. The undesired portion of this partially resolved mixture is flushed from the secondary outlet. This leads to a mechanical separation of fractions of α-pinene and camphene (Fig. 8b), the purity of which is checked by the final thermistor without the necessity of recirculating part of the fractions. If required, the portion flushed from the secondary outlet may be collected and recirculated. This technique should have value if only a small initial amount of sample is available. A reduction in run-time could have been attained had the bypass also been used.

An example in which both the bypass and secondary outlet are used is afforded by the case of a mixture of α-pinene, β-pinene and ocimene, (Fig. 9). β-Pinene plus some α-pinene and ocimene were flushed from the secondary outlet and ocimene was channelled through the bypass, while α-pinene was held stationary in column (M). Compared with a normal run on the above mixture, this technique gave a reduction in run-time of approximately 20 %. The run-time could have been reduced further by allowing α-pinene to travel along column (M) during the flush-out and bypass period.

The use of the modified apparatus allows collection of components of a mixture in less than normal run-time if required. Such a reduction in time may be important in the case of repetitive sample collection. If a reduction in run-time is not of primary importance the components of a mixture may be separated to such a degree that contamination during collection is completely avoided. In general the separation of pure components from partially resolved and multicomponent mixtures can be carried out in a comparatively simple manner. This technique becomes particularly useful if only a small initial amount of sample is available, and where no complete resolution of components has been achieved even by the use of the most efficient available stationary phases and conditions. Furthermore recirculation becomes unnecessary as

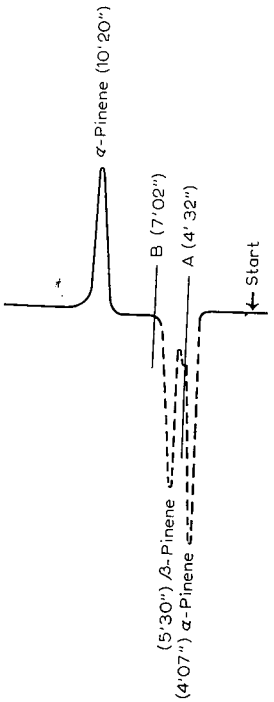

Fig. 6. Chromatogram of a mixture of α- and β-pinene. Conditions as for Fig. 3. (A) m.c. stopcock: Position III; secondary inlet pressure; 2.0 kg/cm^2; secondary outlet: open. (B) Secondary outlet: closed; m.c. stopcock: Position I; secondary inlet: closed.

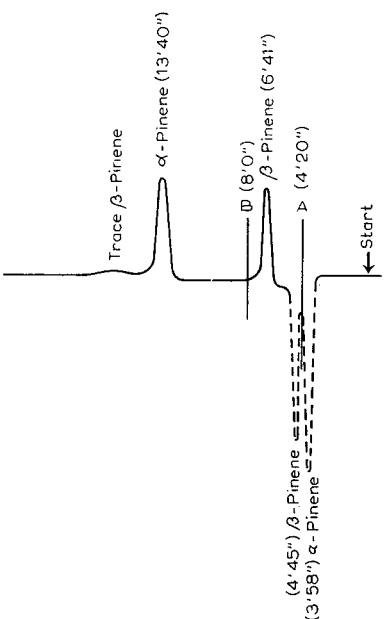

Fig. 7. Chromatogram of a mixture of α- and β-pinene. Conditions as for Fig. 3. (A) m.c. stopcock Position II; (B) m.c. stopcock: Position I.

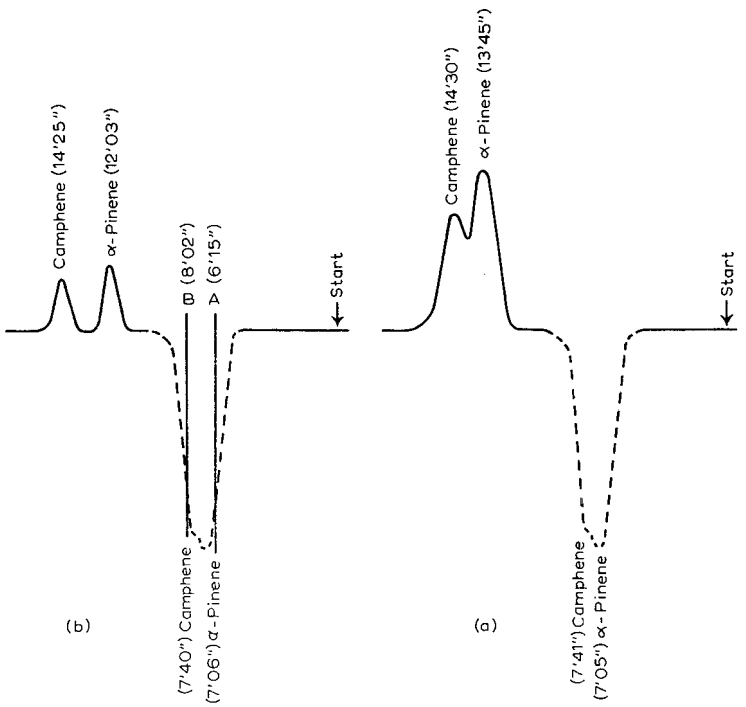

Fig. 8. Chromatogram of a mixture of α-pinene and camphene. (a) Temperature: 133°; normal inlet pressure: 2.5 kg/cm²; column length: 3 m; attenuation: 64; chart speed: 300 mm/h; sample volume: 16 μl. (b) Conditions as for (a). (A) m.c. stopcock: Position III; secondary outlet: open. (B) Secondary outlet: closed; m.c. stopcock: Position I.

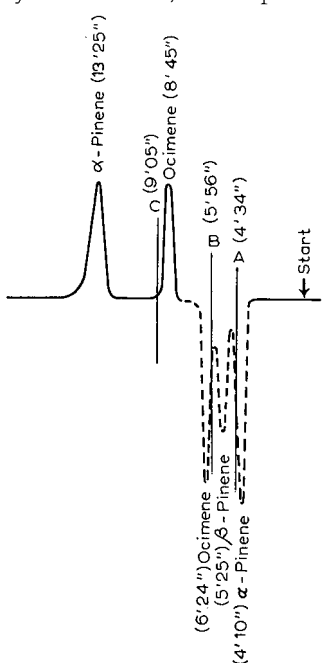

Fig. 9. Chromatogram of a mixture of α-pinene, β-pinene and ocimene. Conditions as for Fig. 3; sample volume: 9 μl. (A) m.c. stopcock: Position III; secondary outlet: open. (B) Secondary outlet: closed; m.c. stopcock: Position II. (C) m.c. stopcock: Position I.

the purity of the component has already been checked by the final thermistor. The modified apparatus may be used for ordinary runs by rearranging the columns.

A disadvantage arises if two components in the system pass over the monitoring and final thermistor at the same time, thus adversely affecting the recorder response. Also, to achieve a successful run, some experience is needed in the manipulation of the various controls.

ACKNOWLEDGEMENTS

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SUMMARY

A series of experiments on volatile samples in a gas chromatography column were carried out to determine the effect of pressure changes at a point intermediate between the normal inlet and outlet, while pressures at the latter were held constant. To facilitate these experiments, a number of modifications were made to a conventional analytical fractometer. No major intrinsic alterations were necessary in modifying the apparatus, and the accessories used were of simple construction.

Of the above series of experiments the only case leading to useful applications was found to be that in which the intermediate pressure was made equal to the normal inlet pressure. The effect of holding a compound stationary under such pressure is discussed in terms of column efficiency and the ratio of hold-up time to travelling time.

From the above study certain applications became evident of which several examples are given. Some of these applications were found to be of considerable value with regard to partially resolved and multicomponent mixtures. The advantages and disadvantages of the modified apparatus are discussed.

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[2] A. I. M. KEULEMANS, *Gas Chromatography*, Reinhold Publ. Corp., New York, 1959, p. 138.

GAS CHROMATOGRAPHY APPLIED TO THE STUDY OF THE LIQUID PRODUCTS OF THE LOW-TEMPERATURE FLUIDIZED-BED CARBONIZATION OF COAL

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INTRODUCTION

Economic considerations in many countries at the present time are not favourable for the large scale conversion of coal into liquid fuels and chemicals. But this situation is bound to change in the future as the proved reserves of coal are many times greater than the most optimistic estimates of petroleum. The Department of Fuel of the University of New South Wales has therefore adopted as one of its research projects the investigation of low-temperature carbonization of coal and the products obtained by various processes for this purpose.

The programme of the Department includes both static- and fluidized-bed carbonization techniques. The latter involves the application of heat to the coal particle "boiling" or suspended in a turbulent state in the reaction chamber by a stream of gas (usually air) for a time interval commensurate with maximum tar-oil yield. The volatile material moves out of the carbonizing zone at a rate that approaches the velocity of the transport gas with the result that the tars produced are considered to represent the most nearly primary tars produced by industrial processes[1].

From the practical point of view fluidized processes have the advantages of high throughput for a given size of plant, high yield of liquid products and better control than is possible with batch processes in static beds.

Various workers have described gas chromatographic examinations of fractions from low-temperature coal tars. KARR *et al.*[2,3] analysed phenols boiling up to 234° from the low-temperature fluidized carbonization of a West Virginia bituminous coal, at 160° on a di-*n*-octyl phthalate column using helium as carrier gas. FITZGERALD[4,5] studied the application of gas chromatography to the analysis of phenols using a number of stationary phases, and concluded that for the best resolution of mixtures both polar and non-polar phases should be used. He found Apiezon L and sodium dodecylbenzene sulphonate (as a spray-dried mixture with sodium sulphate in an industrial detergent) to be the most useful stationary phases and employed them to analyse the lower-boiling phenols from Lurgi brown coal tar. BROOKS[6] studied the relative effectiveness of phosphate esters of various phenols for the separation of close-boiling phenol isomers, *e.g.* 2,4- and 2,5-xylenols and *m*-ethylphenol and *p*-ethylphenol. PAYN[7] used a di-*n*-octyl sebacate column at 178° for routine analysis of phenols.

In the case of the neutral oil most work has been done on the aromatic fraction. This is a natural consequence of earlier work on high-temperature tars. DUPIRE AND BOTQUIN[8] developed a chromatograph which employed silicone grease as the stationary

phase and helium as carrier gas, and this was later used by DUPIRE[9] to determine quantitatively forty-five components of a tar using column temperatures from 130° to 360°. CHANG AND KARR[10] analysed aromatic hydrocarbons boiling up to 218° in the low-temperature tar mentioned above[2,3] at 150° on a 15 ft. long column using Apiezon L as stationary phase.

Although not available to the author until the present work was in its concluding stages, the papers by COPPENS, BRICTEUX AND NEURAY[11] and BOYER *et al.*[12] are of particular interest. COPPENS *et al.* worked on tar obtained by carbonization of a high volatile coal at about 600° in a fixed bed. The fraction of the neutral oil boiling below 280° was separated into paraffins (and naphthenes), olefins and aromatics by a modification of the fluorescent indicator adsorption (F.I.A.) method of column chromatography. The paraffin and olefin fractions were then submitted to gas chromatography at 190° on a column containing silicone grease on firebrick with helium as carrier gas. The paraffin fraction, which consisted of 16.5 % of the neutral oil boiling below 280°, was shown to consist predominantly of normal paraffins from C9 to C16 together with minor components corresponding to five homologous series of different isoparaffins of the same range of carbon numbers. The olefin fraction, which consisted of 11.0 % of the neutral oil boiling below 280°, was found to consist essentially of normal olefins C9 to C16 in three series decreasing in amount in the order 1-, *trans*-2- and *cis*-2-olefins. Members of up to six homologous series of branched olefins also appeared to be present in minor amounts. It should be noted that over two-thirds of this neutral oil fraction was aromatic.

BOYER *et al.* carbonized to 550° vitrinite and exinite concentrates from a Lorraine high-volatile coal in a laboratory apparatus under conditions of high heating rate and low secondary cracking. They considered the tar obtained to be somewhat analogous to that obtained from semi-carbonization in a fluidized bed. The tar was subjected to counter-current extraction to remove polar compounds, distilled, separated into hydrocarbon types by column chromatography on silica gel, and these fractions analysed on a gas chromatograph analogous to that described by DUPIRE[9]. Infrared and ultraviolet spectroscopy were used to identify components trapped out after separation. Paraffins and olefins up to about thirty carbon atoms were identified. Most of these were straight-chain compounds and the authors claimed that these came "almost only from exinite" (a petrographic maceral* group in coals comprising remains of spores, cuticles, algae and resin bodies). They further stated that aromatic compounds were abundant in all the tars, but were more highly substituted in the exinite tar.

The liquid products in the present study were examined on the invitation of the Australian Joint Coal Board, and were produced for the Board in a fluidized-bed pilot plant operated by the United States Bureau of Mines in Denver, Colorado, U.S.A. The coal carbonized was a high-volatile bituminous coal from the Liddell Seam, Foybrook Open Cut, New South Wales, and conditions of the run were adjusted to produce a char of 16.0 % volatile matter.

The general properties of 20 tars obtained from a variety of coals in this pilot plant have been described by GOMEZ, GOODMAN AND PARRY[1]. Six of these were from

* Macerals are defined as the elementary homogeneous microscopic constituents of microlithotypes in coals, and are analogous to minerals in rocks.

TABLE I

	Range	Mean
Primary distillation yield (weight %)	28.0–60.6	43.2
Composition of distillate (volume %)		
Acids	18.0–34.8	26.5
Bases	1.4– 3.4	2.5
Neutral Oil	62.6–80.6	71.0
Composition of neutral oil (volume %)		
Olefins	30.3–69.5	40.3
Aromatics	14.0–37.3	28.6
Paraffins (includes naphthenes)	16.5–39.1	31.1

high-volatile bituminous coals, and some results of interest for them are given in Table I.

The analytical procedures for these determinations were those described by REYNOLDS AND HOLMES[13]. The various fractions thus obtained were not studied in further detail.

EXPERIMENTAL

(a) Samples

(i) *Light oil distillate*

The light oil had been recovered from the secondary condenser of the carbonization plant. It was a dark brown to black mobile liquid with a tarry smell. 100 ml was distilled in an A.S.T.M./I.P. standard distillation apparatus to yield a distillate (93 %) of boiling range 90–285° (60 % between 190° and 280°).

Phenols in light oil distillate. These were extracted in the conventional way with 10 % sodium hydroxide solution. The yield was 18.8 % v/v of the distillate.

Neutral oil from light oil distillate. The phenols-free light oil distillate was extracted with 25 % w/w sulphuric acid to remove bases (0.5 % v/v of the distillate) so that the neutral oil amounted to 80.7 % v/v of the distillate.

(ii) *Tar distillates*

The tar had been recovered from the primary condenser and precipitator of the carbonization plant. It consisted of a mixture of mobile and viscous material which was not homogeneous even after heating at 80° for a week. It was therefore distilled

	Yields of tar distillates (% w/w)		
	(A)	*(B)*	*Original tar (calc.)*
Pitch	62.2	69.5	68.0
Distillate to 310°	35.6	27.0	28.8
Liquor	0.4	0.0	0.1
Loss	1.8	3.5	3.1

in two lots representative of the mobile (A) and viscous (B) portions and the yields for the original tar calculated. The standard apparatus for crude tar distillation was used

with a charge of approximately 4 kg in each case. The specific gravity of distillate (A) was 0.887 (15.5/15.5°).

The softening point of pitch (A) was determined as 70° by the "Ring and Ball" method.

Phenols in tar distillates. These were extracted by 10 % sodium hydroxide solution. Portions of the wet phenols were mixed with ether and dried with anhydrous sodium sulphate. The ether was then allowed to evaporate.

	Yields of phenols (% v/v)		
	(A)	*(B)*	*Composite (calc.)*
Wet phenols	16.6	38.0	33.7

Neutral oil in the tar distillates. Bases were removed by 25 % w/w sulphuric acid. Emulsion formation, interfacial solids and the very dark colour made the separation difficult.

	Yield of neutral oil (% v/v)		
	(A)	*(B)*	*Composite (calc.)*
Tar bases	1.1	6.4	5.3
Neutral oil	79.3	55.6	61.0

(*b*) *Gas chromatograph*

A Beckman GC2 instrument fitted with a fraction collector was used. Hydrogen and, more frequently, helium were the carrier gases employed and detection was by means of a katharometer.

Columns

Silicone. (Beckman 74346) Dow-Corning Silicone Fluid Type 550 on 42–60 mesh C22 Johns Manville firebrick in the ratio 30:100 in a 6 ft. × ¼ in. column.

Apiezon L. (Beckman 70013) Apiezon L on C22 firebrick in the ratio of 30:100 in a 6 ft. × ¼ in. column.

The total weight of column filling material was 22.5 g.

Molecular sieve 5A. (Beckman 70020) 29.2 g in a 6 ft. × ¼ in. column.

(*c*) *Chromatographic procedures*

For analytical separations samples varied in size from 2–20 μl and were injected by Hamilton syringe. The gas inlet pressure was kept constant at 25 lb./sq.in., and the flow rates corresponding to different columns and conditions were measured at the exit. For the phenols the reference was phenol itself. In the case of the neutral oils toluene, mesitylene and diphenyl were used at 100°, 160° and 220° column temperatures respectively so that sufficient accuracy without excessively long retention time was obtained. In all cases the reference was run immediately before and after each unknown mixture or series of known substances.

When collection of components was desired the sample size was increased up to 200 μl (0.2 ml), but the same columns were used. Up to this volume separation was still good, but one test using 300 μl flooded the column. The inlet was heated by a cartridge heater during injection and the exit line to the collector was kept above column temperature to prevent premature condensation. Ice water was found to be adequate for cooling the traps in the Dewar flask, which were inserted on the exit only when the peak concerned was being traced on the recorder. For individual peaks the component condensed almost completely in the inner tube, and was easily washed out with a few drops of spectroscopic grade carbon tetrachloride. For larger cuts the liquid collected in the narrow bottom of the tube and could be sucked into a syringe fitted with a long needle. It was not found necessary to centrifuge the tube.

(*d*) *Infrared spectroscopy*

Most spectra were determined on a Perkin Elmer "Infracord" instrument. Several samples were run on a Perkin Elmer Model 421.

RESULTS AND DISCUSSION

Phenols

Separation of the phenols was found to be reasonable on the silicone column with either hydrogen or helium as carrier gas. The peaks tended to be somewhat tailed. Temperatures of 100°, 160° and 190° were tried. At 100° resolution was good, but the retention times were too long, *e.g.* phenol itself took about 24 min to pass through. The best compromise between resolution and time of analysis appeared to be at 160°. A typical chromatogram for the phenols from the light oil run at this temperature is shown in Fig. 1. The phenols from tar distillate (A) gave a very similar chromatogram. Apiezon L was found much less satisfactory for separation of the phenols mixtures.

Tentative identification of the phenols by means of relative retention times is shown in Table II for the light oil and Table III for tar distillate (A). Several pairs of phenols could not be resolved under the conditions used. Other stationary phases

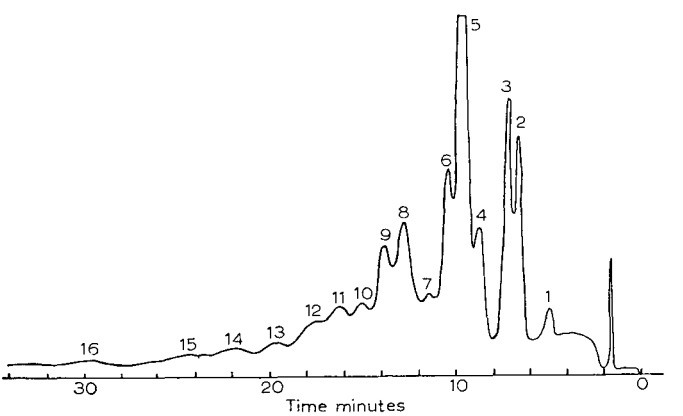

Fig. 1. Chromatogram of phenols from light oil on silicone column at 160°. Sample size: 2 μl; carrier gas: He; inlet pressure: 25 lb./sq.in.; outlet pressure: atmospheric; flow rate: 46 ml/min.

TABLE II

PHENOLS FROM LIGHT OIL DISTILLATE AT 160° ON SILICONE

Peak No.	*R.R.T. to peak No. 1*	*Identification*	*R.R.T. of known compounds*	*Relative strength**
1	1.00	Phenol	1.00	w
2	1.37	*o*-Cresol	1.41	m
3	1.50	*m*- and *p*-Cresol	1.55, 1.52	s
4	1.88	2,6-Xylenol	1.90	w
5	2.07	2,4-Xylenol; 2,5-xylenol	2.14, 2.13	vs
6	2.25	4-Ethylphenol; 3,5-xylenol	2.32, 2.32	m
7	2.49	2,3-Xylenol	2.53	vw
8	2.80	2-*n*-Propylphenol	2.84	m
9	3.05			m
10	3.32	4-*n*-Propylphenol; 3-ethyl-5-methylphenol	3.42, 3.40	w
11	3.60	2,3,5-Trimethylphenol	3.71	w
12	3.92			w
13	4.40			w
14	4.9	4-Indanol	5.08	w
15	5.5			vw
16	6.7			w

* w = weak; m = medium; s = strong; vs = very strong; vw = very weak.

would no doubt be able to do this. Separation was considered insufficient to attempt quantitative analysis. All that was thought justifiable was a rough division of peaks into "very strong" (vs), "strong" (s), "medium" (m), "weak" (w) and "very weak" (vw) as is shown in the tables.

The work demonstrates that with a general purpose column, even though the ultimate in separation is not achieved, much valuable information can be easily

TABLE III

PHENOLS FROM TAR DISTILLATE (A) AT 160° ON SILICONE

Peak No.	*R.R.T. to peak No. 1*	*Identification*	*R.R.T. of known compounds*	*Relative strength**
1	1.00	Phenol	1.00	m
2	1.40	*o*-Cresol	1.41	m
3	1.52	*p*- and *m*-Cresol	1.52, 1.55	s
4	1.90	2,6-Xylenol	1.90	w
5	2.10	2,4-Xylenol; 2,5-xylenol	2.14, 2.13	s
6	2.29	3,5-Xylenol; 4-ethylphenol	2.32, 2.32	m
7	2.5	2,3-Xylenol	2.53	vw
8	2.84	2-*n*-Propylphenol	2.84	m
9	3.09			m
10	3.37	4-*n*-Propylphenol; 3-ethyl-5-methylphenol	3.42, 3.40	m
11	3.65	2,3,5-Trimethylphenol	3.71	vw
12	4.0			vw
13	4.4			vw
14	5.0	4-Indanol	5.08	w
15	5.6			w
16	6.7			w
17	7.5			vw
18	8.0			vw
19	8.7			vw

* For abbreviations see Table II.

obtained from tar acid fractions. In the case of the light oil eleven of the sixteen peaks have been tentatively identified. If "major components" are defined as those of medium strength or stronger then only one major component (No. 9 peak) remains unknown. (From its position it is almost certainly a C9 phenol.)

The same phenols are apparently present in tar distillate (A) and their quantitative distribution follows almost the same pattern. There are three more components in minor amounts at the high end of the boiling point range.

The following phenols could not be detected in either the light oil or tar distillate (relative retention times to phenol given in brackets): O-ethyl-phenol (2.00); 3,4-xylenol (2.71), 1-naphthol (13.0) and 2-naphthol (13.8).

Neutral oils

A typical chromatogram of the neutral oil from tar distillate (A) on the silicone column at 220° is shown in Fig. 2. Separation was quite good at this temperature whilst lower temperatures led to very long retention times and flattened peaks. The neutral oil

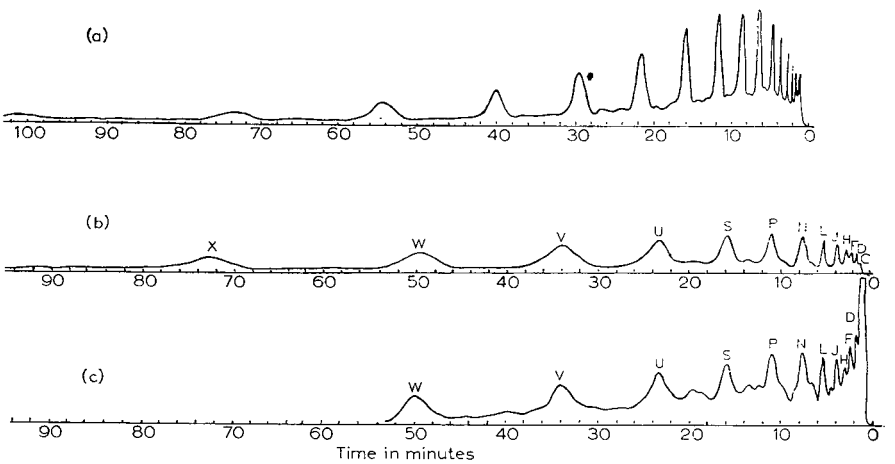

Fig. 2. (a) Chromatogram of neutral oil from tar distillate (A) on silicone column at 220°. Sample size: 4 μl; carrier gas: He; inlet pressure: 25 lb./sq.in.; outlet pressure: atmospheric; flow rate: 41 ml/min. (b) Chromatogram of neutral oil from tar distillate (A) on Apiezon L column at 220°. Sample size: 5 μl; carrier gas: He; inlet pressure: 25 lb./sq.in.; outlet pressure: atmospheric; flow rate: 43.5 ml/min. (c) Chromatogram of neutral oil from tar distillate (B) on Apiezon L column at 220°. Sample size: 20 μl of ethereal solution; carrier gas: He; inlet pressure: 25 lb./sq.in.; outlet pressure: atmospheric; flow rate: 43.5 ml/min.

from the light oil gave a chromatogram similar to the early part of the tar distillate at 220°. If the column temperature was reduced to 160° the same peaks were obtained with better separation.

Comparison with the relative retention times of known neutral substances was not helpful. These reference compounds were mostly aromatic hydrocarbons. Nine of the major components of the tar distillate were collected for examination by infrared spectroscopy. The spectra were all similar (Fig. 3b) showing very strong bands corresponding to aliphatic carbon–hydrogen bonds at about 2800–3000 cm^{-1}, 1460 cm^{-1} and 1370 cm^{-1}. Minor bands were also present, *e.g.* at 965 cm^{-1}, 910 cm^{-1} and 720 cm^{-1}.

The neutral oil itself from tar distillate (A) was very similar except that there were additional weak bands at about 1600 cm^{-1}, 810 cm^{-1} and 745 cm^{-1} (Fig. 3a). For comparison the spectrum of paraffin oil is shown in Fig. 3c. It is evident that the components of the neutral oil are very aliphatic in nature.

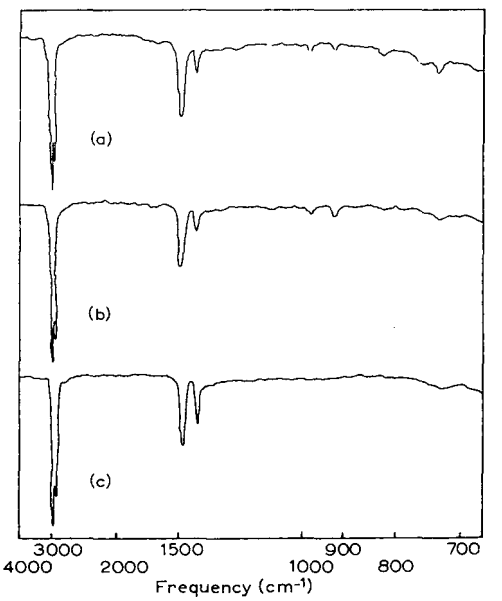

Fig. 3. Infrared spectra of: (a) Neutral oil from tar distillate (A). (b) Typical major peak trapped from tar distillate (A) neutral oil on silicone column at 220°. (c) Paraffin oil.

When the same sample was run on the Apiezon L column at 220° (Fig. 2) the peaks were more spread out, but the same regularity, which suggested an homologous series, was apparent. Fig. 2 also shows part of a run on the neutral oil from tar distillate (B), showing differences from (A) in minor early components, but having all the same major components. (N.B. The very strong front peak is due to added ether as solvent.) If the major components were assumed to be normal paraffins then peaks D, H and U corresponded to *n*-octane, *n*-decane and *n*-hexadecane, by relative retention time to diphenyl as reference. The graph of log (relative retention time × 100) *versus* carbon number on this basis for the major peaks was a straight line as can be seen from Fig. 4. However, collected components above C_{17} were liquids, which was inconsistent with their being normal paraffins, so it appeared that they were either isoparaffins of about the same relative retention time, or mixtures.

As mentioned earlier it was impractical to run the neutral oil from the tar distillate below 220°, and at 160° on silicone the neutral oil from the light oil showed the same peaks as at 220° except that at the lower temperature they were more widely spaced. However, when the latter oil was run on Apiezon L at 160° all the major peaks were split in two, the first of each pair being somewhat stronger. This is shown in Fig. 5a.

The infrared spectrum of the light oil neutral oil (Fig. 6a) showed strong bands corresponding to aliphatic carbon–hydrogen bonds and a number of other bands

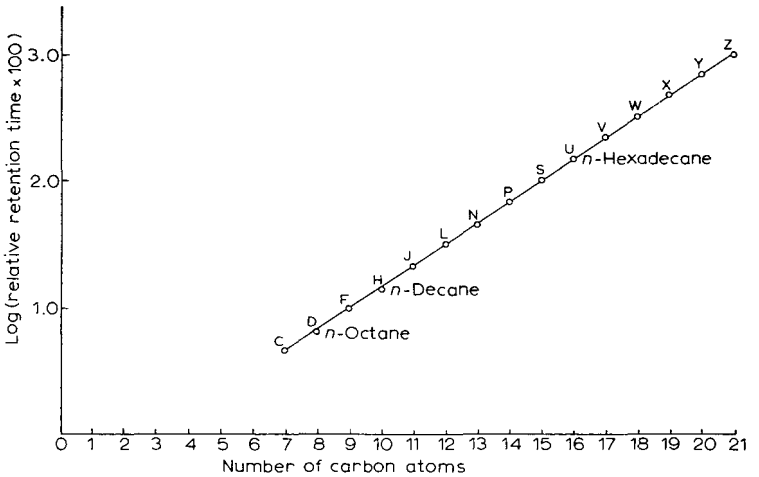

Fig. 4. Graph of log [relative retention time (to diphenyl reference) × 100] *versus* number of carbon atoms for the major peaks from tar distillate (A) neutral oil on Apiezon L column at 220°.

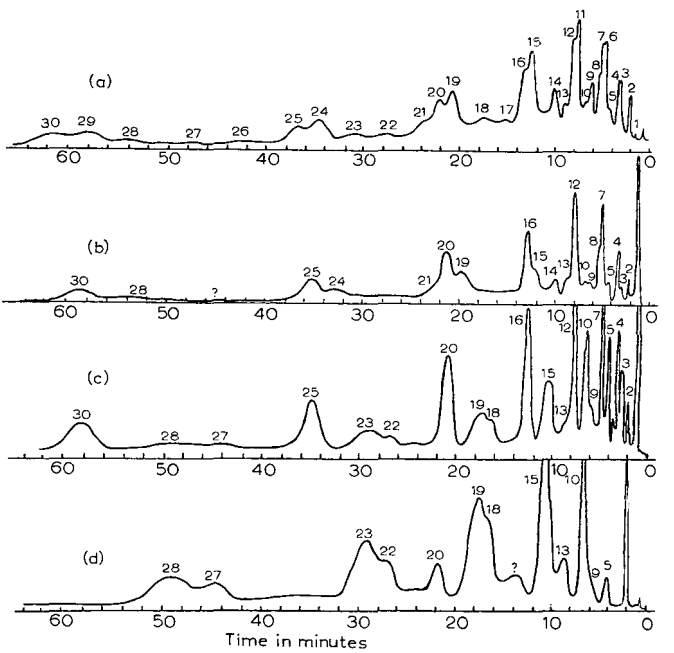

Fig. 5. Chromatograms of light oil neutral oil (a), and the residual oil from it after successive treatment with 80% sulphuric acid (b), 98% sulphuric acid (c), and passage through molecular sieve 5A column at 220° (d). The chromatograms are all on Apiezon L column at 160°. Sample size (a) 5 μl, (b) 8 μl ethereal solution, (c) 10 μl ethereal solution, (d) 6.5 μl benzene solution; carrier gas: He; inlet pressure: 25 lb./sq. in.; outlet pressure: atmospheric; flow rate: 48 ml/min.

consistent with olefinic and aromatic structures. These were also present to varying degree in six components trapped out of it at 160° on silicone (*e.g.* Fig. 6b). Advantage was taken of the splitting of these peaks on Apiezon L at 160° to collect the first of each of a number of pairs separately from the second of each. The spectra are shown in Fig. 6c and 6d. It can be seen that (c) shows bands at about 3050 cm^{-1} (w), 1650 cm^{-1} (m), 995 cm^{-1} (m) and 910 cm^{-1} (s), which are absent or much weaker in (d). These bands are consistent with a vinyl type alkene structure as in a 1-olefin.

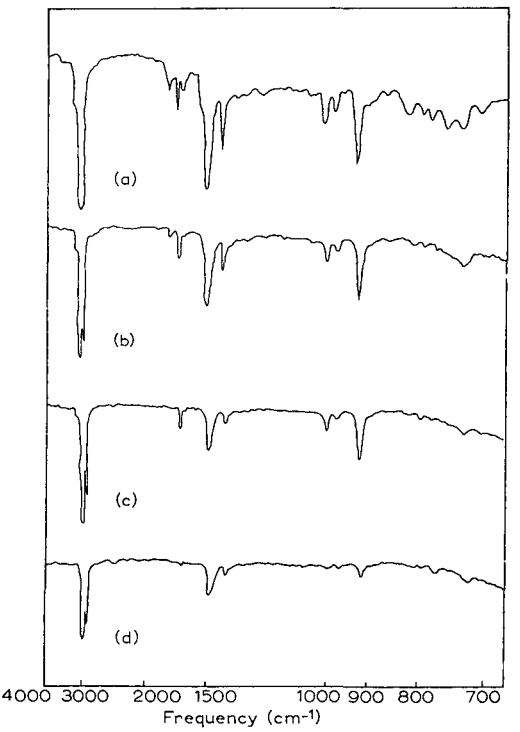

Fig. 6. Infrared spectra of: (a) Neutral oil from light oil. (b) Typical major peak trapped from light oil neutral oil on silicone column at 160°. (c) Combined peaks 11, 15, 19, 24 and 29 trapped from light oil neutral oil on Apiezon L column at 160°. (d) Combined peaks 12, 16, 20, 25 and 30 trapped from light oil neutral oil on Apiezon L column at 160°.

Successive removal of components from light oil neutral oil

This oil was subject to treatment designed to remove successively olefins, aromatics and normal paraffins. The oil was examined after each such treatment. The resulting chromatograms on Apiezon L at 160° are shown in Fig. 5b, 5c and 5d.

The oil was first extracted with 80 % sulphuric acid, giving a volume reduction of 23.3 %. The first of each double peak was either eliminated or drastically reduced. This indicated the presence of a series of olefins (peaks 3, 6, 11, 15, 19, 24, 29 and 31).

Further extraction with 98 % sulphuric acid removed only a few minor peaks (8, 14, 21 and rest of 24), although the reduction in volume corresponded to 51.7 % of the original light oil. This high figure is believed to be the result of the extremely long contact time between the oil and the acid necessitated by emulsion formation,

and is therefore not considered a true indication of the aromatic content. FOWKES *et al.*[14] have noted that a considerable amount of the lower boiling material from the neutral oil from a lignite tar was removed by successive extraction with sulphuric acid of increasing strength.

A sample of 100 μl of the residual oil was next passed through the molecular sieve 5A column at 220° in order to remove the normal paraffins, after the manner of BRENNER AND COATES[15] and WITHAM[16]. The residue was collected and was chromatographed on Apiezon L at 160°. Fig. 5d shows that the peaks eliminated were 3, 4, 7, 12, 16, most of 20, 25, 30 and 32. All of these except 3 were second peaks in the major pairs originally, and were thus shown to correspond to a series of normal paraffins. The part of peak 3 eliminated does not appear to be a normal paraffin, and its origin is unknown.

The graph of log (relative retention time × 100) *versus* carbon number for these peaks is shown in Fig. 7. The straight-line relationship is quite good. On the same graph is a similar line for the peaks 3, 6, 11, 15, 19, 24, 29 and 31, previously shown

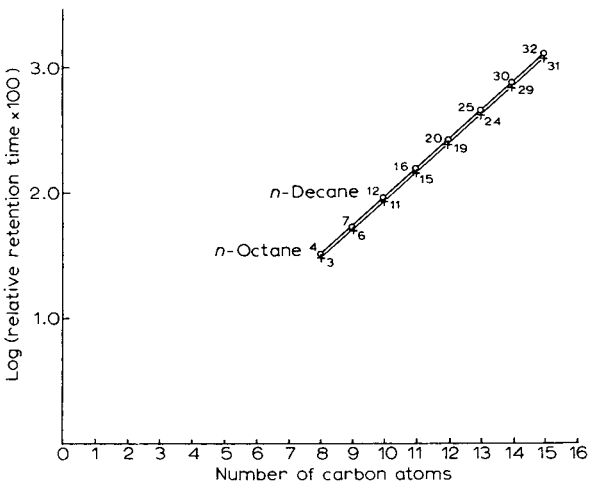

Fig. 7. Graph of log [relative retention time (to mesitylene reference) × 100] *versus* number of carbon atoms for the two series of major peaks from the light oil neutral oil on Apiezon L column at 160°.

to be olefins, on the assumption that the carbon number is the same as the normal paraffin associated with it (no reference substances available). Again the relationship is linear.

The final residual oil showed as major peaks components which were very minor in the original neutral oil. These would be either isoparaffins or naphthenes. It may be noticed that three double peaks, each with the second stronger than the first, are present (18–19; 22–23 and 27–28). These may well be members of two series of isoparaffins.

These results are summarized in Table IV, where the relative retention times of the various peaks to mesitylene as reference are given. Identification of the various components is proposed in the final column. For the branched paraffins and isomeric

TABLE IV

NEUTRAL OIL FROM LIGHT OIL DISTILLATE AT 160° ON APIEZON L

Peak No.	Original relative strength	Relative retention time (mesitylene reference)				Identification proposed
		Originally	After 80% H_2SO_4	After 98% H_2SO_4	After 5A molecular sieve	
1	vw	0.110	Masked by added solvent			?
2	w	0.187	0.184 (w)	0.187 (w)	Masked	*n*-Heptane (0.175) + cyclohexane (0.182) etc.
3	w	0.297	0.273 (w)	0.274 (w)	—	*n*-Octane + ?
4	w	0.319	0.319 (m)	0.314 (m)	—	*n*-Octane
5	vw	0.446	0.447 (w)	0.443 (m)	0.451 (w)	Isononane or naphthene + ?
6	m	0.506	—	—	—	1-Nonene
7	m	0.532	0.531 (m)	0.533 (s)	—	*n*-Nonane
8	vw	0.571	0.570 (vw)	—	—	*m*- and *p*-Xylenes (0.567, 0.570)
9	w	0.685	0.678 (vw)	0.675 (vw)	0.675 (vw)	Isodecene + isodecane or naphthene
10	vw	0.743	0.740 (vw)	0.740 (s)	0.745 (s)	Isodecane or naphthene
11	s	0.858	—	—	—	1-Decene
12	m	0.904	0.897 (s)	0.902 (s)	—	*n*-Decane
13	vw	1.01	0.993 (vw)	1.05 (vw)	1.00 (w)	Isoundecane or naphthene
14	w	1.15	1.15 (w)	—	—	Aromatic *e.g.* pseudocumene (1.16)
15	s	1.44	1.41 (w)	1.25 (s)	1.25 (s)	1-Undecene + isoundecane or naphthene
16	m	1.53	1.52 (s)	1.52 (s)	—	*n*-Undecane
17	vw	1.76	—	—	—	Isododecene
18	vw	2.03	?	2.02 (w)	2.01 (m)	Isododecane or naphthene
19	s	2.43	2.35 (m)	2.09 (m)	2.11 (s)	1-Dodecene + aromatic + isododecane or naphthene
20	m	2.59	2.56 (s)	2.56 (s)	2.65 (m)	*n*-Dodecane + isododecane or naphthene
21	vw	2.76	2.73 (vw)	—	—	Aromatic
22	vw	3.3	?	3.3 (vw)	3.31 (m)	Isotridecane or naphthene
23	vw	3.6	?	3.54 (m)	3.56 (s)	Isotridecane or naphthene
24	m	4.10	3.96 (w)	—	—	1-Tridecene + aromatic
25	w	4.35	4.28 (s)	4.29 (s)	—	*n*-Tridecane
26	vw	5.1	—	—	—	Isotetradecene
27	vw	5.6	?	5.6 (vw)	5.50 (m)	Isotetradecane or naphthene
28	vw	6.4	6.4 (vw)	6.0 (w)	6.05 (m)	Isotetradecane or naphthene
29	w	6.9	—	—	—	1-Tetradecene
30	w	7.3	7.2 (m)	7.20 (s)	—	*n*-Tetradecane
31	vw	11.5	—	—	—	1-Pentadecene
32	vw	12.3	12.0 (w)	12.0 (m)	—	*n*-Pentadecane

olefins the carbon number has been taken in general as the same as the next higher normal member of the series, on the assumption that branched molecules usually have lower boiling points.

An attempt to carry out a similar series of experiments on the tar distillate neutral oil was unsuccessful because the chromatograms could not be run successfully at 160°, which would have been necessary to resolve the peaks. However, the neutral oil from tar distillate (A) was split into three fractions on the silicone column at 220°, and the lowest cut from this was examined by infrared and also chromatographed at 160° on Apiezon L. The spectrum was almost identical to that of the light oil (Fig. 6a) and the chromatogram, part of which is shown in Fig. 8, had the same major peaks as the

light oil except that the second peak (the normal paraffin) in each pair was stronger. These peaks are numbered to correspond with the equivalent peaks in the light oil as shown in Fig. 5a.

The spectra of the two higher boiling fractions of the tar distillate neutral oil tended towards that of paraffin oil which may indicate that olefins are present in

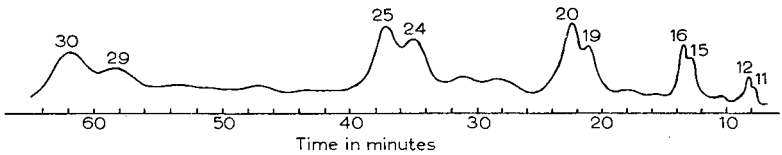

Fig. 8. Part of chromatogram of low-boiling fraction trapped from tar distillate (A) neutral oil on silicone column at 220°, rechromatographed on Apiezon L column at 160°. Sample size: 5 μl; carrier gas: He; inlet pressure: 25 lb./sq.in.; outlet pressure: atmospheric; flow rate: 48 ml/min.

lower amount in these fractions. However, the extent of the decrease is most probably misleading when estimated from the spectra as isolated double bonds would tend to be "swamped" by the long aliphatic chains present in both olefins and paraffins.

CONCLUSIONS

The neutral oils appear to be remarkably aliphatic, consisting of homologous series of normal paraffins and 1-olefins with minor amounts of branched isomers. They resemble the aliphatic fractions from the tars examined by COPPENS, BRICTEUX AND NEURAY[11], but are not associated with a large aromatic fraction as was found by these authors.

The same applies to comparison with the results of BOYER *et al.*[12] while, in addition, the coal carbonized in the present instance has generally a low proportion of the exinite macerals which BOYER *et al.* claimed were responsible for such straight-chain compounds in the tars they obtained.

The composition of the neutral oil would be a most important consideration in its utilization, *e.g.* mild hydrogenation may be used to produce diesel oil.

From the fundamental point of view of coal constitution the presence of such long carbon chains in such a "primary" tar gives rise to interesting questions, *e.g.*: Are there still longer chains in the pitch or is it aromatic in nature? Are such chains present in the coal itself, contrary to present opinion, or are they formed by polymerization of small fragments during carbonization? If so, is low-temperature tar from fluidized beds really so "primary" after all?

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SUMMARY

Acidic and neutral fractions of a light oil distillate and tar distillates produced by the low-temperature fluidized-bed carbonization of a New South Wales bituminous coal were examined by gas chromatography using silicone, Apiezon L and Molecular sieve 5A columns up to 220°.

The separation of the phenols was incomplete, but eleven peaks were tentatively identified. The distribution of phenols in the light oil distillate and in the tar distillate was very similar, the major components being the cresols and certain xylenols.

The neutral fractions were highly aliphatic as shown by infrared spectra of the whole oils and of components collected after chromatographic separation. Successive removal of olefins, aromatics and normal paraffins from the light oil followed by chromatography of the residue in each case showed that normal paraffins and 1-olefins ranging from C7 to C15 (mainly C9 to C13) were the major constituents. The tar distillate appeared to be similarly constituted except that the proportion of olefins was somewhat less. The range was C7 to C21 approximately (mainly C11 to C20). Iso-paraffins, isomeric olefins and perhaps naphthenes were also present in minor amounts in both cases. Very little aromatic material appeared to be present.

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SOLVENT EFFECTS IN INORGANIC CHROMATOGRAPHY

I. FACTORS AFFECTING THE ADSORPTION FRONT

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INTRODUCTION

ZIMMERMANN[1] and SOMMER[2] have investigated the effect of varying the concentration and type of acid and alcohol in various solvent mixtures employed in the determination of the R_F values of a number of metal ions. They also investigated the influence of the variation in the physical properties of various types of papers commonly used in paper chromatography. KERTES AND LEDERER[3] and DECARVALHO[4] have extended their work and have shown the relationship between the R_M value and alcohol concentration, on the one hand, and water concentration on the other. No other significant quantitative approach to the effect of these factors appears in the literature. This paper describes part of an attempt to link the effects of acid concentration, water–alcohol ratio, nature of the acid anion, and the identity of the alcohol (and the interactions between these) on the R_F values of a selected group of metal ions. As there is evidence that at least some metal ions move in a way which is governed by the acid adsorption front, the first step was to study the above effects on the R_P value of this front, and this report is confined to that aspect of the investigation; the R_P value being the ratio—height of adsorption front/height of liquid front—as proposed by POLLARD *et al.*[5].

The approach is through a factorial design, in a series of experiments, in which the above-mentioned factors were varied in a regular manner and the results treated by analysis of variance. This allows the variations in the property of interest (here the R_P value) to be assigned to the different causes of variation and allows the variability due to particular causes to be compared with the experimental error. Unless this is done on a statistical basis interesting effects may not be observed because of the magnitude of uncontrolled experimental variation. The overall design of the experiment and method of treating the data are described in greater detail below.

EXPERIMENTAL

Apparatus and technique employed

Cylindrical glass jars of approximately 30 cm height and 13.5 cm diameter with ground edges and fitted with heavy glass plates as covers were used as development tanks for the running of the chromatograms. The alcohol component of the solvent was used to saturate the vapour of the tank prior to the running of the chromatograms. An empty Petri dish, of approximately 70 ml capacity, was placed in the

alcohol on the bottom of the tank in such a way that the alcohol surrounded it. The tank was covered with the glass plate and allowed to stand for one hour. The solvent was then added to the Petri dish. The chromatograms were made with sheets of Schleicher and Schüll No. 598 paper, 31 × 21 cm formed into a cylinder 21 cm high. The paper was immersed to a depth of 1 cm in the solvent. The tank was covered and left for ¾ to 12 hours depending on the nature of the solvent used. The chromatograms were run to a uniform height of 18 cm above the surface of the solvent pool using the ascending technique of WILLIAMS AND KIRBY[6]. The temperature throughout the experiments was 25 ± 3°. The papers were not equilibrated before the chromatograms were made as MARTIN[7] has shown this to be unnecessary.

Metal ions were also run in these chromatograms but details of the results with these ions will be reported later.

Solvent reagents employed

The first four alcohols of the aliphatic series, methanol, ethanol, propan-1-ol and butan-1-ol and their four isomers, propan-2-ol, 2-methylpropan-1-ol, butan-2-ol, 2-methylpropan-2-ol, were used as the basis of the solvents.

The acids chosen were hydrochloric, hydrobromic, nitric and perchloric. All the alcohols were anhydrous and all the reagents were of A.R. grade.

Individual mixtures of each alcohol, each acid, and water, were made so that the following ratios were obtained:

Alcohol/water: 0.30, 0.35, 0.40, and 0.45 mole fraction of water. To these sufficient of each acid was added to make solutions which were 0.30, 0.60, and 0.90 M with respect to the added acid. The water in these mixtures included the water normally present in each acid used. A minimum accuracy of ± 1.0 % was used in all measurements of volume in the preparation of the solvents. The R_P values of all these solvents are given in Table I.

Design of the experiment

A factorial design was adopted for the experimental arrangement, since this permits not only the average effects of the factors to be determined but also their "interactions" *i.e.* effects due solely to particular combinations of factors. These experimental designs are described in works on statistical analysis and methods[8].

In this investigation the characteristics of the experimental design are:

(i) Four factors designated for convenience by capital letters, *viz.*, A = alcohols, B = acid anions, C = water content, and D = acid concentration.

(ii) Several levels of each factor (by variation of which the effect of the factor as such is determined) designated by a number with a lower case letter for generality, *viz.*, $a = 1, 2 \ldots 8$ for eight individual alcohols, $b = 1, 2, 3, 4$ for the anions, $c = 1, 2, 3, 4$ for the water contents, and $d = 1, 2, 3$ for the acid concentrations.

(iii) Single replication, by which one value of the property of interest (R_P) is obtained for each and every combination of factor levels, $8 \times 4 \times 4 \times 3 = 384$ in all.

(iv) A "fixed effect" or "regression" model, interest being devoted to the actual factor levels tried, *i.e.* the specific alcohols, anions and concentrations.

According to the model adopted we may write for any individual value in any such table:

$$X_{abcd} = \mu + \alpha_a + \beta_b + \gamma_c + \delta_d + (\alpha\beta)_{ab} + \ldots + (\alpha\beta\gamma)_{abc} + \ldots + \varepsilon_{abcd}$$

TABLE I

R_P VALUES FOR ALL SOLVENTS

The letters *A*, *B*, *C*, *D*, refer to, *A* identity of alcohol, *B* identity of anion, *C* mole fraction of water in solvent, and *D* molar concentration of acid in solvent. The alcohols are given in the following order indicated under the heading "Solvent reagents employed": 1 = methanol; 2 = ethanol; 3 = propan-1-ol; 4 = propan-2-ol; 5 = butan-1-ol; 6 = 2-methylpropan-1-ol; 7 = butan-2-ol and 8 = 2-methylpropan-2-ol.

A	*B*	*C* = 0.35			0.40			0.45			0.50 *mf*		
		D = 0.3	0.6	0.9	0.3	0.6	0.9	0.3	0.6	0.9	0.3	0.6	0.9 *M*
1	Cl	0.85	0.86	0.95	0.83	0.85	0.88	0.85	0.82	0.81	0.85	0.88	0.90
	Br	0.85	0.84	0.86	0.86	0.90	0.89	0.88	0.88	0.86	0.83	0.88	0.90
	NO_3	0.84	0.87	0.92	0.94	0.95	0.89	0.86	0.93	0.92	0.83	0.92	0.93
	ClO_4	0.83	0.96	0.88	0.85	0.87	0.88	0.93	0.86	0.86	0.85	0.91	0.88
2	Cl	0.80	0.88	0.88	0.90	0.85	0.88	0.85	0.85	0.88	0.88	0.84	0.86
	Br	0.89	0.87	0.90	0.85	0.84	0.87	0.85	0.84	0.87	0.82	0.86	0.88
	NO_3	0.82	0.88	0.92	0.93	0.88	0.93	0.84	0.88	0.92	0.88	0.90	0.90
	ClO_4	0.87	0.89	0.94	0.88	0.91	0.92	0.88	0.90	0.97	0.85	0.88	0.90
3	Cl	0.80	0.83	0.80	0.73	0.80	0.83	0.76	0.79	0.82	0.78	0.80	0.83
	Br	0.75	0.83	0.87	0.80	0.81	0.82	0.77	0.87	0.88	0.81	0.86	0.89
	NO_3	0.77	0.90	0.86	0.86	0.85	0.90	0.80	0.85	0.92	0.81	0.85	0.87
	ClO_4	0.83	0.92	0.97	0.80	0.90	0.91	0.86	0.88	0.91	0.90	0.88	0.94
4	Cl	0.74	0.82	0.83	0.80	0.83	0.82	0.76	0.82	0.88	0.81	0.88	0.87
	Br	0.77	0.80	0.92	0.74	0.81	0.90	0.82	0.90	0.89	0.77	0.87	0.93
	NO_3	0.78	0.87	0.91	0.84	0.89	0.90	0.80	0.85	0.88	0.82	0.88	0.96
	ClO_4	0.84	0.94	0.96	0.87	0.92	0.95	0.92	0.92	0.95	0.90	0.95	0.96
5	Cl	0.65	0.75	0.82	0.66	0.70	0.83	0.72	0.77	0.84	0.68	0.72	0.87
	Br	0.76	0.74	0.77	0.70	0.75	0.77	0.68	0.83	0.75	0.73	0.76	0.79
	NO_3	0.72	0.79	0.85	0.73	0.88	0.92	0.76	0.88	0.93	0.84	0.81	0.83
	ClO_4	0.77	0.88	0.81	0.78	0.86	0.90	0.81	0.87	0.86	0.79	0.85	0.88
6	Cl	0.65	0.68	0.73	0.66	0.67	0.78	0.62	0.68	0.75	0.67	0.70	0.78
	Br	0.72	0.70	0.75	0.66	0.74	0.77	0.70	0.73	0.77	0.65	0.73	0.76
	NO_3	0.72	0.77	0.83	0.84	0.79	0.84	0.80	0.78	0.81	0.76	0.84	0.82
	ClO_4	0.77	0.85	0.92	0.78	0.87	0.87	0.79	0.82	0.88	0.78	0.84	0.89
7	Cl	0.76	0.74	0.82	0.63	0.72	0.78	0.69	0.73	0.79	0.69	0.82	0.79
	Br	0.78	0.76	0.84	0.72	0.77	0.86	0.72	0.77	0.86	0.73	0.88	0.82
	NO_3	0.83	0.82	0.88	0.78	0.83	0.88	0.76	0.82	0.86	0.80	0.85	0.87
	ClO_4	0.83	0.90	0.95	0.84	0.88	0.95	0.82	0.90	0.94	0.84	0.89	0.94
8	Cl	0.70	0.77	0.70	0.72	0.73	0.91	0.72	0.78	0.90	0.76	0.80	0.88
	Br	0.72	0.87	0.96	0.75	0.83	0.93	0.80	0.87	0.95	0.78	0.85	0.94
	NO_3	0.79	0.88	0.96	0.80	0.92	0.94	0.81	0.88	0.94	0.83	0.93	0.70
	ClO_4	0.83	0.95	0.98	0.87	0.94	1.00	0.85	0.94	1.00	0.82	0.92	1.00

where μ is a general constant estimated by the grand mean of all the values in the table, α_a etc. are particular corrections for individual factors, $(\alpha\beta)_{ab}$ etc. are corrections for particular combinations of factors (there are six such first order interaction terms), $(\alpha\beta\gamma)_{abc}$ etc. are corrections for particular combinations of levels of three factors (there are four such second order interaction terms), and ε_{abcd} is a random error distributed normally with zero mean and a variance (σ^2), which is independent of particular factor levels. In the analysis of the data, the total variability of the values

of X, measured by the sum of the squares of their deviations from the grand mean *i.e.* $\Sigma(X_{abcd} - \overline{X})^2$, is divided into parts which can be attributed to the factors alone and in combination, and a remainder which cannot be so accounted for and is largely due to ordinary experimental errors of measurement. The analysis of variance table (Table II) illustrates this partitioning. A comparison of the mean variability due to change of levels within a factor with the mean residual variability (an estimate of the distribution of errors, σ^2) indicates whether or not such changes in factor levels have a significant effect (*i.e.* greater than can be explained by random variation) on the values of the variable of interest.

DISCUSSION OF THE EXPERIMENT

The main restriction on the observations which could be made was the desire to work within a single batch of paper. This limited the number of separate chromatograms to about 400, but it permitted a considerable number of interesting alcohols to be included as well as several different acids. In the case of quantitative factors, such as C and D, three equally spaced levels are sufficient to detect a curvilinear relationship and this was the number of acid concentrations adopted, but four different water contents were included because it was desired to cover the range of water content as well as possible and it was not certain that the top level could be achieved in all cases. Testing for every combination of the eight alcohols, four anions, four water concentrations and three acid concentrations involved 384 individual chromatograms. These were run in random order of time (the order being taken from a table of non-repeating random numbers[9]) so as to prevent minor variations in experimental conditions from influencing the effects of the actual factors under investigation.

Choice of experimental pattern

The general pattern of inorganic chromatography has been formed around a choice of organic solvents in which a considerable amount of water (at least 10 %) would dissolve and which, on the addition of an acid or complexing agent, would differentiate between some inorganic cations. This pattern has been continued in this study with certain limitations imposed partly by the nature of the materials chosen and partly by the necessity of keeping the experimental design within bounds which could be handled with reasonable ease.

The first four of the saturated aliphatic alcohols and their isomers were chosen as they were readily available in a high degree of purity and have a suitable solvency for water. Other types of solvent (*e.g.* ketones and ethers) were excluded so as to avoid too diverse a range of chemical effects and to limit these to purely structural changes. Moreover alcohols have been the basis of most solvents. The ratio of water to alcohol was limited, at the highest concentration by mutual solubility and at the lowest by the maximum concentration of acid which could be obtained. It did, however, cover the range of the majority of normal solvents.

Acid concentration was limited by the experimental design, as the strongest aqueous hydrobromic acid was only $8M$ solution and so did not permit of much greater concentration in the solvent than the $0.9M$ solution used. The limitations of the experimental framework still permitted the ratio of water to acid to range from 57 to 1 down to 6 to 1 on a molar basis.

The four acids were all "strong" acids. The halogen acids were capable of forming anionic complexes with some of the metals chosen while in the case of nitric and perchloric this was not likely.

DISCUSSION OF RESULTS

We concentrate on the R_P value here, since the complete treatment of the R_F has not yet been undertaken, nor have we yet investigated the relationship between the R_F and the R_P values.

The analysis of variance table (Table II) shows that the R_P values are very considerably affected by changes in alcohol, in anion, in acid concentration, and to a lesser extent in water concentration. There is a dubious connection between anion and water concentration, but the effect of acid concentration varies, highly significantly, from alcohol to alcohol (alcohol–acid concentration interaction).

TABLE II

ANALYSIS OF VARIANCE OF R_P VALUES

The level of significance is indicated by the number of asterisks. One asterisk (*) indicates significance at better than the 5% level, two asterisks at better than the 1% level, and three asterisks at better than the 0.1% level.

Source of variation	*Sum of squares*	*Degrees of freedom*	*Mean square*	*Ratio to residual*	*Significance*
A Main effect	5994.33	7	856.33	109.1	***
B Main effect	5500.35	3	1833.45	233.6	***
C Main effect					
linear	73.63	1	73.63	9.381	**
quadratic	0.85	1	0.85	0.108	
cubic	0.25	1	0.25	0.032	
D Main effect					
linear	4865.06	1	4865.06	619.8	***
quadratic	18.75	1	18.75	2.389	
AB Interaction	1349.99	21	64.29	8.191	***
AC Interaction	188.44	21	8.97	1.143	
AD Interaction					
linear	855.56	7	122.22	15.57	***
quadratic	47.54	7	6.79	0.865	
BC Interaction					
linear	55.72	3	18.57	2.365	
quadratic	48.84	3	16.28	2.074	
cubic	77.86	3	25.95	3.306	*
BD Interaction	31.28	6	5.21	0.664	
CD Interaction	56.58	6	9.43	1.201	
ABC Interaction	459.07	63	7.29	0.929	
ABD Interaction	466.64	42	11.11	1.415	
ACD Interaction	335.50	42	7.99	1.018	
BCD Interaction	254.83	18	14.16	1.804	*
Residual	988.92	126	7.85		
Total	21670.00	383			

Fig. 1 (a) shows the overall variation of R_P values from alcohol to alcohol. The values are much lower for the butanols (except *tert.*-butanol) than for the remainder. This is believed to be related to the fact that *n*-butanol, isobutanol and *sec.*-butanol are only partly miscible with water, while all other alcohols are completely miscible.

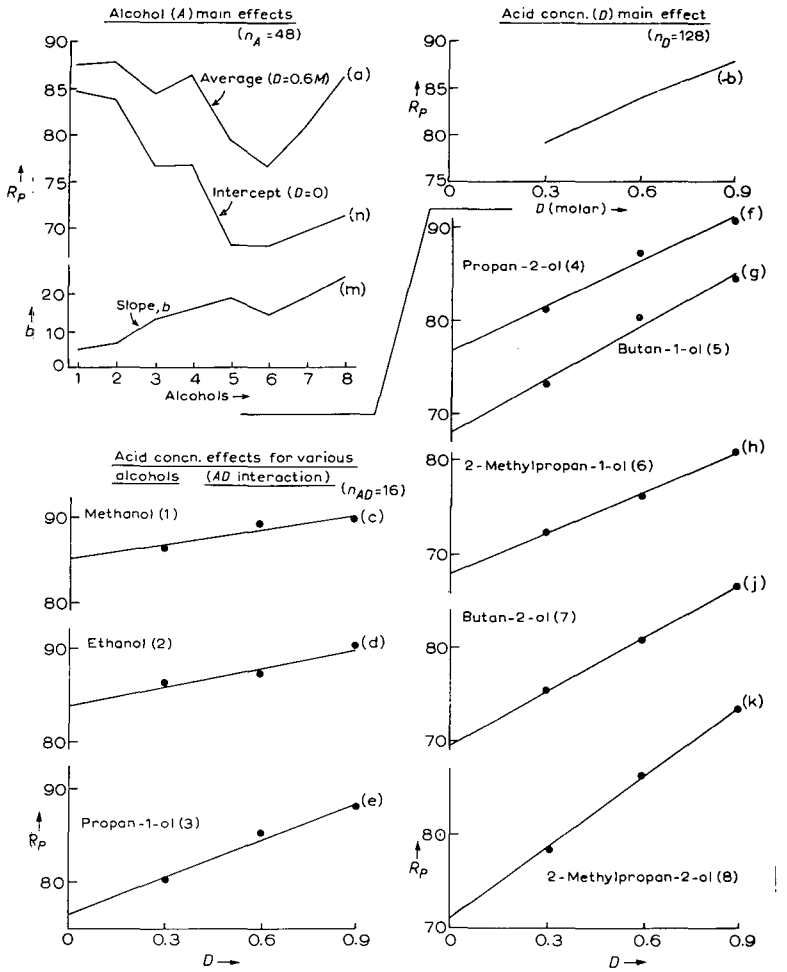

Fig. 1. Graphical representation of the R_P values showing the effects of (a) change in alcohol (means of 48 values for each of the eight alcohols in order: methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol, 2 methylpropan-1-ol, butan-2-ol, 2 methylpropan-2-ol); (b) acid concentration (means of 128 values for each of three concentrations); (c) to (k) acid concentration for individual alcohols (means of 16 values); (n) R_P values extrapolated to zero acid concentration for the eight alcohols (intercepts of curves (c) to (k)); (m) rate of change of R_P with acid concentration for the eight alcohols (slopes of curves (c) to (k)).

The upper right-hand curve, Fig. 1 (b), shows the average effect of acid concentration, the relationship to R_P being strictly linear over the range of concentration studied. The analysis of variance indicates, however, a very strong interaction between alcohol and the linear component of acid concentration which means that the actual slope of the R_P *versus* concentration lines varies significantly from alcohol to alcohol. The eight lines, Fig. 1 (c) to Fig. 1 (k), are shown on the lower part of the figure. The intercepts (in effect R_P extrapolated to zero acid concentration) and the slopes of these significantly different lines are plotted in the upper left-hand graph of Fig. 1. The

intercepts, curve Fig. 1 (n), show the same kind of general variation from alcohol to alcohol as the overall averages, except that all the butanol values are now low by comparison with the simpler alcohols. The slopes, curve Fig. 1 (m), on the other hand show a general rise over the alcohol series. That is to say, the effect of higher concentration in raising the acid front is the greater, the greater the complexity of the alcohol molecule.

The linear relationship of R_P to acid concentration is explicable on the assumption that the range of concentrations used lies on a linear or nearly linear portion of a Langmuir isotherm (MARTIN[10]).

The upper left-hand graph, Fig. 2 (a), shows the very marked differences in the overall averages for the four anions of the acids employed, the values following the lyotropic series, frequently quoted from ZECHMEISTER[11], except that the positions of Cl^- and Br^- are in reverse of the usual order (which goes Br^-, Cl^-, NO_3^-, ClO_4^- in strengths of adsorption). Nor does the order found quite agree with the relative strengths of the acids in glacial acetic acid as reported by BELL[12] although in this case it is the nitrate and bromide which are transposed.

The effect of changes in the water to alcohol ratio (in the regions investigated) is relatively slight, although statistically very significant by comparison with the random error. The overall averages change linearly with R_P, curve Fig. 2 (b). The analysis shows, however, a significant anion–water concentration (cubic) interaction. This means that the up-down alternation, rather than slope or curvature, of the averages for the four concentrations differ between anions by more than can be explained by the random error. In spite of this statistical significance we incline to the belief that the effect is actually spurious and can offer no explanation for such behaviour.

Lastly, the analysis indicates a highly significant alcohol–anion interaction, *i.e.* the effect of a particular anion on R_P value differs from alcohol to alcohol and *vice versa*. The lower curves, Fig. 2 (c) and (d), attempt to show this. The values plotted are not the actual average R_P for particular alcohol–anion combinations, but those values adjusted for general alcohol and general anion variations. These represent particular discrepancies due to the isolated effect of alcohol–anion combinations above and below the grand mean (centre lines). The lines equidistant above and below the grand mean line represent the extent of variations which could be accounted for by ordinary experimental error. The nature of the interaction can be clearly seen—a fall over the 8 alcohols in the presence of chloride changing over to a rise in the presence of perchlorate—a fall over the four anions, in the presence of methanol and ethanol, changing over to a rise in the presence of the more complex butanols.

Attention must again be drawn to the proportions of the solvent constituents, there being a 10-fold range of water to acid when considered in molar proportions. There seems little doubt that the proton will have at least one molecule of water associated with it, and previous work (MARTIN[10]) indicates that it may be greater than one in the case of *n*-butanol. Of the total quantity of water in the solvent some is directly adsorbed by the cellulose. A further indefinite quantity accompanies the proton or hydronium ion or hydrated hydronium ion. This leaves a greatly diminished quantity available for hydration of the anion with the possible situation of this position of the solvent being solvated partly by water and partly by alcohol. This may explain the very significant alcohol–anion type interaction (A–B interaction) and the alcohol–anion concentration interaction (A–D interaction).

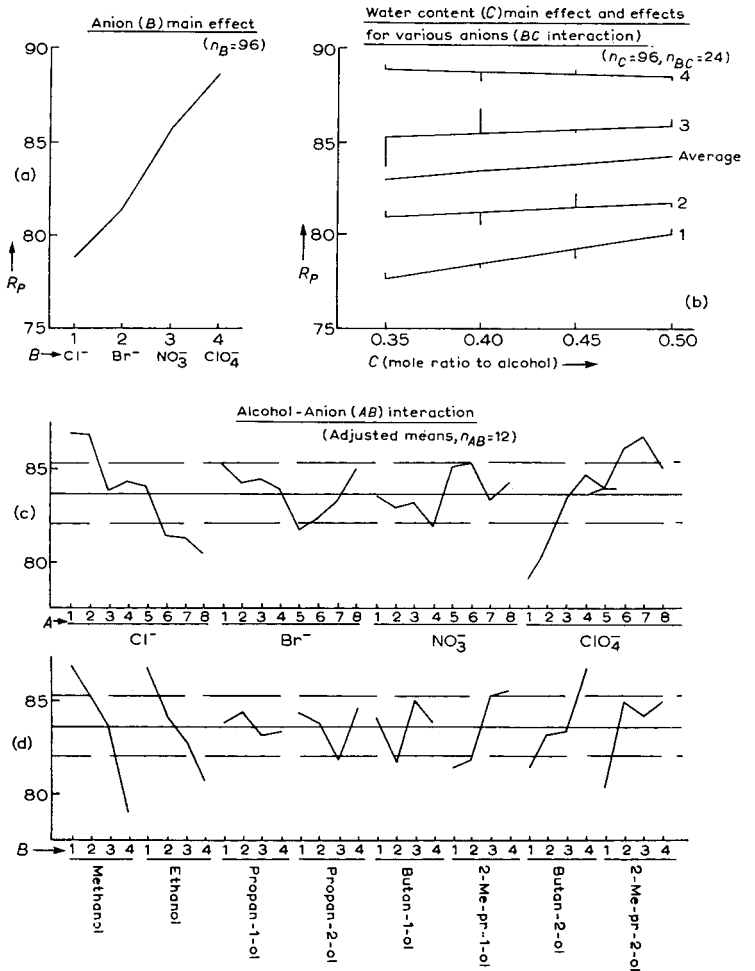

Fig. 2. Graphical representation of the R_P values showing the effects of (a) change in acid anion (means of 96 values for the four acid anions in order: Cl^-, Br^-, NO_3^-, ClO_4^-); (b) water/alcohol ratio (means of 96 values showing overall variation (central curve) and means of 24 values for each of the four anions (1 = Cl^-, 2 = Br^-, 3 = NO_3^-, 4 = ClO_4^-); (c) change in alcohol for individual anions (adjusted means of 12 values); (d) change in anion for individual alcohols (adjusted means of 12 values).

The type of experimental design adopted is that best fitted for elucidating the average changes in values of a property caused by changes in factors and for distinguishing those changes from the effects of combinations of factors. In this case the most significant features revealed are:

(i) the close interdependence of the effects of anions of the acids and the alcohols,

(ii) the variation of the effect of acid concentration from alcohol to alcohol.

SUMMARY

This paper describes a series of tests designed to elucidate the effects of varying the

alcohol and acids as well as the concentration of water and acid on the R_P value of solvents consisting of these three constituents. The first four aliphatic alcohols and their four isomers propan-2-ol, 2-methylpropan-1-ol, butan-2-ol, 2-methylpropan-2-ol, and hydrochloric, hydrobromic, nitric, and perchloric acids were used. The acid concentration was varied from 0.3 to 0.9 M and the water to alcohol ratio from 0.35 to 0.50 mole fraction. The results obtained from 384 chromatograms were treated by analysis of variance to show that the R_P value was linearly related to the acid concentration and water concentration although the latter had little effect on the result. The interactions between the acid anion and the alcohol and the acid concentration and the alcohol were highly significant.

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THE DISTRIBUTION OF ACIDS, WATER AND BUTAN-1-OL ON CHROMATOGRAMS MADE BY THE ASCENDING TECHNIQUE

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In a previous paper[1] the author has described the way in which butanol and water were distributed on an ascending chromatogram. As most chromatographic solvents contain an acid as well as the other constituents, the factors governing the distribution of acid are of importance and the aim of this paper is to show some of the relations which hold during chromatography.

EXPERIMENTAL

The work was divided into two broad groups. The first of these dealt with solvents made by varying the proportions of butan-1-ol, hydrochloric acid and water, while the second was concerned with variations in the nature of the acid while still using butanol and water as the basis of the solvent.

The method used in making the chromatograms was the same for both groups and has been described previously[1]. However, there were differences between the two groups in regard to the paper used and the height to which the chromatogram was ran. In the first group the chromatograms were 18 cm long and made on Schleicher and Schüll 598 paper. For the other group Munktell's 20/150 paper was used and the height of the chromatogram was 20 cm.

Butanol–water–hydrochloric acid series

The solvents were of two types for the butanol–water–hydrochloric acid series. The first type was made by shaking equal volumes of aqueous acid and butanol together for a period of 4–5 min and then using the butanol layer as solvent and the aqueous layer to saturate the atmosphere of the development vessel. Four solvents were made based on aqueous solutions of hydrochloric acid of the following concentrations, 0.1, 0.5, 1.0 and 2.0 *M*. The other type of solvents was made by taking a butanol–water mixture of 0.3 mol fraction of water and adding dry HCl gas to form a series of six solvents which had acid concentrations, in the solvent itself, varying from 0.04 *M* to 1.19 *M* (see Table I).

Series using butanol–water and various acids

The second group of solvents was prepared by shaking equal volumes of butanol and 2 *M* aqueous acid. The acids were tartaric, acetic, hydrochloric, hydrobromic, nitric and perchloric acid. The actual concentrations of these acids in the solvents is given in Table II.

Butanol for the solvents was prepared from a commercial product by fractional distillation. The fraction boiling between 116° and 118° was taken. It was shown to be free from significant quantities of acids and aldehydes. The water was freshly distilled from glass apparatus. All acids used were of analytical reagent grade.

The pattern of sampling and methods for the determination of water and total solvent were similar to those described in a previous paper[1]. However the following modifications were necessary because of the acid in the solvent. Extra samples were taken immediately below and above the adsorption front which was apparent as a brown line on the paper. The acid concentration in the solvent and on the paper was determined by titration with sodium hydroxide solution to a potentiometric end point appropriate to the acid being titrated. Where water concentrations were determined, two separate chromatograms were made. The water content was determined on one, and, on the other, the total solvent and acid concentration.

The results of these tests are reported in a series of figures in which the concentration of each solvent constituent on the paper is plotted against the mean height of the sample taken from the chromatogram. Conductivities were determined with a Phillips conductivity bridge and cell. These measurements were made at 25°.

DISCUSSION

The distribution of water and butanol

Where the relative properties of butanol and water in the solvents were similar to those of solvents previously described, it was found that the distribution of these constituents on the chromatogram was almost the same as for the previous solvents. One modification, however, was significant. A dip in the curve representing water concentration *versus* height occurred at or near the height corresponding to the adsorption front. The lower concentration ahead of the adsorption front was presumably due to the absorption of some water with the hydronium ion. The extent of the change

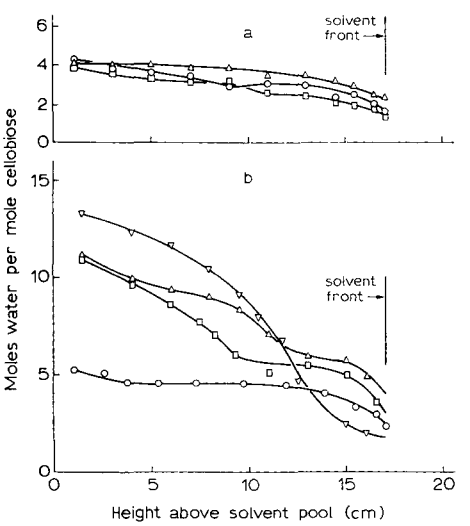

Fig. 1. The distribution of water on paper chromatograms made using both series of solvents.

was indefinite and did not seem to bear a constant relationship to the hydronium ion concentration in the solvent. This was true of both series of experiments though the change in aqueous concentration was smaller for the series in which the total butanol–water ratio in the solvent was fixed. Examples for each series are given in Fig. 1.

Acid distribution

Although the general pattern of acid distribution was the same for both series of experiments (see Fig. 2), the relationship between R_P value and acid concentration differed. For the first series, in which the solvent was made by shaking butanol with

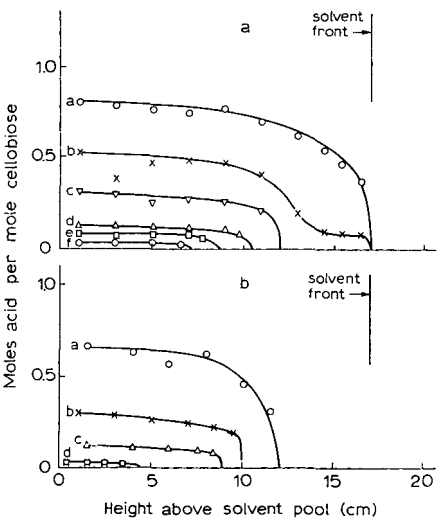

Fig. 2. The distribution of hydrochloric acid on paper chromatograms made using both series of solvents.

an aqueous acid until at equilibrium, the R_P values were related to the logarithm of the actual concentration of the acid in the solvent by the relationship:

$$R_P = n + k \log C_{acid}$$

as is shown by the following data:

Aqueous acid concentration (M)	0.1	0.5	1	2
Actual concentration in solvent (M)	0.014	0.11	0.27	0.84
Log (acid concentration $\times$ 10^2)	1.146	2.041	0.431	2.924
R_P	0.25	0.50	0.59	0.70

Later experiments showed that the linearity of this relationship is limited to R_P = 0.85. Above this value the region of complete miscibility of aqueous acid and butanol is approached. It was found that the constants n and k were, at least partly, properties of the filter paper used.

No simple relationship was found for the second series of solvents.

Nature of acid adsorption

The hypothesis that the hydronium ions of the solvent are adsorbed to produce the adsorption or acid front may be tested as follows. As it has been found[1] that the paper swells during the course of a chromatogram approximately in proportion to the water concentration of the solvent, the series of solvents which have a constant butanol–water ratio were chosen for this study. It can be assumed that all chromatograms were swollen to about the same amount.

The total weight of acid A_t at any height on a chromatogram is then equal to the sum of the weights of adsorbed acid (A_a) and the acid remaining in solution (A_s), the quantities being referred to 1 g of cellulose. The solvent is assumed to travel sufficiently slowly for equilibrium to be reached for the adsorption process.

Thus:

$$A_t = A_s + A_a \tag{1}$$

The weight of acid in any section may then be taken as $A_s = V_s \times C_a$, where V_s is the volume of solvent and C_a the acid concentration.

Further, if it is assumed that the hydronium ions are held on the cellulose by some type of adsorption the adsorbed acid can be described by an equation of the Langmuir isotherm type.

$$A_a = \alpha\beta C_a/(1 + \beta C_a) \tag{2}$$

where α and β are constants.

Hence:

$$A_t = V_s C_a + \alpha\beta C_a/(1 + \beta C_a) \tag{3}$$

Eqn. (2) may be arranged to give:

$$\frac{C_a}{A_a} = \frac{1}{\alpha\beta} + \frac{C_a}{\alpha}$$

a standard procedure for testing such data.

Table I shows the values of some of the terms involved in the latter relationship calculated, where necessary, from the data given in Fig. 2. α here is taken as the number of moles of acid required completely to cover 1 g of cellulose (paper).

From the data in Table I, a regression line was calculated which had a slope of 1900 and intercepted the ordinate ($C_a = 0$) at 1100. The correlation coefficient of

TABLE I

VALUES OF SOME TERMS INVOLVED IN EQN. (2)

C_a is molar concentration; A_t, V_sC_a and A_a are moles acid/g of paper $\times 10^5$.

C_a	A_t	V_sC_a	$A_a = A_t - V_sC_a$	$C_a/A_a \times 10^{-3}$
0.0412	10.5	7	4	1.03
0.0986	28.7	18	11	0.90
0.181	41.4	31	10	1.81
0.445	103	79	24	1.85
0.766	165	140	25	3.06
1.19	252	214	38	3.12

0.902 lies between the 0.02 and 0.01 levels of significance for four degrees of freedom. The Langmuir isotherm can therefore be claimed satisfactorily to present the data.

The parameters α and β are related to surface area and heat of adsorption respectively so the adsorption hypothesis may be tested further through these values.

From the slope of 1900 a value of $5.2 \cdot 10^{-4}$ is obtained for α. This gives an area of 33 m^2/g of paper when calculated using 10.5 $Å^2$ (calculated from ionic radii) as the area occupied by an hydronium ion. It lies within the range of 10–100 m^2/g found by FORZIATI *et al.*[2]. This is not inconsistent with the area obtained by calculation, assuming the 10.8 Å × 8.4 Å surface of the cellulose crystal is exposed with its six hydroxy groups available for hydrogen bonding. Further, MASON[3] has shown that the "freeness" of a paper pulp is related to the surface area of the fibre and freeness determinations made on a sample of the paper used in these experiments suggested a value of 11 m^2/g; a value in reasonable agreement with that calculated above considering the diverse conditions of determination.

Heat of adsorption

An approximate heat of adsorption may be calculated from DE BOER's[4] expression:

$$\beta = \frac{N\tau}{\sigma_0\sqrt{2\pi MRT}}$$

where N, M, R, T are, respectively, Avogadro's number, the molecular weight of the adsorbed particle, the gas constant and the absolute temperature. τ is the time of adsorption and σ_0 the number of particles which can occupy 1 cm^2 of surface. Calculated from α, this gave a value lying between $6.5 \cdot 10^{11}$ and $2.9 \cdot 10^{12}$ for σ_0, while β, obtained from the intercept of the regression line mentioned above, was 1.9.

Q, the heat of adsorption, may be calculated from Frenkels relationship (*vide* DE BOER[4])

$$Q = \tau_0 \exp. Q/RT$$

τ_0 being the period of vibration of the adsorbed particle. It was taken as 10^{-13} sec in this case, a value given by DE BOER for the water molecule and probably not greatly in error for the hydronium ion.

These calculations give a heat of adsorption of 0.9 kcal/mol which, as a net value representing the heat change between solution and adsorption, is not inconsistent with the hypothesis of a hydrogen-bonded hydronium ion.

Composition of solvent on the chromatogram

It is interesting to compare the concentrations of the constituents in the solvent at various heights on the chromatogram, with their concentrations in the original solvent.

No data could be found in the literature on the partial pressures for the ternary system butanol–water–HCl. It seems reasonable to assume that the presence of hydrochloric acid in the butanol–water mixture would lower the partial pressure of the water in much the same way as the partial vapour pressure of aqueous hydrogen chloride is lowered by increasing the concentration of acid[5]. Weight balances were calculated on the basis of the acid concentrations and in most cases a slight loss of water was found. This was greater in the series where water was controlled in the

solvent and almost zero for the other series. The butanol loss, however, was much greater than the water losses in both cases and in this a comparison may be drawn with the results of the work with butanol and water solvents.

Fig. 3 shows a considerable variation between the acids. The curves for the strong acids, HCl, HBr, $HClO_4$ and HNO_3 are similar in outline and such differences as

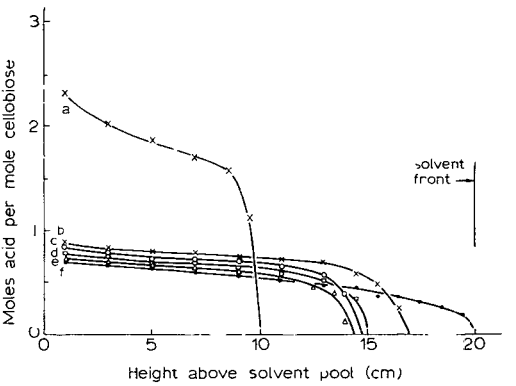

Fig. 3. The comparative distributions of acids on chromatograms using solvents made by shaking butanol with 2 N aqueous acid.

appear could be attributed to differences in concentration (Table II) were it is not noticed that HBr ($R_P = 0.75$) takes a position below nitric acid ($R_P = 0.79$) although the concentrations would suggest that they should be placed in the opposite order. No clear reason for this has been established.

There is a marked difference between the behaviour of the solutions containing tartaric acid and those made with acetic acid. The tartaric acid solution, of a concentration comparable with the strong acids, shows from two to three times the adsorplion by the paper. This resulted in all the tartaric acid being removed by an R_P value tess than 0.5, compared with the 0.7–1.0 of the other acids.

TABLE II

R_P VALUES FOR SEVERAL ACIDS

Acid	*Concn. acid in solvent (N)*	R_P
HCl	0.689	0.72
HNO_3	0.813	0.79
HBr	0.831	0.75
$HClO_4$	0.892	0.85
Acetic acid	1.091	1.00
Tartaric acid	0.893	0.47

Acetic acid solutions on the other hand gave R_P values of 1.0 even when the concentration of acid was reduced to 0.25 M in the original solvent.

In aqueous solution tartaric acid is dissociated about 150 times as strongly as

acetic acid. This does not appear to be the case in the butanol–water mixtures where the difference in equivalent conductance is small, 0.4 compared with 0.06. The equivalent conductance for hydrochloric acid is 350 in butanol–water solution under the same conditions. It is concluded that the difference between the acetic and tartaric acids lies not in differences of dissociation, but in the mode of their adsorption, the tartaric acid being adsorbed by hydrogen bonding through one of its hydroxyl groups, while acetic acid, possibly hydrogen bonded internally, is only very weakly adsorbed.

ACKNOWLEDGEMENT

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SUMMARY

The distribution of hydrochloric acid, water and butan-1-ol on paper chromatograms is described for solvents made by saturating the butanol with aqueous acid and by adding dry HCl to solvents of fixed butanol–water ratios. It is shown that the hydronium ion is adsorbed onto the cellulose, probably by hydrogen bonding, thus giving rise to the adsorption fronts. The adsorption process may be described by the Langmuir isotherm. The variation to be found between a number of both weak and strong acids is also described.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

V. TAUTOMERISM. THE DETERMINATION OF TAUTOMERIC EQUILIBRIUM BY PAPER CHROMATOGRAPHY. THIENOL AND *p*-NITROSOPHENOLS

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INTRODUCTION

If the introduction of a group X into a compound produces a new structure that can exist in tautomeric forms, its effect on R_M will be different from the homologous increment $\Delta R_M(X)$.

Tautomerism is possible in many types of compound, although its existence is often difficult to establish with certainty. It is most clearly established when both tautomers are sufficiently stable to be capable of independent isolation. Several classic cases of keto–enol and nitroso–oxime tautomerism fall into this category. In certain instances—as with 1,4-naphthaquinol, described below—the individual tautomers are exceptionally stable and, chromatographically, can be dealt with as two separate species; but, in other compounds, interconversion is more rapid and can be expected to occur during chromatography. Most compounds in which tautomerism is postulated are not in fact separable into two forms at all, and the tautomerism is implied by either chemical or physical evidence, sometimes both. (Chemical and physical evidence often fail to agree as to the existence of tautomerism: sometimes they agree but lead to diametrically opposed conclusions as to the nature of the tautomeric form; *cf.* the conflicting evidence as to keto–enol forms in phloroglucinol, and HODGSON[1,2] and ANDERSON AND YANKE[3] on the structure of *p*-nitrosophenol.)

The problem of tautomerism cannot be neglected in any discussion on the relation between chromatography and chemical structure. It is, in many ways, a more complex problem than other "constitutive" effects on R_M. Indeed, it is not strictly speaking a constitutive effect at all, since if a compound is tautomeric it must be treated from the theoretical point of view as a mixture of two compounds, in each of which additional constitutive effects on R_M(hydrogen bonding, steric and polar interaction) may occur. There are three reasons why tautomerism may be of especial importance in chromatographic studies. Since tautomerism in most cases involves the mobility of a hydrogen atom the two forms can be expected to differ chromatographically to a large extent. Secondly, chromatographic separation and identification is particularly valuable in the study of complex molecules, precisely those in which the possibility of tautomerism is increased and in which tautomers may have increased stability because of increased possibilities of resonance stabilisation. Finally, tautomeric equilibrium is

markedly affected by solvents: for example, ethyl acetoacetate contains 0.4% of enol form in water, but 46.4% in hexane[4]. Therefore, under the conditions of partition chromatography, providing interconversion of tautomers can take place rapidly enough, each phase may contain the two tautomeric forms in different proportions.

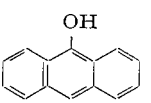
THE NATURE OF TAUTOMERISM IN PHENOLS AND *p*-NITROSOPHENOLS

The problem of tautomerism in phenols has been reviewed by THOMSON[5]. The chemical and physical properties of phenol itself indicate that it exists almost exclusively in the enol form (I), although the existence of a minute proportion of keto form (II) cannot be ruled out.


(I)


(II)

Although a keto form is intrinsically more stable than an enol form by about 18 kcal/mole, the energetic advantage in ketonisation of (I) is more than offset by the loss in resonance energy involved, about 35 kcal/mole. Polyhydroxybenzenes can be expected to contain a greater proportion of keto forms, since in them the gain in energy on ketonisation may more nearly compensate for the loss of resonance energy. Nevertheless, although in the most favourable compounds, such as phloroglucinol, there is some chemical evidence for ketone formation, the existence of such forms in the ground state is highly speculative. Bicyclic and polycyclic phenols show greater tendency to ketonisation, since the loss of resonance energy involved becomes progressively smaller as the number of aromatic rings increases. Thus, with 9-anthranol (III), the loss of resonance energy in converting the central "aromatic" ring to a quinonoid structure is so small (about 12 kcal/mole) that the keto form is energetically favoured and this indeed is the simplest carbocyclic monophenol to exist in both tautomeric forms, the keto form being 9-anthrone (IV).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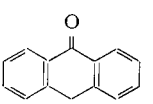
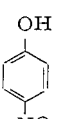

(IV)

In polycyclic dihydroxy compounds ketonisation is still more favourable energetically, and the bicyclic compound, 1,4-naphthaquinol exists in a stable diketo form.

The problem of tautomerism in nitrosophenols is also still open. Theoretically *p*-nitrosophenols can exist in three forms (V), VI) and (VII), although form (VI) is usually ignored.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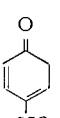
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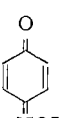

(VII)

Chemical evidence indicates tautomerism in these compounds, while the physical evidence is inconclusive. HODGSON[1,2] has amassed strong chemical and physical evidence in favour of the nitroso form as the chief component. ANDERSON AND YANKE[3], from a spectral study, concluded that *p*-nitrosophenol existed as an oxime (VII), but their interpretation of the evidence has been critized by HODGSON[2]. More recently SCHORS, KRAAIJEVELD AND HAVINGA[6] have re-examined the spectra and estimated that *p*-nitrosophenol contains about 20 % of (V) in methanol and 30 % in acetone. 4-Nitrosonaphthol, on the other hand, exists exclusively as oxime form (VII) owing to the stability of the naphthaquinone structure. There is no evidence, either chemical or physical, for the existence of a nitroso form in this compound[7].

In order to study tautomerism chromatographically it is essential to eliminate (or to be able to account for) all other molecular factors influencing R_M. This is usually a difficult problem. Thus resorcinol and hydroquinone, in both of which the possibility of limited tautomerism exists, separate chromatographically[8]; but the effect of tautomerism in these molecules (if it exists) cannot be studied without taking into account the general problem of the separation of *m*- and *p*-isomers[9]. We have therefore limited ourselves here to a study of tautomerism in (a) the one monohydric phenol in which it has been shown unequivocally to occur and in which other constitutive effects on chromatography are non-existent and (b) some *p*-nitrosophenols, whose tautomerism is well established also.

THEORETICAL

The chromatographic behaviour of a tautomeric mixture theoretically depends on the rate of interconversion of the tautomers and on the position of the tautomeric equilibrium. If the position of equilibrium is such that one tautomer exists only to a small extent, the substance will, from the practical point of view, behave as if it were a single form. If sufficient of a second form is present to affect R_M, chromatographic behaviour will be governed by the kinetics of interconversion. If the rate is slow, as in 1,4-dihydroxynaphthalene or naphtharesorcinol[5], the two tautomers should be separable and their individual R_M values can be calculated from MARTIN's equation, with due consideration to other constitutive effects (see the separation of the two forms of 1,4-naphthaquinol, described below). When the rate of interconversion is more rapid, however, the tautomeric change may interfere with the establishment of chromatographic equilibrium and, consequently, the solute will migrate, not as a zone, but as a diffuse streak. Such a compound is truly impossible to chromatograph in any system in which such rates occur. (We have found what appears to be a genuine example in *o*-nitroso-*p*-chlorophenol. On reversed phase chromatography with olive oil as stationary phase and aqueous ethanol as mobile phase, it gives only elongated streaks, although it is readily soluble in both phases. The compound was also impossible to chromatograph in direct phase trigol/ether systems although once again it was soluble in both phases. The effect could not have been due to adsorption as other nitrosophenols could be run satisfactorily in these systems.) Finally, when the interconversion of tautomers is very rapid, the solute will migrate as a discrete spot and its R_M value will not be the theoretical R_M value of either tautomer but will be determined by the relative amounts of each form in each phase.

The effect of tautomerism on R_M can be studied by the following treatment,

which also makes it possible to determine the position of tautomeric equilibrium. Consider two tautomeric forms, A and B, of a compound. Let x and y be the concentration of form A and z and u the concentration of form B in the stationary and mobile phases respectively, during the development of the chromatogram. The condition for chromatographic migration as a discrete zone is that all these concentrations are at equilibrium. According to CONSDEN, GORDON AND MARTIN[10]

$$\alpha = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right) \tag{1}$$

where α is the partition coefficient of the solute and A_L and A_S are the cross-sectional areas of the moving and stationary phases respectively. Then, at equilibrium,

$$\frac{x}{y} = \frac{A_L}{A_S}\left(\frac{1}{R_{F(x,y)}} - 1\right) \tag{2}$$

$$\frac{z}{u} = \frac{A_L}{A_S}\left(\frac{1}{R_{F(z.u)}} - 1\right) \tag{3}$$

and

$$\frac{x+z}{y+u} = \frac{A_L}{A_S}\left(\frac{1}{R_{F(\mathrm{exp})}} - 1\right) \tag{4}$$

where $R_{F(\mathrm{exp})}$ = the experimentally found R_F value of the tautomeric compound, $R_{F(x,y)}$ = the hypothetical R_F value of A, and $R_{F(z,u)}$ = the hypothetical R_F value of B.

By rearranging and combining eqns. (2), (3) and (4), we obtain the following expression for the position of the equilibria.

In the stationary phase,

$$\frac{x}{z} = \left[\frac{\frac{1}{R_{F(x,y)}} - 1}{\frac{1}{R_{F(z,u)}} - 1}\right]\left[\frac{\frac{1}{R_{F(z,u)}} - \frac{1}{R_{F(\mathrm{exp})}}}{\frac{1}{R_{F(\mathrm{exp})}} - \frac{1}{R_{F(x,y)}}}\right] \tag{5}$$

In the mobile phase,

$$\frac{y}{u} = \frac{\frac{1}{R_{F(z,u)}} - \frac{1}{R_{F(\mathrm{exp})}}}{\frac{1}{R_{F(\mathrm{exp})}} - \frac{1}{R_{F(x,y)}}} \tag{6}$$

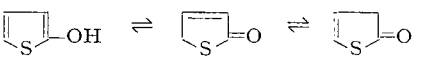
The solvent effect on the tautomeric equilibrium is given by the empirical equation of DIMROTH[11], which was verified by MEYER[12]:

$$\frac{C_A}{C_B} = \frac{L_A}{L_B} \cdot G$$

where C_A and C_B are the concentrations of A and B and L_A and L_B their solubilities, respectively. G is a constant, which is characteristic for the system and is independent of the nature of the solvent.

This expression indicates that tautomeric equilibria can be studied chromatographically when the ratios of the solubilities of A and B in the stationary and mobile phases are considerably different, as in the case of systems such as keto–enol, but not when the solubility ratios are similar.

If the hypothetical R_F value[8] for the two forms can be calculated by means of the group additivity principle, the position of tautomeric equilibrium can be calculated from the experimental R_F value of the tautomeric compound. For this study we have used the heterocyclic phenol, thienol (2-hydroxythiophen) (VIII).



(VIII)

This substance was recently prepared by HURD AND KREUZ[13] who offered clear chemical and physical evidence for its existence in keto and enol forms. There are, as shown, two keto forms possible, the 3- and 2-butenolactones; but the ultra-violet absorption spectrum indicates that the 2-butenolactone form is predominant, and in the following calculations we have assumed that only this keto form is present. Thienol is the only known monohydric mononuclear phenol that has been unequivocally shown to be tautomeric. This is due to the markedly diminished aromatic character of the thiophen ring.

The tautomeric equilibrium was also determined in *p*-nitrosophenol and 4-nitroso-1-naphthol.

EXPERIMENTAL

Compounds

With the exception of *p*-(2-thiophenylmethoxy)-phenol, all the compounds are known and were either obtained commercially or prepared by literature methods.

p-(2-Thiophenylmethoxy)-phenol was prepared by heating hydroquinone (20 g), sodium (1 g), chloromethylthiophen (5 g) and ethanol (250 ml) under reflux for 3 h. The product was extracted into ether, washed with water and then chromatographed in benzene on 50 g of Decalso F. Elution with benzene gave a fraction that coupled with diazotized *o*-dianisidine to give a red-brown colour. Crystallisation from light petroleum–benzene gave the required product as white crystals, m.p. 100–101°. It analysed correctly.

Chromatography

Whatman No. 4 papers (23 × 57 cm) were impregnated with trigol (triethylene glycol, 10% in chloroform) and dried for 1 h. The mobile phase was either (A) di-isopropyl ether or (B) di-*n*-butyl ether, the ether being saturated with trigol. Chromatograms were developed by the descending method in an atmosphere of nitrogen. Solutes were spotted as thin lines 3 cm long, 60 μg of each substance being used. Development times of 1 h, 2 h, and 4 h were used, giving solvent front migrations of 21, 32 and 46 cm respectively. The R_F values were shown to be independent of the development time. Some of the key compounds (such as *p*-benzoquinone and *p*-nitrosoanisole) were relatively volatile and were chromatographed by a special technique. The paper was placed between two glass sheets (56 × 10 × 0.5 cm), so that the starting line was between the sheets and the end of the paper dipped into the solvent trough. Chroma-

tography was carried out horizontally. (This technique is a modification of our tankless method, previously described[14], and is suitable if the solvents are relatively non-volatile and anhydrous and only a few compounds are to be chromatographed. As pointed out earlier, glass sheets cannot be used in a pile, as they distort under pressure.) R_F values for standard substances were found to be the same by the two techniques.

Over-running

To obtain the necessary information on tautomerism it is often necessary to chromatograph substances with widely differing speeds of migration. Thus, quinol migrates very slowly in the trigol/dibutyl ether system. To obtain the R_F value of this compound accurately, therefore, the over-running technique was used. Quinol was spotted side by side with a marker substance of known R_F, *p*-benzoquinone dioxime, and the chromatogram developed by the descending method for 28 h. During this time the solvent over-ran the paper and the marker substance had migrated nearly the whole length of the sheet. Since R_F was shown to be independent of development time, the R_F(quinol) could be determined from the R_F value of the marker.

Spot visualization

(a) Hydroquinones and their mono-ethers with a mixture of equal volumes of 0.2 % ethanolic ferric chloride and 0.5 % ethanolic 2,2-dipyridyl. (b) Phenols and oximes with 5 % aqueous K_2CO_3 followed by diazotised *o*-dianisidine. (c) Quinones with 50 % aqueous ethylamine. (d) *p*-Nitrosoanisole with a 20 % (v/v) solution of aniline in acetic acid. (e) Dihydro-1,4-naphthaquinone with conc. H_2SO_4.

RESULTS, CALCULATIONS AND DISCUSSION

Table I gives the R_F and R_M values of the compounds required for the various calculations. Two systems were used to obtain data, as certain key compounds migrate too far in one system. Since eqns. (5) and (6) are only obeyed if chromatography is by pure partition, it was essential to eliminate the possibility of adsorption playing a part. Several compounds, therefore, were run in systems A and B, supported on glass paper. They were found to have the same R_F values as on cellulosic paper, and it was assumed that adsorption effects, if they occurred were small enough to be ignored.

In the following calculations, the numerical value of ΔR_M parameters for groups substituted in an aromatic ring arbitrarily includes a contribution due to the replacement of one hydrogen atom. Thus, ΔR_M(OH) includes the unknown but small subtraction of ΔR_M(aromatic H). This procedure eliminates several tedious repetitions, and no error is introduced into the calculations, as the hydrogen atoms cancel out. The ΔR_M value for the divalent group (CH=CH—CH=CH) includes the loss of two hydrogen atoms.

(a) Calculation for thienol

The R_F value of thienol is (eqns. (5) and (6)) determined by the chromatographic equilibrium of two tautomeric forms. To calculate the position of equilibrium, the R_F values of these two forms must be found. To do this, ΔR_M(OH) is calculated first and then ΔR_M for the ketonisation of a phenol. Thienol is then converted to a derivative of its pure enol form, which by comparison with a similar derivative of a

TABLE I

R_F* AND R_M VALUES OF PHENOLS, *p*-ALKOXYPHENOLS, QUINONES, OXIMES AND *p*-NITROSO COMPOUNDS ON PAPER IMPREGNATED WITH TRIGOL

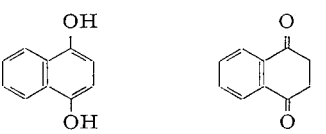
The mobile phase was either (A) di-isopropyl ether or (B) di-*n*-butyl ether

Compound	*System A*		*System B*	
	R_F	R_M	R_F	R_M
Phenol	0.725	—0.420	0.52	—0.027
p-Methoxyphenol	0.50	0.000	0.36	+0.255
1-Naphthol	0.76	—0.509	0.64	—0.252
2-Naphthol	0.685	—0.337	0.50	0.000
Quinol	0.10	+0.954	0.02	+1.644
p-Benzoquinone	0.75	—0.468	0.55	—0.092
1,4-Naphthaquinone	0.86	—0.802	0.80	—0.592
2,3-Dihydro-1,4-naphthaquinone	0.715	—0.398	0.67	—0.312
1,4-Naphthaquinol	0.15	+0.764	—	—
2-Phenanthrol	0.645	—0.260	0.49	+0.029
3-Phenanthrol	0.645	—0.260	0.49	+0.029
2-Anthrol	0.645	—0.260	0.49	+0.029
9-Phenanthrol	0.73	—0.428	0.62	—0.220
1-Anthrol	0.73	—0.428	0.62	—0.220
p-Benzoquinone dioxime	—	—	0.115	+0.883
1,4-Naphthaquinone dioxime	—	—	0.29	+0.380
1,4-Naphthaquinone monoxime	—	—	0.56	—0.106
p-Nitrosophenol	—	—	0.20	+0.602
p-Nitrosoanisole	—	—	0.85	—0.759
Thienol	0.71	—0.397		
p-Benzyloxyphenol	0.835	—0.703		
p-(2-Thiophenylmethoxy)-phenol	0.725	—0.421		

* R_F values were calculated from R_M values and not *vice versa*. (See Part III[15].)

non-tautomeric phenol will yield the hypothetical R_M value of the enol of thienol. ΔR_M for the ketonisation of a phenol will then give the R_M value of the keto form.

The value for ΔR_M(OH) can be determined by comparing R_M(phenol) with R_M(quinol). However, it was considered best to obtain the value of this parameter from at least two comparisons as it is of vital significance for the accuracy of the calculation. (In any case, it could not be taken as axiomatic that quinol itself is non-tautomeric, since certain tautomeric properties are observed in other polyhydroxybenzenes.) A further comparison was therefore made with 1,4-naphthaquinol (IX). This substance is readily separable from its tautomeric keto form, 2,3-dihydro-1,4-naphthaquinone (X), and both tautomers are stable under laboratory conditions[5].



(IX) (X)

However, since in (IX) each OH group is subject to the *ortho*-effect of a *peri*-CH group, the magnitude of this effect must be determined first.

(*i*) ΔR_M(*CH=CH—CH=CH fused to aromatic ring*). This parameter was calcu-

lated (see a previous example[15]) by comparing the R_M values of (a) phenol and 2-naphthol, and (b) 2-naphthol and 2- or 3-phenanthrol. The values were in excellent agreement and

$$\Delta R_M = + 0.080 \pm 0.003 \text{ in System A and}$$
$$+ 0.023 \pm 0.001 \text{ in System B}$$

(*ii*) ΔR_M(*ortho-effect due to peri-CH*). This was calculated by comparing (a) 1- and 2-naphthol, (b) 1- and 2-anthrol, and (c) 2- and 9-phenanthrol. The agreement was excellent.

$$\text{Mean } \Delta R_M = -0.170 \pm 0.002 \text{ in System A and}$$
$$-0.251 \pm 0.002 \text{ in System B}$$

(Comparison of *p*-benzoquinone and 1,4-naphthaquinone using the expression,

$$R_M(p\text{-benzoquinone}) + \Delta R_M(\text{CH=CH—CH=CH}) + 2 \times \Delta R_M(\textit{ortho}\text{-effect}) = R_M(\text{naphthaquinone})$$

gives

$$\Delta R_M(\textit{ortho}\text{-effect}) = -0.207 \text{ in System A and}$$
$$-0.308 \text{ in System B}$$

The *ortho*-effect thus appears to be remarkably constant in this system, even when the functional group varies.)

(*iii*) ΔR_M(OH). Calculation (a) from R_M(quinol) and (b) from R_M(1,4-naphthaquinol).

$$\text{(a) } \Delta R_M(\text{OH}) = R_M(\text{quinol}) - R_M(\text{phenol}) = + 1.451 \text{ in System A}$$

$$\text{(b) } \Delta R_M(\text{OH}) = R_M(\text{1,4-naphthaquinol}) - \Delta R_M(\text{CH=CH—CH=CH}) - R_M(\text{phenol}) - 2 \times \Delta R_M(\textit{ortho}\text{-effect}) = + 1.444 \text{ in System A}$$

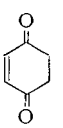
The agreement between the two calculations confirms that there is no tautomerism in quinol and that the ΔR_M parameters of the two *p*-substituted OH groups in quinol and 1,4-naphthaquinol are additive.

(*iv*) ΔR_M(*ketonisation of OH*). The two key compounds for the calculation of this parameter are the stable tautomers (IX) and (X).

$$\Delta R_M(\text{ketonisation of OH}) = \frac{R_M(\text{X}) - R_M(\text{IX})}{2}$$
$$= -0.581 \text{ in System A}$$

The value of this parameter in System A can be checked independently by another calculation involving R_M(quinol). The latter calculation also yields the parameter in System B, which cannot be found directly as 1,4-naphthaquinol (IX) does not migrate appreciably in the latter system.

Thus, the R_M value for the hypothetical diketo tautomer of quinol (XI) is found from the following expression.



(XI)

$$R_M(\text{XI}) = \Delta R_M(\text{X}) - \Delta R_M(\text{CH=CH-CH=CH}) - 2 \times \Delta R_M(\textit{ortho}\text{-effect})$$
$$= -0.138 \text{ in System A and}$$
$$+ 0.165 \text{ in System B}$$

Then

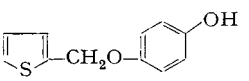
$$\Delta R_M(\text{ketonisation of OH}) = \frac{R_M(\text{XI}) - R_M(\text{quinol})}{2}$$
$$= -0.584 \text{ in System A and}$$
$$-0.739 \text{ in System B}$$

There is complete agreement between the two values for System A.

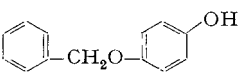
(*v*) ΔR_M(*enol form of thienol*). To calculate the R_M value of the pure enol form of thienol, we need to know ΔR_M(OH), which has already been calculated, and the hypothetical ΔR_M value for the thiophenyl radical. By the processes of formal summation (described in more detail previously[3]),

$$R_M(\text{enol form of thienol}) = \Delta R_M(\text{thiophenyl radical}) + \Delta R_M(\text{OH})$$

(This summation is legitimate as the constitutive relationships are identical in both halves of the equation.) The value of ΔR_M(thiophenyl radical) can be obtained by chromatographing a suitable 2-thiophenyl compound with an analogous phenyl compound and calculating from their R_M values by the method shown below. We used *p*-(2-thiophenylmethoxy)-phenol (XII), which could be readily prepared from the available 2-chloromethylthiophen, and compared it in System A with *p*-benzyloxyphenol (XIII).



(XII)



(XIII)

Then, the usual formal processes lead to

(a) $R_M(p\text{-benzyloxyphenol}) = \Delta R_M(\text{phenyl}) + \Delta R_M(\text{OH}) + \Delta R_M(\text{CH}_2\text{OPh})$
(b) $R_M(p\text{-thiophenylmethoxyphenol}) = \Delta R_M(\text{thiophenyl}) + \Delta R_M(\text{OH}) + \Delta R_M(\text{CH}_2\text{OPh})$
(c) $R_M(\text{phenol}) = \Delta R_M(\text{phenyl}) + \Delta R_M(\text{OH})$
(d) $R_M(\text{enol form of thienol}) = \Delta R_M(\text{thiophenyl}) + \Delta R_M(\text{OH})$

Therefore,

$$R_M(\text{enol form of thienol}) = R_M(p\text{-thiophenylmethoxyphenol}) - R_M(p\text{-benzyloxyphenol}) + R_M(\text{phenol})$$
$$= -0.138 \text{ in System A}$$

The R_F value of the enol form is thus 0.579.

(*vi*) R_M(*keto form of thienol*). This R_M value can be obtained by using the value in (iv) above for ΔR_M(ketonisation).

In System A, therefore,

$$R_M(\text{keto form of thienol}) = -0.138 - 0.581 = -0.719$$

The corresponding R_F value is 0.840.

(vii) The position of equilibrium in thienol can now be calculated from eqns. (5) and (6), since the R_F values of both forms and the tautomeric mixture are known. In the mobile phase of System A (di-isopropyl ether), the amount of enol form is found to be 40 % and keto form 60 %. In the stationary phase (trigol) there is 70 % enol form and only 30 % keto form. There is thus a higher proportion of keto form in the less polar phase. This is the reverse of what is found in acetoacetic ester and similar keto–enol tautomers. But this can be taken as confirmatory of the theoretical treatment, since the enol form of acetoacetic ester is stabilised by internal hydrogen bonding (which is maximal in non-polar solvents such as hexane and minimal in water because of competitive solvation); in thienol, however, no internal hydrogen bonding is possible and it is to be expected that the enol form would be more soluble in more polar solvents, in agreement with the above findings. Our results agree well with the prediction of HURD AND KREUZ[13], who stated that thienol could be expected to contain roughly equal amounts of keto and enol form.

(b) p-Nitrosophenol

To determine the tautomeric equilibrium in this compound a similar procedure to that outlined above can be used. First it is necessary to select compounds closely similar to *p*-nitrosophenol but which contain pure non-tautomeric nitroso and oximino groups. By processes of formal addition and subtraction the R_F values of the two tautomeric forms and hence the equilibrium can be calculated. *p*-Nitrosoanisole was selected as a suitable compound containing a non-tautomeric nitroso group.

(i) $\Delta R_M(OCH_3)$

$$\begin{aligned}\Delta R_M(\mathrm{OCH_3}) &= R_M(p\text{-methoxyphenol}) - R_M(\text{phenol})\\ &= +\ 0.282 \text{ in System B}\end{aligned}$$

(ii) The R_M value for the hypothetical nitroso form (*V*) of *p*-nitrosophenol is found as follows:

$$\begin{aligned}R_M(\text{nitroso form}) &= R_M(p\text{-nitrosoanisole}) - \Delta R_M(\mathrm{OCH_3}) + \Delta R_M(\mathrm{OH})\\ &= +\ 0.630 \text{ in System B}\end{aligned}$$

(ΔR_M(OH) for System B was calculated from quinol and phenol.)

(iii) The R_M value of the hypothetical oxime form (*VII*) of *p*-nitrosophenol was calculated from *p*-benzoquinone and its dioxime.

$$\begin{aligned}R_M(\text{oxime form}) &= R_M(\text{benzoquinone}) + \frac{R_M(\text{benzoquinone dioxime}) - R_M(\text{benzoquinone})}{2}\\ &= +0.396 \text{ in System B}\end{aligned}$$

(iv) The R_M value of the hypothetical keto-nitroso form (*VI*) of *p*-nitrosophenol can be calculated as follows:

$$\begin{aligned}R_M(\text{keto-nitroso form}) &= R_M(\text{nitroso form}) + R_M(\text{ketonisation of OH})\\ &= -0.109 \text{ in System B}\end{aligned}$$

The experimental R_M value for *p*-nitrosophenol in System B is + 0.602. This is, within experimental error, indistinguishable from the R_M value calculated for the

pure nitroso form (V). There would thus appear not to be more than a few percent of oxime form present. These findings thus support the views of HODGSON[1,2].

(c) 4-Nitroso-1-naphthol

The R_M value of the oxime form (1,4-naphthaquinone monoxime) can be calculated, as was the oxime form of *p*-nitrosophenol.

$$R_M(\text{oxime form}) = R_M(\text{naphthaquinone}) + \frac{R_M(\text{naphthaquinone dioxime}) - R_M(\text{naphthaquinone})}{2}$$
$$= -0.592 + 0.486$$
$$= -0.106 \text{ in System B}$$

This is identical with the experimental R_M value for this substance, thus supporting all the other chemical and physical evidence[7] for a 100% oxime structure.

(d) Ethyl acetoacetate

It would have been desirable to test the equations described above on a compound whose tautomerism had been quantitatively studied by classical methods. The most promising type of compound appeared to be ethyl acetoacetate, whose tautomeric equilibrium in several solvents[4] has been extensively studied. Attempts were made to chromatograph this substance in two systems, trigol/iso-octane and olive oil/70% (v/v) aqueous ethanol, but only diffuse bands were obtained and R_F values could not be measured. This may have been due to the interconversion of keto and enol forms in ethyl acetoacetate being relatively slow (as it is known to be in the absence of catalysts[4]). Although interconversion is rapid in the presence of an acid or base[4], the addition of either to one phase would introduce a new variable. The enol form of ethyl acetoacetate is known to be stabilised by internal hydrogen bonding. As enolization is affected by pH, the resultant chromatographic effect would be too complex for study.

SUMMARY

The effect of tautomerism on R_M has been studied. The general nature of the effect on chromatography is discussed and equations obtained by means of which the existence of tautomerism can be verified chromatographically. If sufficient data are available from the study of archetypal compounds in which each form of the tautomeric group occurs free from other constitutive effects, it is possible, by these equations, to calculate the position of tautomeric equilibrium in a given compound. The procedure is illustrated with reference to the tautomerism of thienol (2-hydroxythiophen), *p*-nitrosophenol and 4-nitroso-1-naphthol. The procedure followed rests on the adherence of many group and structural ΔR_M parameters to the additivity principle of MARTIN's equation.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

VI. TAUTOMERISM AND INTRAMOLECULAR HYDROGEN BONDING IN THE SAME MOLECULE. *o*-NITROSOPHENOLS

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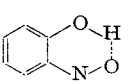
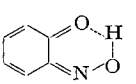
INTRODUCTION

In the preceding parts[1–5] it has been shown that MARTIN's equation is rigorously obeyed if molecular increments are free from constitutive effects. Several types of molecular interaction, which lead to constitutive effects on R_M, have already been discussed, and, in the two preceding papers[4,5], two of these, internal hydrogen bonding and tautomerism, have been studied in some detail. In this communication, we wish to present a study on a series of compounds in which both these phenomena can be expected to occur.

Such a group of compounds is the *o*-nitrosophenols. MARCINKIEWICZ AND GREEN[6] have already reported on the separation of the *o*-nitrosotocopherols by reversed phase chromatography, and GREEN, MCHALE, MARCINKIEWICZ, MAMALIS AND WATT[7] prepared some nitroso derivatives of tocol and its higher homologues. In both these studies, a serious lack of obvious correlation with the requirements of MARTIN's equation was noted. We have now prepared and chromatographed the *o*-nitroso derivatives of some phenols, naphthols, phenanthrols, alkoxyphenols, coumaranols and chromanols. All these compounds can be regarded as tautomeric mixtures of nitroso (I) and oxime (II) forms.



(I)



(II)

As already indicated[5], it is likely that two such different structures would always be found to exhibit a considerable difference in R_F if they could be isolated. In practice, however, the nitroso and oxime forms of compounds can only rarely be obtained as distinct compounds, and then only when there is a particular structural configuration present that effects a stabilisation of one or other form. Thus HENRICH[8] was able to isolate both forms of nitrosoresorcinol monomethyl ether. Usually, either one form exists to the exclusion of the other (as in 4-nitroso-1-naphthol, which exists exclusively as the oxime of 1,4-naphthaquinone), or the two forms have such a rapid rate of interconversion that neither form can be isolated singly. The latter appears to be

the case with *p*-nitrosophenol and its derivatives and also with 1-nitroso-2-naphthol[9].

o-Nitrosophenols, in contrast to the *p*-nitroso compounds, have received little study. In the simple mononuclear phenol series they are difficult to prepare, and CRONHEIM[10], who prepared most of the known ones by a modification of BAUDISCH's[11] reaction, obtained such minute yields that he was only able to identify them by their ultra-violet spectra. *o*-Nitroso derivatives of polynuclear phenols are obtained more readily, however, and alkoxyphenols, and cyclic mono-ethers such as coumaranols and chromanols, also yield *o*-nitroso derivatives. Pronounced hydrogen bonding can be expected to occur in both the nitroso and the oxime form (I and II) of all these compounds. Thus the two phenomena, tautomerism and chelation, will co-exist and they will affect each other in a complex fashion, for the degree of bonding will affect the position of the tautomeric equilibrium and *vice versa*. Furthermore, the two phenomena can be expected to be influenced both sterically and electronically by additional substitution in the aromatic ring, especially if this occurs *ortho* to either the nitroso or hydroxy group.

EXPERIMENTAL

Compounds

The nitrosonaphthols and 10-nitroso-9-phenanthrol are known compounds. *1-Nitroso-2-phenanthrol* is a new compound. It was made by the standard procedure for preparing nitroso-*β*-naphthol[12] and was obtained from aqueous ethanol or light petroleum–benzene as a dark brown micro-crystalline powder, m.p. 170°. It analysed correctly. The *o*-nitroso derivatives of the alkoxyphenols, coumaranols and chromanols are unknown, and compounds of this type have only been prepared in the analogous tocopherol series[6,7,13]. These compounds (which can all be regarded as substituted *o*-nitrosoquinols) are even less accessible than the *o*-nitrosophenols. Their preparation was desirable, however, for systematic correlation with the parent phenols and mono-ethers we have already studied[1–5]. They cannot be prepared by CRONHEIM's method, which is too drastic, but can be obtained on a submilligram scale by QUAIFE's original procedure for the nitrosotocopherols[13]. Accordingly, as we have described elsewhere[7], pure specimens of each nitroso derivative in amounts sufficient for chromatography were prepared and characterized by their infra-red (and where possible) their ultra-violet spectra. Table I records their ultra-violet maxima and minima.

In three compounds (Nos. 5, 10 and 11 in Table I) there are two non-equivalent *ortho*-positions available for nitrosation. Each of these compounds thus gave two isomeric *o*-nitroso derivatives, which could be separated by adsorption chromatography on $ZnCO_3$-impregnated paper[6], as they differ in the screening effect of the nitroso group on the phenolic OH (a similar phenomenon was observed in the nitrosation of *δ*-tocopherol[6]).

Chromatography

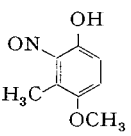
The tank descending method was used. Whatman No. 4 papers were impregnated with the stationary phase. The systems were (A) 50 % (v/v) aqueous ethanol against liquid paraffin (5 % v/v in light petroleum); (B) 40 % (v/v) aqueous ethanol against olive oil (5 % v/v in light petroleum); and (C) di-*n*-butyl ether against trigol (triethylene glycol, 10 % v/v in chloroform). The compounds were all visible on sodium fluorescein-treated paper under ultra-violet light. With systems (A) and (B) 4 h runs

were used and the front moved about 40 cm. With system (C), the same migration was achieved in 1½–2 h.

RESULTS AND DISCUSSION

In Table I the R_F and R_M values are given for *o*-nitroso derivatives of alkoxyphenols, chromanols and coumaranols, chromatographed in the liquid paraffin/ethanol system. It is at once apparent that these values cannot be readily correlated with MARTIN's equation. Consider first the derivatives of *p*-methoxy-, *p*-ethoxy- and *p*-propoxyphenol. $\Delta R_M(CH_2)$ in this system (determined from a homologous series not shown here) is about + 0.450. Since the nitroso derivatives of *p*-ethoxy- and *p*-propoxyphenol have almost the same R_M value, it is clear that even a minute structural change in the *para*-position is sufficient to influence the relationship of the two *ortho*-groups to each other and to produce a constitutive effect on R_M in cancellation of the molecular weight difference.

Compare now compounds 4 and 5 (Table I). 4-Methoxy-3-methylphenol yields two nitroso derivatives (III) and (IV), which are separable chromatographically in an



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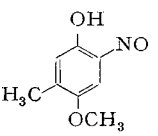

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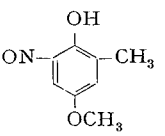


(V)

exactly analogous fashion to the separation of the nitroso derivatives of β- and γ-tocopherol[6], and they both have an R_F value different from that of the single nitroso derivative of 4-methoxy-2-methylphenol (V).

TABLE I

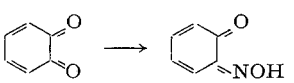
R_F AND R_M VALUES OF *o*-NITROSO DERIVATIVES OF ALKOXYPHENOLS, CHROMANOLS AND COUMARANOLS IN REVERSED PHASE SYSTEM A

No.	*Nitroso derivative of*	λ_{max} (mμ)	λ_{min} (mμ)	R_F	R_M
1	*p*-Methoxyphenol	310	279; 390–395	0.81	—0.620
2	*p*-Ethoxyphenol	326	395	0.77	—0.523
3	*p*-Propoxyphenol	317	396	0.76	—0.500
4	4-Methoxy-2-methylphenol	323	400	0.65	—0.261
5	4-Methoxy-3-methylphenol (a)	350	399	0.75	—0.469
	4-Methoxy-3-methylphenol (b)	327	393	0.70	—0.366
6	2,3-Dimethyl-4-methoxyphenol	337	295; 401	0.52	—0.031
7	2,5-Dimethyl-4-methoxyphenol			0.92	—1.046
8	2-Propyl-4-methoxyphenol	322	286; 290	0.37	+0.279
9	4-Benzyloxy-2-methylphenol			0.28	+0.408
10	4-Benzyloxy-3-methylphenol (a)	333	399	0.42	+0.139
	4-Benzyloxy-3-methylphenol (b)	315–323	391	0.42	+0.139
11	2,2-Dimethyl-6-chromanol (a)	325	400–405	0.83	—0.678
	2,2-Dimethyl-6-chromanol (b)	335	400–405	0.67	—0.301
12	2,5,8-Trimethyl-6-chromanol	350–355	297; 407	0.69	—0.356
13	4-Methyl-5-coumaranol	331	294; 408	0.81	—0.620
14	6-Methyl-5-coumaranol			0.63	—0.222
15	2,4,7-Trimethyl-5-coumaranol	345	299; 415–203	0.68	—0.330

We regard it as unlikely that these pronounced differences are purely spatial in origin: the *ortho*-effect of a CH_3 group vicinal to OH is negligible in this system (*cf.* System 5 described previously[3]). They must be considered as being produced by the total electronic configuration of the molecule, which must affect the tautomeric equilibrium and chelation of the *ortho*-groups. Such an effect is even more clearly discerned in compounds 6 and 7, which exhibit a remarkable R_F difference (although here steric forces may reinforce the polar interactions, because in 6-nitroso-2,5-dimethyl-4-methoxyphenol, the nitroso–hydroxy chelate ring is under steric compression from a number of substituent groups). A similar phenomenon is observed with compounds 9 and 10. The two nitroso derivatives of 4-benzyloxy-3-methylphenol have the same R_F value in the partition system. Compound 11 also yields two nitroso derivatives, as there are two non-identical *ortho*-positions in this molecule. Compounds 12 and 15 have virtually the same R_M value, although one is a chromanol and the other a coumaranol, (the parent phenols, it should be noted, are easily separable[2]: in the nitroso derivatives, the slight difference in the electronic effect of the fused 5- and 6-membered rings is apparently sufficient to affect the equilibrium in the chelated ring and cancel out the molecular weight difference). Finally, compounds 13 and 14 illustrate in the coumaranol series exactly the same phenomenon as we have described in the separation of the tocopherols[6].

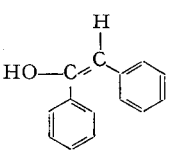
In order to pursue the investigation further, the relation between the R_M values of some *o*-nitrosophenols and the relevant bond order of the double bond (in the parent hydrocarbon) bearing the nitroso and hydroxy groups was studied. The oxime form of an *o*-nitrosophenol is formally derived from an *o*-quinone (VI) and the stability of *o*-quinones is related to the relevant bond order (hence the stability of *o*-naphthaquinones compared to *o*-benzoquinones).

By analogy, the stability of quinone oximes might also be expected to depend on relevant bond order, and we have already shown[4] that the degree of intramolecular hydrogen bonding is so related. Since the phenomena of tautomerism and chelation are intimately connected in *o*-nitrosophenols, it was thought possible that a relation between R_M and bond order might be discerned in these compounds. In order to test this hypothesis, several *o*-nitrosophenols were prepared and chromatographed in



(VI)

reversed phase system B and in the trigol/ether system C, in both of which the parent phenols could also be run. Table II gives their R_F and R_M values and also the relevant bond orders in the parent hydrocarbons (calculated by the L.C.A.O. approximation method[14]). The compound described as α-hydroxystilbene (VII) is of special



(VII)

interest; this substance is, in fact, unknown; but its hypothetical R_F values in the two systems could be expected not to be too dissimilar from those of 9-phenanthrol, and these are the figures shown in parentheses in Table II.

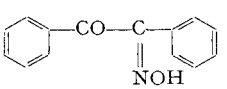
TABLE II

R_F AND R_M VALUES OF PHENOLS AND THEIR NITROSO DERIVATIVES IN SYSTEMS B AND C. ΔR_M(NO) VALUES AND RELEVANT BOND ORDER IN CORRESPONDING HYDROCARBONS

Compound	*System B*			*System C*			*Relevant bond order*
	R_F	R_M	ΔR_M(NO)	R_F	R_M	ΔR_M(NO)	
Phenol	0.83	−0.70			−0.27		
o-Nitrosophenol	0.45	+0.08	+0.78	0.53	−0.06	+0.21	0.67[a]
1-Naphthol	0.45	+0.08			−0.25		
2-Nitroso-1-naphthol	0.73	−0.45	−0.53	0.37	+0.23	+0.48	0.73[b]
2-Naphthol	0.55	−0.08			+0.00		
1-Nitroso-2-naphthol	0.59	−0.17	−0.09	0.58	−0.14	−0.14	0.73[b]
2-Phenanthrol	0.22	+0.54			+0.03		
1-Nitroso-2-phenanthrol	0.10	+0.95	+0.41	0.74	−0.44	−0.47	0.70[c]
9-Phenanthrol	0.15	+0.76			−0.22		
10-Nitroso-9-phenanthrol	0.22	+0.55	−0.21	0.69	−0.34	−0.12	0.78[d]
(α-Hydroxy-stilbene)	(0.15)	(0.76)			(−0.22)		
α-Benzil monoxime	0.64	0.25	−1.01	0.66	−0.27		0.88[e]
β-Benzil monoxime	0.69	0.35	−1.11	0.66	−0.27	−0.05	0.88[e]

[a] 1,2 bond in benzene.
[b] 1,2 bond in naphthalene.
[c] 1,2 bond in phenanthrene.
[d] 9,10 bond in phenanthrene.
[e] α, β-bond in stilbene.

Its value for the present purposes lies in the fact that the bond order of the α,β-bond in stilbene (the corresponding parent hydrocarbon) is exceptionally high and, although (VII) is hypothetical, its nitroso derivative, which is formally derived from it does exist in pure oxime form. This compound, benzil monoxime, exists in two stereochemical modifications, the α-oxime (VIII) and the β-oxime (IX), which are *cis-trans* isomers.



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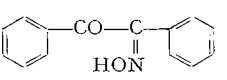

(IX)

The results in Table II confirm the indications of Table I, that ΔR_M(NO) is exceedingly variable, there being no clear relation between structure and chromatography. Although in System B there seems to be a general trend for ΔR_M(NO) to decrease as the bond order increases, this is not confirmed in System C. Indeed, there is no correlation between the values of ΔR_M(NO) in the two systems. It is noteworthy that the stereochemical difference between benzil α-oxime and the β-oxime has a relatively insignificant effect on R_M or on ΔR_M(NO), compared to the major effects of hydrogen bonding and tautomerism. These results show most clearly how complex is the combined effect of tautomerism and internal hydrogen bonding. Not only is a mathematical correlation difficult even in one system, but it is likely that every system will produce

somewhat different results, since the ratio of nitroso–oxime forms probably differs in different solvents.

SUMMARY

A series of *o*-nitroso derivatives of phenols, alkoxyphenols, coumaranols and chromanols have been chromatographed. Two reversed phase and one direct phase system have been used. The correlation of the R_M values of these compounds with their structure is at present impossible, being complicated by the combined occurrence of tautomerism and internal hydrogen bonding. Since these factors are interdependent and since they also depend on the nature of the solvents, the chromatography of *o*-nitrosophenols cannot be satisfactorily interpreted in terms of the electronic structure of the parent phenols or the relevant bond orders in the parent hydrocarbons.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

VII. THE SEPARATION OF *meta*- and *para*-DERIVATIVES OF BENZENE

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INTRODUCTION

In preceding papers[1–6] we have discussed several aspects of the relationship between chromatography and chemical structure. This communication deals with the chromatography of *m*- and *p*-derivatives of benzene. Little information concerning this problem appears to exist. It did not appear to be known whether all *m*- and *p*-isomers differ in R_F or whether only certain pairs do. Although the separation of a few isomeric pairs has been described, other pairs have been found to have identical R_F values. How much of this is due to system differences is unclear, as no detailed chromatographic study of *m*- and *p*-isomers has been made nor has the theoretical basis for any differences been investigated. This study attempts to answer the following questions. Can *m*- and *p*-isomers be separated in neutral, partition systems? Do they obey MARTIN's equation? What factors determine their separation, if any? The chromatography of *o*-derivatives was not considered in detail, although a few such compounds were examined. It is well established that *o*-derivatives often have different R_F values from their *m*- and *p*-isomers, but this arises from different causes, particularly those due to steric crowding, internal hydrogen bonding and polar interactions of the types we have already discussed[2,4].

PERPAR, TIŠLER AND VRBASKI[7] separated *m*- and *p*-nitroaniline in a reversed phase neutral system. TURI[8] was able to separate *m*- and *p*-aminophenol in an acid system, but not in a neutral one. This suggests that, under conditions in which salt formation is possible, separation of isomers with basic groups may be increased. The results of ETTEL, POSPISIL AND DEYL[9] seem to confirm this, for they were unable to separate the aminophenols in ammoniated systems. TURI[8] separated *m*- and *p*-phenylenediamine, however, even in a neutral system and EKMANN[10] also separated these isomers. ETTEL, POSPISIL AND DEYL[9] separated *m*- and *p*-nitrobenzoic acid, but used ammoniated solvents, which must markedly influence salt formation of acids. The *m*- and *p*-isomers of nitrophenol, however, could be separated even in neutral systems[9]. BATE-SMITH AND WESTALL[11], WAGNER[12] and GASPARIC[13] have all described the separation of *m*- and *p*-dihydroxybenzene in both neutral and acid systems. GESSNER AND SMITH[14] achieved a slight separation of *m*- and *p*-chlorophenol in two out of eight systems investigated, but the nature of the stationary phase in these two systems makes it uncertain whether purely partition conditions existed.

It is clear that a theoretical analysis of the separation of *m*- and *p*-isomers (in

common with the analysis of other constitutive effects) cannot be easily made in compounds where other complicating factors may affect chromatography. Thus the separation of acids, as shown by BARK AND GRAHAM[15] is complicated by adsorption, and so might the chromatography of strong bases be. Phenols such as the nitrophenols must be regarded with caution. Thus, *m*- and *p*-nitrophenol differ exceedingly in acid strength, and they may even exist in different tautomeric forms (see later). Considerations such as these seemed to provide a ready explanation for many of the literature separations, except for the case of resorcinol and quinol, which were clearly separable in several partition systems and could not be regarded as compounds in which other phenomena are present. (A possibility, in the case of resorcinol and quinol, was that tautomerism might exist. But many physical studies on these compounds have shown that tautomerism is virtually absent in them. A calculation according to our previously described method[5] showed that, in order to account for the observed separation of resorcinol and quinol on tautomeric grounds, the latter would have to exist as about 20–30 % of diketo form, obviously impossible in view of other physical data.) There appeared to be no way of deciding, *ab initio*, whether any given pair of *m*- and *p*-isomers would have different R_M values nor whether group addition to benzene compounds obeyed MARTIN's equation[15].

In this study, a series of *m*- and *p*-derivatives of benzene were chromatographed in several systems. The following general stipulations were observed.

1. The system must consist of two neutral phases.

2. The effects of adsorption must be shown to be non-existent.

3. If at all possible, each pair of isomers must be run in at least two systems, one of which should preferably be reversed phase. Before it can be safely concluded that two isomers have identical R_F values they should be shown to be inseparable in as many systems as possible. The systems must be such that (a) chromatography is near ideal (demonstrated by the use of substances of known R_F as standards) and (b) the R_F values are, as far as possible, in the range (0.2–0.8), most sensitive to small differences.

EXPERIMENTAL

Chromatography

Whatman No. 4 papers were used for all systems. Confirmatory runs were made on glass paper. The techniques used were, in general, similar to those described earlier[5]. The following systems were used: (A) trigol (triethylene glycol, 10 % w/v in chloroform)/2,2,4-trimethylpentane (iso-octane); (B) trigol (as above)/di-isopropyl ether; (C) (reversed phase) olive oil (10 % w/v in light petroleum)/85 % (v/v) aqueous ethanol; (D) (reversed phase) ethyl oleate (5 % w/v in light petroleum)/25 % (v/v) aqueous ethanol. Some other systems were used with certain compounds and are described in the text.

Visualisation

The aniline derivatives were visualised by exposing the papers to nitrous fumes for a few minutes, then spraying with an alkaline solution of β-naphthol. Other compounds were detected by incorporating a small amount of sodium fluorescein into the trigol phase (5 mg/100 ml $CHCl_3$ solution). After development these papers were sprayed with 5 % (w/v) sodium carbonate solution and then dried, when nearly all the compounds investigated appeared as quenching spots under ultra-violet light. As pre-

viously described[2,3], R_M values were calculated directly from the migration data and R_F values were derived from them. The R_M values of one or two compounds with specially low R_F values, such as phloroglucinol, were determined by the over-running technique, as described previously[5].

RESULTS

Tables I and II give the R_F and R_M values of several *m*- and *p*-derivatives of benzene, and also of some 1,3,5-trisubstituted derivatives (no other trisubstituted isomers were studied as, in all other structural isomers, *ortho*-effects must be present). If system A is considered first, it is seen that only some of the pairs of *m*- and *p*-isomers differ in R_M. The results show that, in order for there to be a difference in the R_M values of the two

TABLE I

R_F AND R_M VALUES OF *meta*- AND *para*-SUBSTITUTED BENZENE DERIVATIVES IN SYSTEM A (TRIGOL/ISO-OCTANE)

Compound	*Isomer*	R_F	R_M
Dinitrobenzene	*m*	0.35	+0.275
	p	0.35	+0.275
Nitroanisole	*m*	0.715	−0.398
	p	0.555	−0.097
Nitrotoluene	*m*	0.84	−0.721
	p	0.81	−0.620
Nitrobenzyl bromide	*m*	0.64	−0.252
	p	0.59	−0.161
Nitro-N-dimethylaniline	*m*	0.785	−0.569
	p	0.335	+0.207
Nitrophenyl acetate	*m*	0.50	0
	p	0.50	0
Nitrophenyl benzyl ether	*m*	0.705	−0.377
	p	0.53	−0.051
Chloronitrobenzene	*m*	0.81	−0.620
	p	0.795	−0.585
Cyanonitrobenzene	*m*	0.205	+0.591
	p	0.205	+0.591
Related compounds			
Nitrobenzene		0.68	−0.332
sym.-Trinitrobenzene		0.11	+0.909

isomers, at least one substituent must be an *ortho/para*-directing group; that is, activating with respect to these positions in the nucleus (INGOLD's + M effect[17]). Thus the two isomers of dinitrobenzene, cyanonitrobenzene, and nitrophenyl acetate, which contain only *meta*-directing groups, had identical R_M values. In marked contrast to the nitrophenyl esters, the benzyl *ethers* of *m*- and *p*-nitrophenol were easily separable in this system (the ether group being *ortho/para*-directing). If the isomers had different R_M values, the *meta*-isomer always migrated faster, irrespective of the type of substituents present. Isomers that showed no chromatographic difference in System A were then rigorously examined in several other systems, but in none of these was separation effected. Thus, the two dinitrobenzenes and the two cyanonitrobenzenes

TABLE II

R_F AND R_M VALUES OF *meta*- AND *para*-SUBSTITUTED BENZENE DERIVATIVES AND SOME *sym*-TRISUBSTITUTED DERIVATIVES IN SYSTEM B (TRIGOL/DI-ISOPROPYL ETHER) AND CALCULATED R_M VALUES

Compound	*Isomer*	R_F	R_M	*Calc.* R_M	R_M — *Calc.* R_M
Dinitrobenzene	*m*	0.81	—0.629		
	p	0.81	—0.629		
Nitrophenol	*m*	0.64	—0.244	+0.182	—0.426
	p	0.525	—0.041	+0.182	—0.223
Nitroaniline	*m*	0.55	—0.081	+0.182	—0.263
	p	0.29	+0.393	+0.182	+0.216
Dihydroxybenzene	*m*	0.100	+0.954	+0.993	—0.039
	p	0.085	+1.031	+0.993	+0.038
Phenylenediamine	*m*	0.044	+1.342	+0.993	+0.349
	p	0.027	+1.556	+0.993	+0.563
Aminophenol	*m*	0.069	+1.300	+0.993	+0.137
	p	0.0475	+1.342	+0.993	+0.207
Chloroaniline	*m*	0.725	—0.420	—0.585	+0.165
	p	0.705	—0.377	—0.585	+0.208
Chlorophenol	*m*	0.795	—0.585		
	p	0.795	—0.585		
Cresol	*m*	0.795	—0.585		
	p	0.795	—0.585		
Cyanophenol	*m*	0.46	+0.068	+0.036	+0.032
	p	0.46	+0.068	+0.036	+0.032
Cyanonitrobenzene	*m*	0.86	—0.775		
	p	0.86	—0.775		
Nitrobenzaldehyde	*m*	0.78	—0.553		
	p	0.78	—0.553		
Hydroxybenzaldehyde	*m*	0.47	+0.057	+0.258	—0.201
	p	0.35	+0.267	+0.258	+0.009
Hydroxybenzoic acid methyl ester	*m*	0.68	—0.319		
	p	0.65	—0.276		
Toluidine	*m*	0.795	—0.585	—0.585	0
	p	0.795	—0.585	—0.585	0
Hydroxybenzyl alcohol	*m*	0.10	+0.954		
	p	0.085	+1.031		
Amino-N-dimethylaniline	*m*	0.635	+0.237		
	p	0.485	—0.009		
N,N-Tetramethylaniline	*m*	>0.95			
	p	>0.95			
Nitro-N-dimethylaniline	*m*	>0.95			
	p	0.78	—0.553		
sym-Trisubstituted derivatives and others					
p-Ethylphenol		0.86	—0.796		
p-Ethylaniline		0.86	—0.796	—0.796	0
3,5-Dinitroaniline		0.069	+1.130	+0.784	+0.346
3,5-Dinitrophenol		0.14	+0.785	+0.784	0
3,5-Dichloroaniline		0.76	—0.500	—0.750	+0.250
3,5-Xylenol		0.85	—0.747	—0.750	+0.003
Phenol		0.725	—0.420		
Aniline		0.725	—0.420		
Phloroglucinol*		0.004	+2.350	+2.406	—0.06

* Over-running technique used.

had identical R_F values in reversed phase System C (0.57 and 0.59 respectively). The two dinitrobenzenes, furthermore, had identical R_F values in the following systems:

0.30 in dimethylformamide/cyclohexane
0.24 in dimethylformamide/iso-octane
0.67 in formamide/cyclohexane–hexane (2:1)
0.18 in formamide/iso-octane

As a result of these examinations it was concluded (and the conclusion is an important one, as much of the later work hangs on it) that *m*- and *p*-dinitrobenzene are chromatographically indistinguishable in neutral partition systems. Nitrobenzene and *sym*-trinitrobenzene were then chromatographed and $\Delta R_M(NO_2)$ was calculated as + 0.615. As will be seen from Table I, these four nitrobenzenes (in which steric effects are absent) all obey MARTIN's equation with respect to the additive nature of the nitro group. This means that, whatever the chromatographic effects of interaction of the NO_2 group with the ring, they can be considered identical in these four compounds.

In System B (Table II), many more derivatives could be chromatographed. Once again it can be seen that, whatever the nature of the substituents, if separation occurred the *m*-isomer always migrated faster. Furthermore, as in System A, only when at least one substituent was *ortho/para*-directing did the isomers differ in R_M. However, the converse was not true: some *m*- and *p*-derivatives did not separate, even if one or sometimes both groups were *ortho/para*-directing.

CALCULATIONS AND GROUP ADDITIVITY

It is axiomatic that, if a given pair of *m*- and *p*-isomers have different R_M values, in at least one of them MARTIN's equation for group additivity cannot be obeyed. It is not excluded that this may be so for both isomers. Since the two dinitrobenzenes were shown to have identical R_M values in every system examined, it was useful to take this pair as a starting point. Table III gives the calculations of a series of group ΔR_M

TABLE III

CALCULATED ΔR_M PARAMETERS IN SYSTEM B (TRIGOL/DI-ISOPROPYL ETHER)

Group	*Calculation*	ΔR_M
NO_2	R_M(trinitrobenzene) — R_M(dinitrobenzene)	+0.602
Benzene	R_M(dinitrobenzene) — 2 × $\Delta R_M(NO_2)$	—1.833
OH	R_M(phenol) — R_M(benzene)	+1.413
NH_2	R_M(aniline) — R_M(benzene)	+1.413
CN	R_M(cyanonitrobenzene) — R_M(benzene) — $\Delta R_M(NO_2)$	+0.456
CHO	R_M(nitrobenzaldehyde) — R_M(benzene) — $\Delta R_M(NO_2)$	+0.678
Cl	R_M(chlorophenol) — R_M(phenol)	—0.165
CH_3	R_M(cresol) — R_M(phenol)	—0.165
C_2H_5	R_M(*p*-ethylphenol) — R_M(phenol)	—0.376

parameters, obtained only from compounds whose *m*- and *p*-isomers had identical R_M values: that is no assumptions were made as to which compound obeyed MARTIN's equation. (In each case the substitution of the benzene ring by a group X replaces one

aromatic hydrogen atom, whose ΔR_M value is unknown. We therefore include the latter in the former, and $\Delta R_M(X)$ includes the $\Delta R_M(H)$ value. Since all the calculations are made in the same way, no error is incurred.) $\Delta R_M(NO_2)$ was found to be constant in System B. From this, it was possible to calculate the hypothetical R_M value of benzene itself in System B, and hence all the other group ΔR_M parameters as shown in Table III. Using these group parameters, it was possible to calculate what would be the theoretical R_M values of many of the compounds of Table II, if MARTIN's equation was obeyed. These are shown in Table II where they can be directly compared with experimental values.

Several points emerge from these calculations:

1. With the exception of the nitrophenols and aminophenols, most phenol derivatives obeyed MARTIN's equation reasonably. The R_M value of *p*-hydroxybenzaldehyde was in agreement with theory, but that of the *m*-isomer was not.

2. There was a considerably discrepancy in the experimental and calculated R_M values for all the substituted anilines, both di- and tri-substituted.

3. *sym*-Trisubstituted phenol derivatives have R_M values in excellent agreement with calculated values, even when derived from a *m*-substituted phenol whose R_M value is in gross disagreement with theory (*cf. sym*-3,5-dinitrophenol and *m*-nitrophenol).

4. $\Delta R_M(NH_2)$ and $\Delta R_M(OH)$ are identical in this system, and $\Delta R_M(CH_3)$ is identical in both *p*-toluidine and *p*-cresol. Differences in the mesomeric moments of the latter molecules must have little effect on $\Delta R_M(CH_3)$.

Study of the R_M values of the aniline derivatives reveals that—in confirmation of a trend already observed—the *m*-substituted isomer usually shows a greater discrepancy than the *p*-isomer; and, indeed, the difference between the calculated and observed R_M values for *p*-nitroaniline, *p*-aminophenol and *p*-chloroaniline is approximately constant, and the difference is approximately twice as large for *p*-phenylenediamine. This suggests that $\Delta R_M(NH_2)$ could, with advantage, be calculated from the latter compound, as follows:

$$\Delta R_M(NH_2) = \frac{R_M(p\text{-phenylenediamine}) - R_M(\text{benzene})}{2} = +1.694$$

If this value is now used to re-calculate the R_M values of substituted anilines (Table IV), aniline and the toluidines show a constant discrepancy, but all the other *p*-substituted anilines fall into line, as do the *sym*-trisubstituted compounds. The *m*-compounds, on the other hand, cannot be correlated so, and clearly they do not obey MARTIN's equation.

Dimethylaniline derivatives also do not obey the additivity principle and a constant additive value for $\Delta R_M[N(CH_3)_2]$ cannot be obtained from the data of Table II, as the following calculations show.

$$\Delta R_M[N(CH_3)_2] = R_M(p\text{-aminodimethylaniline}) - R_M(\text{benzene}) - \Delta R_M(NH_2)$$
$$= +0.411$$

If this value is now used to calculate the R_M value of *p*-nitro-N-dimethylaniline, a value of + 0.182 is found, in pronounced disagreement with the observed R_M value of this compound, — 0.553.

TABLE IV

EXPERIMENTAL AND CALCULATED R_M VALUES OF ANILINES IN SYSTEM B

Compound	*Experimental* R_M	*Calculated* R_M	*Difference*
Aniline	—0.420	—0.139	—0.281
p-Toluidine	—0.585	—0.304	—0.281
m-Toluidine	—0.585	—0.304	—0.281
p-Ethylaniline	—0.796	—0.515	—0.281
p-Nitroaniline	+0.398	+0.463	—0.065
m-Nitroaniline	—0.081	+0.463	—0.544
3,5-Dinitroaniline	+1.130	+1.065	+0.065
p-Aminophenol	+1.300	+1.274	+0.026
m-Aminophenol	+1.130	+1.274	—0.144
p-Chloroaniline	—0.377	—0.304	—0.073
m-Chloroaniline	—0.420	—0.304	—0.116
3,5-Dichloroaniline	—0.500	—0.469	—0.031
m-Phenylenediamine	+1.342	+1.556	—0.214

EFFECTS OF SYSTEM CHANGE ON SEPARATION OF *meta*- AND *para*-ISOMERS

A possibility to be considered at this stage was that the separation of certain *m*- and *p*-isomers could have been due to adsorption effects of the paper. In accordance with a practice we have used previously[3,5], this was tested by chromatographing key compounds in the same partition system as used above, but supported on glass paper. No difference in R_M was found for any compound and this confirms our previous conclusions that R_M values of most compounds (except acids) in systems using an impregnated stationary phase are unaffected by the support. As a further check on the adsorption problem, several compounds were chromatographed in reversed phase system C — since if any adsorption were present it must surely affect the R_M values in reversed phase and direct phase systems in the opposite direction. Although not many simple benzene derivatives run in this system, Table V gives the R_F values of those that do.

TABLE V

R_F VALUES OF *meta*- AND *para*-ISOMERS IN REVERSED PHASE SYSTEM C (OLIVE OIL/85% ETHANOL)

Compound	*m-Isomer*	*p-Isomer*
Dinitrobenzene	0.57	0.57
Chloronitrobenzene	0.67	0.67
Cyanonitrobenzene	0.59	0.59
Nitroanisole	0.70	0.74
Nitro-N-dimethylaniline	0.56	0.64
N,N-Tetramethylphenylenediamine	0.71	0.76
Nitrophenyl acetate	0.78	0.78

In confirmation of the results in other systems, the nitrobenzenes, cyanobenzenes and nitrophenyl acetates were still inseparable. The nitroanisoles, nitrodimethylanilines and tetramethylphenylenediamines (all containing *ortho/para*-directing substituents)

had different R_F values, and as expected, the *p*-isomers of the latter compounds ran *faster* than the *m*-isomers; this again suggests that any difference between the R_F values of *m*- and *p*-isomers is unlikely to be due to adsorption effects.

HALOPHENOLS

The isomeric chlorophenols (although they each contain two *ortho/para*-directing substituents) could not be separated in any of our systems. GESSNER AND SMITH[14], using a formamide/hexane system, found the R_F values of the *m*- and *p*-isomers to be 0.7 and 0.6 respectively, but the separation appeared to depend on the method of impregnation. We compared the two compounds in their system and obtained R_F values of 0.67 and 0.63, but the *p*-chlorophenol spot was diffuse and difficult to locate exactly. When the system was used on glass paper instead of Whatman No. 4 paper, both substances migrated just behind the front and did not separate. With hexane as a solvent, R_F values might be especially sensitive to the amount of moisture in the paper; or, alternatively, adsorption might just begin to play a part when the mobile phase is completely non-polar. In any case, any difference between these two compounds must be considered as marginal and perhaps doubtful except in special systems.

The apparent chromatographic identity of *m*- and *p*-chlorophenol was of especial interest as it indicates that the phenolic ionisation strength has (as we have already suggested[2]) little affect on R_M. It also indicates that the inductive effect of the halogen group (which must produce a different mesomeric displacement in the aromatic ring, depending on whether it is *meta* or *para* to the OH group) cannot be directly and simply related to R_M differences. In order to study this question in more detail all twelve mono-halophenols were chromatographed in reversed phase System D (neither of the trigol systems were suitable for all twelve compounds). The results are given in Table VI (together with the cresols, for comparison) and show that *all three* isomers of each

TABLE VI

R_F AND R_M VALUES OF HALOPHENOLS IN SYSTEM D (ETHYL OLEATE/25% AQUEOUS ETHANOL)

Compound	R_F	R_M
o-, *m*-, *p*-Fluorophenol	0.89	−0.906
o-, *m*-, *p*-Chlorophenol	0.725	−0.417
o-, *m*-, *p*-Bromophenol	0.54	−0.070
o-, *m*-, *p*-Iodophenol	0.385	+0.204
o-Cresol	0.78	−0.547
m-, *p*-Cresol	0.85	−0.767

halophenol have identical R_F values in this system. There is thus not only no separation of *m*- and *p*-isomers, but there is no *ortho*-effect in these compounds. The significance of these results is discussed further below.

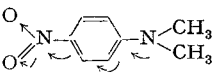
DISCUSSION

Analysis of the problem of *m*- and *p*-isomers revolves round four paramount questions. First, why do some pairs of isomers have different R_M values, while others have the

same R_M value? (Although it is true that non-separation in any finite number of systems cannot, theoretically, be used as an argument for universal non-separation in all other systems, there is sufficient distinction between the two classes of compounds to comprise a valid effect. As shown later, our interpretation of the phenomenon does not in any case exclude the eventual separation of certain of these identical pairs.) Secondly, can the *degree* of separation be correlated with molecular interactions? Thirdly, why, with certain exceptions, do *p*-substituted and *sym*-trisubstituted compounds obey MARTIN's equation, whereas *m*-substituted compounds often do not? Finally, why do *m*-compounds run faster than *p*-compounds (in direct phase), irrespective of the substituents?

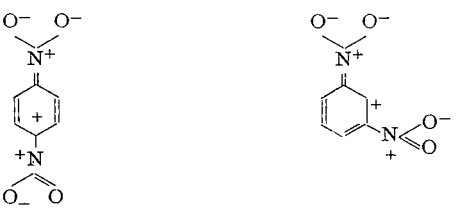
The only previous suggestion that appears to have been made to account for the separation of a pair of *m*- and *p*-isomers has been by FRANC AND JOKL[18]. They considered that MARTIN's equation was, in fact, not obeyed and substituted for it an equation which included a parameter derived from the molecular dipole moment of the compound under study. As we have already shown[2], in alkylbenzenes where the deviation from MARTIN's equation arises simply from polarization in the alkyl group, which must be directly related to the moments of such compounds, such a correction is useful. However, FRANC AND JOKL used it to correlate the R_F values of di- and polyhydroxybenzenes, particularly to explain the separation of quinol and resorcinol. Their view that vectorial dipole moments affect R_F values cannot be supported, for both practical and theoretical reasons. FRANC AND JOKL calculated the R_F values of quinol and resorcinol by assuming they had dipole moments of zero and 1.6 D respectively. In fact, this is an error. Quinol, because it contains two angular groups *para* to each other, has a vectorial dipole moment whose magnitude is dependent on the direction of the O–H bonds[19]. Its value has been variously determined as 2.47 D[20] and 1.40 D[21]. In either case, it is not appreciably different from the dipole moment of resorcinol (1.6 D) and the calculations of FRANC AND JOKL cannot be justified. It seems unlikely, indeed, that the vectorial dipole moment of a compound should affect its partition coefficient. DIKSTEIN[22] has shown that the cohesion energy of a solute enters the solubility parameter and has an important effect in determining R_F, and KETELAAR[23] shows that the Keesom energy of a molecule (that part of the cohesion energy affected by group polarizations) is influenced only by the individual group moments and not by the total molecular dipole, since the former are operative only over small distances of the order of atomic radii. As a consequence, the cohesion energies of molecules such as *o*-, *m*- and *p*-dichlorobenzene (and hence their boiling points, solubilities in regular solutions, etc.) are almost identical, although their molecular dipole moments are quite different. INGOLD[17], considering chemical reactions, remarks that solvation energy differences must be nearly independent of those parts of the molecule that do not change in the reaction, solvation forces being highly localized on solute molecules. The results in Tables I and II offer unequivocal evidence that the dipole moment of a compound cannot affect its R_F value appreciably. The moment of *m*-dinitrobenzene[24] is 4.07 D, and that of *p*-dinitrobenzene is close to zero (both NO_2 groups lying in the plane of the ring)[25]: this difference is perhaps the largest possible between two isomers, and yet these compounds have identical R_F values. The fact that $\Delta R_M(NO_2)$ is additive for *sym*-trinitrobenzene confirms that dipole effects do not directly influence R_M, and much similar evidence can be adduced from Tables I and II. The cyanobenzenes are exactly analogous to the nitrobenzenes: the *m*-compound

must have a large moment and *p*-dicyanobenzene has a moment of zero, as the CN group is linear. Table V shows that both *m*-nitro-N-dimethylaniline and *m*-N,N-tetramethylphenylenediamine run more slowly (reversed phase system) than their respective *p*-isomers. Since the directions of the interaction moments in the two *p*-isomers are quite different, the nitro group reinforcing while the dimethylamino group opposes the mesomeric moment of the other dimethylamino group (I), one would certainly not expect both *m*-isomers to run faster, if the vectorial dipole moment was of importance. In fact, the reinforcing interaction moment of the nitro and dimethylamino groups *para* to each other is as much as + 1.86 D[26], without apparent influence on R_M.



(I)

The separation of *m*- and *p*-isomers cannot be a simple function, either, of the electronic interactions of substituents with the aromatic ring. Such interactions must occur, and indeed they must markedly influence the resonance structures of *m*- and *p*-derivatives, but (as we have concluded previously as a result of our study of alkylated benzenes and ethers[2]) charge separation *by itself* does not seem to affect R_M unless there is a secondary effect on intramolecular or intermolecular hydrogen bonding. Thus the resonance forms of *m*- and *p*-dinitrobenzene involve contributions from markedly different charged structures (II)



(II)

and similar examples are to be found in Tables I and II. Furthermore, the variations in R_M cannot be simply correlated with the inductive effects of substituents. If the electronegativity or electropositivity of substituents were important it would certainly not be possible for compounds as different as the chlorophenols, cyanophenols and di- and trinitrobenzenes all to obey the additivity principle (see Table II), whereas *m*-nitrotoluene which has a different R_F value from *p*-nitrotoluene, clearly does not. The best illustration of this is in the study of the twelve halophenols. DIKSTEIN's thermodynamic derivation[22] of MARTIN's postulate has shown that the partition coefficient of a substance is partly determined by its molar volume. In Fig. 1, we have plotted the R_M values of the four *p*-halophenols against the molar volumes of the various *p*-substituents. Included in the plot is the R_M value of a hypothetical "*p*-cresol" obtained from the R_M value of *p*-cresol, corrected for the hyperconjugation effect of the CH_3 group attached to the aromatic ring, as follows:

System D in this series is identical with System 1 described previously[2] and the hyperconjugation effect of the CH_3 group can be corrected for by considering the

three α-hydrogen atoms of *p*-cresol to be δ-hydrogen atoms. In System D, ΔR_M(α-hydrogen) is + 0.014 and ΔR_M(δ-hydrogen) is + 0.096 and the correction is therefore 3 × 0.082 or + 0.246. Thus, if *p*-cresol contained no electronic effects, its R_M value would be not — 0.767, but — 0.521, and this is the value included in the plot of Fig. 1. The plot is closely linear, although *p*-fluorophenol runs slightly faster than

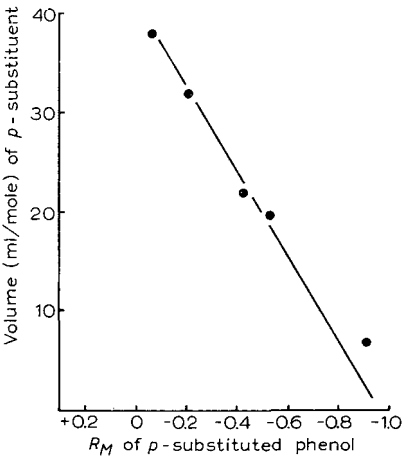
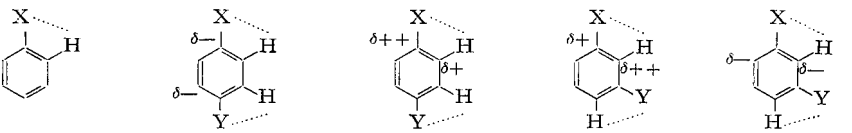


Fig. 1. Relationship between R_M values of *p*-substituted halophenols and molar volume of the halogen atom.

predicted. (The molar volume of *p*-fluorophenol might in fact be rather smaller than calculated. There are some grounds for believing that the C–F bond in aromatic compounds is somewhat shorter than in alkyl fluorides because of the exceptional electronegativity of the fluorine atom[27].) Since the inductive effect of the "corrected" CH_3 group can be considered as zero (or at any rate slightly positive) it is clear that the negative inductive effects of the halogen atoms can play little part in affecting R_M since the only determinant in this series would appear to be molar volume. This is reinforced by the further observation that, although the mesomeric polarizations in *o*-, *m*- and *p*-halophenols differ, the three isomers, in each case, have identical R_F values. It can be concluded, therefore, that the separation of *m*- and *p*-isomers cannot be a simple function of either the direction or magnitude of the electronic polarizations in these molecules, although it is clear that some factor must exist that is related to the electronic effects of groups attached to the ring. Molar volume differences, although they partly determine absolute R_M values (*cf.* DIKSTEIN[22] and above), cannot account for the difference between *m*- and *p*-isomers and, especially, they cannot be used to explain why some pairs separate and some do not.

In preceding parts[2,4–6], we have shown that many deviations from the strict additivity of groups required by MARTIN's equation can be explained in terms of intra- and intermolecular hydrogen bonding and the competition between them. In a study of alkylated phenols and aromatic ethers[2] it was suggested that electronic polarizations operated by affecting the strength of C–H bonds sufficiently to influence solvent-solute interactions. We believe that this is the factor that also determines the separation of *m*- and *p*-benzene derivatives. The energy of a hydrogen bond is usually

considered to be mainly electrostatic in origin, and is highest when the bond joins two strongly electronegative elements, such as O, N or F. In substituted aromatic compounds it is possible for hydrogen bonding to occur as shown (III).



(a) (b) (c) (d) (e)

(III)

The energy of the various C-H···X bonds depicted in (III) can be considered to be a function of (1) the distance C–H···X, (2) the electronegativity of X (or if X is a polyatomic group, of the atom nearest to the hydrogen atom and included in the bond) and (3) the electronic state of the C atom included in the bond. The formation of such an internal hydrogen bond, if it occurs, must have a chromatographic effect, for it will compete with the formation of intermolecular hydrogen bonds between solute and solvents. The energy of an intramolecular C–H···X bond of this sort must normally be low if the carbon atom is uncharged or bears a fractional positive charge. However, under conditions in which the carbon atom bears a fractional negative charge, the energy of the bond may be considerably increased. In a monosubstituted benzene (IIIa), any effect of this kind is automatically included in ΔR_M for the group X. In a *p*-disubstituted compound, two conditions can be distinguished. If Y is a group that is *ortho/para*-directing, that is it repels electrons into the ring by either an inductive or mesomeric mechanism, a fractional negative charge is induced on the *para*-C atom (IIIb), but the *meta*-C atom is hardly affected[17,28] and so the C–H···X bond is relatively unaffected. In this case the chromatographic effect will be similar to that in a monosubstituted derivative and will be included in the parameter ΔR_M(X). Deviation from MARTIN's equation will thus be minimal. If Y is electron-withdrawing, there is a general de-activation of the ring (IIIc) and since the *meta*-C atom bears a fractional positive charge under these conditions, the C–H···X bond is too weak to compete with the intermolecular hydrogen bond. *Para*-compounds can thus be expected to obey MARTIN's equation, unless other effects are present. Consider now a *m*-disubstituted benzene. If group Y is electron-withdrawing (IIId), the C atom involved in the C–H···X bond is relatively unaffected, as above. If, however, Y is *ortho/para*-directing (IIIe), then the C atom involved in the C–H···X bond bears a fractional negative charge whose strength will depend on the total inductive and mesomeric displacements produced by Y. This will, according to our hypothesis, increase the strength of the intramolecular hydrogen bond, thus affecting the partition coefficient. This hypothesis seems to us to be the only one capable of accounting for the chromatography of *m*- and *p*-isomers. It explains why one *ortho/para*-directing group is necessary for separation and the fact that *m*-derivatives (if separation occurs) always run faster than *p*-derivatives in direct phase systems.

The hypothesis is supported by the possibility of demonstrating a relationship between the magnitude of the charge induced on a *para*-carbon atom by a substituent group or atom and the difference between the R_M values of the *m*- and *p*-isomer.

Table VII lists the differences in R_M between *m*- and *p*-substituted nitrobenzenes (calculated from the data of Table I) and Hammett's *para*-constants for the various substituents[29]. Those constants are the best measurement of the fractional charge induced on an aromatic C atom by a *para*-substituent, due to the sum total of reso-

TABLE VII

DIFFERENCES BETWEEN R_M VALUES OF *meta*- AND *para*-SUBSTITUTED NITROBENZENES (DATA FROM TABLE I) AND HAMMETT'S SUBSTITUENT CONSTANTS

Substituent in nitrobenzene	$R_M(meta)-R_M(para)$	$\sigma(para)$
NO_2	0	+0.788
OCH_3	−0.301	−0.268
CH_2Br	−0.091	+0.184*
$N(CH_3)_2$	−0.776	−0.600
$OCH_2C_6H_5$	−0.326	−0.415
Cl	−0.035	+0.227
CN	0	+0.628
CH_3	−0.101	−0.170

* Figure for CH_2Cl.

nance and polar effects of the substituent on the aromatic ring. (Since the nitro group in aromatic systems obeys MARTIN's equation, by studying substituted nitrobenzenes, we eliminate the difficulty of studying variations due to both substituents together.) Fig. 2 shows the plot of these R_M differences against Hammett's constants, which lie on a smooth curve.

It would follow that, providing a fractional negative charge is induced on the *para*-C atom of a *meta*-isomer, any factor that decreases the C–H···X distance will

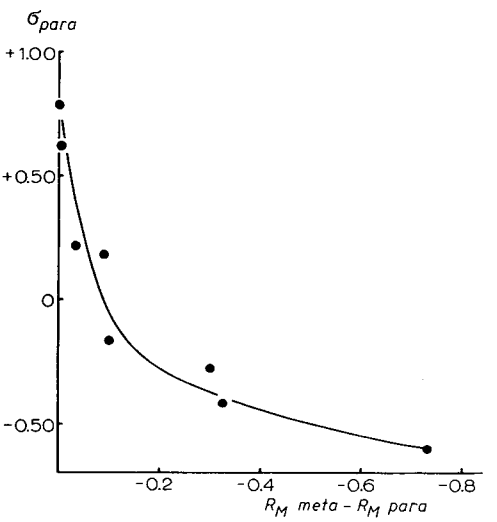
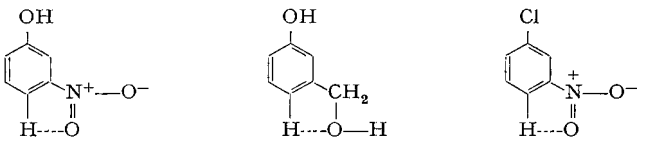


Fig. 2. Relationship between Hammett's $\sigma(para)$ constants and $R_M(meta) - R_M(para)$.

operate in the same direction as the induced charge and still further increase the strength of the intramolecular hydrogen bond and hence the separation of isomers. This is supported by the results in Tables I and II, which show that the largest differences between isomers occur with compounds such as the nitrophenols, nitroanilines, nitrodimethylanilines and hydroxybenzaldehydes. In the *m*-isomers of these compounds, the C–H···X distance is decreased and bonding facilitated by the possibility of 5-membered ring formation (IVa) and (IVb).



(a) (b) (c)

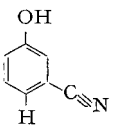
(IV)

The effect of this phenomenon can be observed by comparing the various chloro-substituted derivatives in Tables I and II. Thus, although quinol and resorcinol have different R_M values, *m*- and *p*-chlorophenol do not, because the C–H···Cl hydrogen bond is much weaker than the C–H···O bond. (The marginal separation of chlorophenols in the formamide/hexane system may be partly due to the fact that intermolecular hydrogen bonding between the aromatic C atom and hexane is even weaker.) Although the Cl atom is itself an *ortho/para*-directing group and might be expected to induce a charge as in (V), this must be very weak, as the Cl atom is reluctant to take part in the implied increase in covalency which is shown in the fully mesomeric structure (VI).

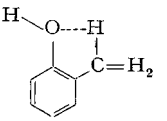
(V) (VI)

The electron-repelling effect of halogen atoms attached to the benzene ring by mesomeric interaction of the lone pair is in fact opposed by the inductive effect of the halogen, which is electronegative. This is why, in aromatic substitution, halogen substituents are uniquely *ortho/para*-directing but deactivating to the ring. Similar conditions must occur in the ground states and lead to uncertainty about the strength of the negative charge induced in the *para*-position by halogens. The general weakness of the effect of Cl is indeed substantiated by the fact that $\sigma(para)$ for this group is positive, whereas for nearly all other *ortho/para*-directing groups it is negative[30]. Unlike *m*- and *p*-chlorophenol, however, *m*- and *p*-chloronitrobenzene do show a slight difference in R_M value. This, we believe, may be due to a slight effect on internal hydrogen bonding due to the possibility of 5-membered ring formation in the *m*-nitro compound as shown in (IVc); the *m*- and *p*-isomers of chloroaniline also show a very slight R_M difference in System B: this we attribute to the powerful electron-repelling

effect of the NH_2 group, compared to the OH group[17], which may strengthen the C–H···Cl bond sufficiently to begin to compete with intermolecular hydrogen bonding. Cyanophenols have identical R_M values, however, since the —C≡N group is linear and any C—H···N bond must be very weak indeed (VII), because of the distance involved.



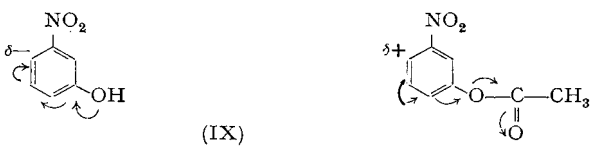
(VII)



(VIII)

(BAKER AND CARUTHERS[30] found no evidence of intramolecular hydrogen bond formation in *o*-hydroxybenzonitrile and attributed this to a similar reason. They state that the distance between the nitrogen atom of the nitrile group and the oxygen atom in this compound is more than 3 Å, too great for normal hydrogen bonding.) The absence of an *ortho*-effect in the *o*-halophenols (Table VI) may also be ascribed to the absence of hydrogen bonding between the two *ortho*-groups. The clear separation of *o*- and *p*-cresol in System D shows that an *ortho*-effect, if it is present, can certainly be observed in this system. Hydrogen bonding is known to be possible in *o*-cresol (VIII) but probably does not occur in *o*-chlorophenol (*cf.* BAKER[31]). This result confirms our earlier opinion[2] that the *ortho*-effect in chromatography is unlikely to be purely spatial in character.

The hypothesis also accounts for the fact that although *m*- and *p*-nitrophenol and their corresponding benzyl ethers show a large separation (Table I), *m*- and *p*-nitrophenyl esters have identical R_M values. This can be ascribed to the fact that the carbonyl group of the acetoxy group in (IX) competes for the lone pair of electrons on the oxygen atom and converts the + M effect of OH and OR into a — M effect[17].

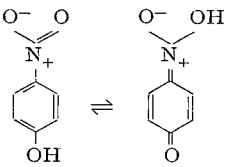


(IX)

It is also possible to account for the fact that *sym*-trisubstituted benzenes obey the group additivity principle even when the corresponding disubstituted derivatives do not. The best example is 3,5-dinitrophenol, which obeys MARTIN's equation with respect to all three groups exactly although *m*-nitrophenol shows an exceptionally large deviation (Table II). In the former molecule, the negative charge induced on the *para*-C atom by the OH group is likely to be neutralized by the unusually strong electron-withdrawing powers of the two *m*-substituted NO_2 groups, which deactivate all positions of the ring. Similar arguments apply to 3,5-dinitroaniline and 3,5-dichloroaniline.

The *p*-substituted anilines obey the group additivity principle less strictly than the other compounds investigated. It is not clear why this is so, but a possible reason is that some compound formation may take place between anilines and hydroxylic

solvents. The presence of even small amounts of compounds of the type, $RNH_3^+OH^-$, would, of course, affect the experimental R_M values considerably. Nitroanilines contain a potentially acidic group and may exist partly as solvated zwitterions, which would behave anomalously. Apart from amino compounds, the only major exception



(X)

to the additivity rule among *p*-substituted benzenes is *p*-nitrophenol. It is difficult to see why this should be so unless, like *p*-nitrosophenol, it too can exist as a tautomeric mixture (X). There is some evidence for this[32].

SUMMARY

The separation of *m*- and *p*-disubstituted benzene derivatives has been studied in four systems. It is shown that the R_M values of these compounds cannot be correlated with the existence of charge separations or with the vectorial dipole moment. The *p*-isomers generally obey the group additivity principle, whereas *m*-isomers, when they differ, depart from this principle. The separation of *m*- and *p*-isomers is explained in terms of a competition between intra- and intermolecular hydrogen bonding. It is shown how in *m*-isomers, the presence of an *ortho/para*-directing group can affect hydrogen bonding by inducing a fractional negative charge on the *para*-carbon atom. Benzene derivatives containing two *meta*-directing groups appear to be inseparable by partition chromatography, and this is discussed. Certain compounds, such as substituted anilines, have anomalous R_M values and may contain, in hydroxylic solvents, small amounts of compounds of the type, $RNH_3^+OH^-$. The chromatography of halophenols shows no influence of the inductive effect of the halogens; they separate according to their molar volume. There was no *ortho*-effect in halophenols in the reversed phase system studied, confirming that this effect is not spatial but mainly polar in origin.

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PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

VIII. HYPERCONJUGATION

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INTRODUCTION

In a preceding part[1], we discussed the chromatography of alkylated phenols and showed that these substances obeyed MARTIN's equation[2] if atomic ΔR_M parameters were used for calculation purposes. A variation in the value of ΔR_M(H) was observed, depending on the proximity of CH_2 and CH groups to the aromatic ring and it was suggested that this variation was a result of the loosening of C–H bonds brought about by the polarization of alkyl groups attached to an aromatic structure. The polarization was regarded as possibly being produced by two mechanisms, carbon–hydrogen hyperconjugation and carbon–carbon hyperconjugation (the latter, according to BERLINER AND BONDHUS[3], being the origin of the inductive effect in alkylbenzenes). Although it did not seem possible clearly to distinguish these two mechanisms chromatographically, the study described here sheds some further light on the problem.

EXPERIMENTAL AND RESULTS

Sheets of Whatman No. 4 paper were impregnated with olive oil (5 % v/v in light petroleum) and the phenols were chromatographed by the descending method, using

TABLE I

CHROMATOGRAPHY OF SUBSTITUTED PHENOLS IN A REVERSED PHASE SYSTEM (OLIVE OIL/70 % ETHANOL)

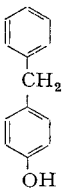
Compound	R_F	R_M	ΔR_M*(phenyl)*
Phenol		—1.142*	
p-Cresol		—1.008*	
p-*n*-Propylphenol	0.85	—0.740	
p-*n*-Butylphenol	0.80	—0.618	
p-*n*-Amylphenol	0.75	—0.483	
p-Phenylphenol	0.805	—0.620	+0.522
p-Benzylphenol (I)	0.805	—0.620	+0.388
p-Hydroxytriphenylmethane (II)	0.64	—0.250	+0.370
p-Hydroxytetraphenylmethane (III)	0.52	—0.036	+0.214

* Phenol and *p*-cresol both run rather too fast in this system for their R_M values to be found experimentally with the required degree of accuracy. They can be calculated with great precision, though, by extrapolation from the other homologous phenols, $\Delta R_M(CH_2)$ being + 0.134 in this system.

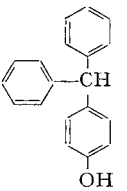
70 % (v/v) aqueous ethanol as mobile phase. After drying, the phenols were observed under ultra-violet light[1]. Table I gives the R_F and R_M values of the compounds. As already described[1], R_M values were calculated directly and R_F values (to two significant figures) from them.

DISCUSSION

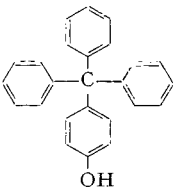
The three substances under investigation are phenyl-substituted phenols, as shown.



(I)



(II)



(III)

They can all be considered as derived from *p*-cresol by successive substitution of one α-hydrogen atom by a phenyl group.

R_M(phenyl) can be obtained by comparing R_M (*p*-phenylphenol) with R_M(phenol) and is equal to + 0.522. As *p*-phenylphenol and polynuclear phenols obey MARTIN's equation with respect to atomic ΔR_M(C) and ΔR_M(H) parameters[1], this value for ΔR_M(phenyl) can be considered as the additive increment for the phenyl group. (The loss of one hydrogen atom by the substitution in phenol to give *p*-phenylphenol can be considered as arbitrarily included in the parameter.) It is now possible to calculate the value of ΔR_M(phenyl) obtained by adding successive phenyl groups to *p*-cresol. Table I shows that the value of this parameter is not constant, but decreases with increased substitution. The comparison between *p*-benzylphenol and cresol is, of course, an alternative expression of what we have previously referred to as the "allyl" effect and is produced by hyperconjugation of the benzyl group with the other aromatic ring. The fact that the value of ΔR_M(phenyl) continues to decrease even in *p*-hydroxytetraphenylmethane (III), *which contains no α-hydrogen atoms for carbon–hydrogen hyperconjugation*, must mean that carbon–carbon hyperconjugation plays a predominant role in determining the nature of polarization in these molecules. A molecule such as (III) is approaching in structure certain compounds in which dissociation into stable free radicals occurs, and the aliphatic-aromatic carbon bonds in (III) must be subject to definite stretching. The relative decrease in ΔR_M(phenyl) in these compounds —in (III) the whole phenyl group increases R_M by little more than one aliphatic CH_2 group—supports our previous suggestion[1] that hyperconjugation causes the deviations from MARTIN's equation in alkylated and aryl-alkylated benzenes. It also supports the contention of BERLINER AND BONDHUS[3] that carbon–carbon hyperconjugation may be more important than carbon–hydrogen conjugation in the ground state. Finally, it may be noted that there is no suggestion that the steric crowding in (II) or (III) produces any effect; if this existed it must produce an *increase* in R_M in a reversed phase system, that is, in the opposite direction to that found. This provides a further example of the apparent lack of importance of spatial effects compared to electronic effects in chromatography.

SUMMARY

The chromatography of a series of phenylated *p*-cresols has been studied. It provides evidence that carbon–carbon hyperconjugation is of importance in determining the R_M values of these compounds.

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Short Communications

Chromatographische Trennungen von Radionukliden mit Eisen(III)-hydroxid auf Papierträger

Die Wechselwirkung von Kationen und Anionen in wässrigen Lösungen mit Eisen-(III)-hydroxid ist seit langem bekannt. Bei der Darstellung von ^{32}P aufgrund der Reaktion $^{32}S(n,p)^{32}P$ kann z.B. das trägerfreie $^{32}PO_4^{3-}$ durch Eisen(III)-hydroxid-Fällungen von Makromengen SO_4^{2-} abgetrennt werden[1,2]. Diese Trennung wurde auch mit Eisen(III)-hydroxid, das auf organische Kationenaustauscher aufgezogen war[3], und mit pseudomorphem Eisen(III)-hydroxid[4] in Säulen beschrieben. Trennungen von Kationen mit Eisen(III)-hydroxid-Säulen sind bisher nicht bekannt geworden.

Geringe Substanzmengen (z.B. trägerfreie oder trägerarme Radionuklide) in kleinen Lösungsvolumina lassen sich papierchromatographisch einfacher auftrennen als mit Säulen. Von verschiedenen Autoren wurden einige anorganische Verbindungen mit ionenaustauschenden Eigenschaften in der Kombination mit Papier als Träger zu Trennungen von Radionukliden verwendet[5–7]. Es lag nahe, in ähnlicher Weise die Vorteile des Eisen(III)-hydroxids mit der papierchromatographischen Arbeitsweise zu kombinieren. Eisen(III)-hydroxid wurde auf Chromatographiepapier aufgezogen und Trennungen durch aufsteigende Chromatographie durchgeführt.

Herstellung des Eisen(III)-hydroxid-Papiers (EH-Papier)

Eisen(III)-hydroxid wird nach folgendem Verfahren auf Whatman 3MM-Papier aufgebracht: Papierstreifen (4.5 × 50 cm) werden mit einer 20%igen Eisen(III)-nitratlösung getränkt. Nach Trocknen an der Luft wird das Eisen(III)-nitrat in Ammoniaklösung (konz.) zu Eisen(III)-hydroxid umgesetzt. Die Streifen werden anschliessend mit fliessendem, destilliertem Wasser bis zur vollständigen Entfernung des Ammoniaks gewaschen und wieder an der Luft getrocknet. Die durchschnittliche Flächenbeladung beträgt 2–2.5 mg Eisen(III)-hydroxid/cm² Whatman 3MM-Papier.

Versuchsdurchführung

Die Versuche wurden mit der üblichen Arbeitstechnik in Glaszylindern durchgeführt. EH-Papierstreifen mit den Abmessungen 2 × 35 cm gelangten zur Verwendung. Zur Messung der Aktivitätsverteilung wurden die Chromatogramme in kleine Querstreifen (0.5 bzw. 1 cm) zerschnitten und die Aktivität dieser Streifen mit einem Methan-durchflusszähler gemessen. Die Laufmittelfront ist bei verdünnten Laufmitteln nach 1 h ca. 18 cm, nach 2 h ca. 26 cm gewandert; bei höheren Laufmittelkonzentrationen (z.B. 1 *N* NaOH, 5 *N* NH_4NO_3) sind diese Werte kleiner.

Trennungen

Anionen. An der Eisen(III)-hydroxidsäule war das Verhalten der Anionen PO_4^{3-}, SO_4^{2-}, Cl^- und J^- untersucht worden[8]. Die Arbeit ergab Trennvorschriften für

wägbare und unwägbare Mengen dieser Ionen. Zur Untersuchung der Eigenschaften von EH-Papier wurden Bedingungen zur Trennung der gleichen Anionen ausgearbeitet. Als Laufmittel kamen Natronlauge und $NaHCO_3$-Lösung zur Anwendung.

TABELLE I

R_F-WERTE* VON PO_4^{3-}, SO_4^{2-}, Cl^-, J^- IN ABHÄNGIGKEIT VON DER KONZENTRATION VERSCHIEDENER LAUFMITTEL

Laufmittel	$^{36}Cl^-$	$^{131}J^-$	$^{35}SO_4^{2-}$	$^{32}PO_4^{3-}$
0.01 *M* $NaHCO_3$	—	0.45	0.28	—
0.05 *M* $NaHCO_3$	0.8	0.52	0.60	0.0
0.1 *M* $NaHCO_3$	—	0.85	0.77	—
0.5 *M* $NaHCO_3$	—	0.92	1.0	—
1 *M* $NaHCO_3$	—	0.95	1.0	—
0.001 *N* NaOH	0**	0.24	0.05	0.0
0.01 *N* NaOH	0.6***	0.33	0.35	0.0
0.1 *N* NaOH	0.90	0.48	0.8	0.2
1 *N* NaOH	0.90	0.76	1.0	0.7
Dest. Wasser	—	—	0.0	0.0

* $R_F = \frac{\text{Abstand des Maximums vom Startpunkt}}{\text{Abstand der Laufmittelfront vom Startpunkt}}$

** Starker Aktivitätsvorlauf.

*** Kein scharfes Maximum.

Die Versuche wurden mit trägerfreiem $^{32}PO_4^{3-}$, $^{35}SO_4^{2-}$, $^{131}J^-$ und niedrigspezifischem $^{36}Cl^-$ (689 μCi/g Cl^-) durchgeführt. Die Ergebnisse sind in Tabelle I zusammengefasst.

Aus den R_F-Werten der Tabelle I lässt sich ablesen, dass sowohl mit Natronauge, als auch mit $NaHCO_3$-Lösung als Laufmittel Trennungen möglich sind; dies

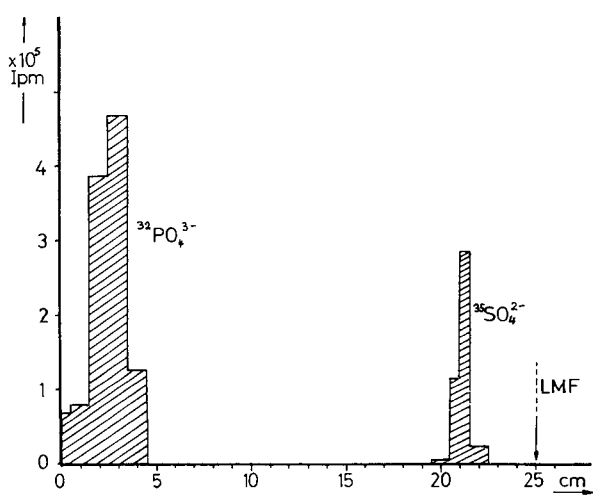

Fig. 1. Trennung von trägerfreiem $^{32}PO_4^{3-}$ und $^{35}SO_4^{2-}$ mit Natronlauge (0.1 *N*) als Laufmittel auf EH-Papier.

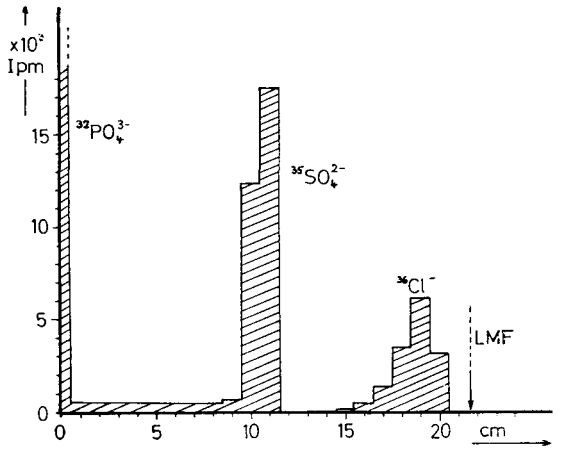

Fig. 2. Auftrennung von Bestrahlungsprodukten von KCl ($^{32}PO_4^{3-}$, $^{35}SO_4^{2-}$, $Cl^-(^{36}Cl)$) mit $NaHCO_3$ (0.05 *N*) als Laufmittel auf EH-Papier.

konnte experimentell bestätigt werden. Fig. 1 zeigt eine typische Aktivitätsverteilung von $^{32}PO_4^{3-}$ und $^{35}SO_4^{2-}$ bei Verwendung von 0.1 *N* Natronlauge als Laufmittel. Fig. 2 zeigt eine Auftrennung von Bestrahlungsprodukten von KCl ($^{32}PO_4^{3-}$, $^{35}SO_4^{2-}$, $Cl^-(^{36}Cl)$), die 10 Tage nach Bestrahlungsende erfolgte. Zu diesem Zeitpunkt waren alle Aktivitäten ausser ^{32}P, ^{35}S und ^{36}Cl abgeklungen. Da die ^{36}Cl-Aktivität unter den gegebenen Bedingungen im Verhältnis zu den übrigen Aktivitäten sehr gering war, wurde zusätzlich $^{36}Cl^-$ zur Ausgangslösung zugegeben. Die beschriebenen Trenneffekte sind auf Wechselwirkungen zwischen den genannten Ionen und Eisen(III)-hydroxid zurückzuführen. Das Verhalten der Ionen ist auf Eisen(III)-hydroxid-freiem Whatman 3MM-Papier grundlegend anders; so hat trägerfreies $^{35}SO_4^{2-}$ mit destilliertem Wasser auf Whatman 3MM-Papier einen R_F-Wert von 1, auf EH-Papier einen R_F-

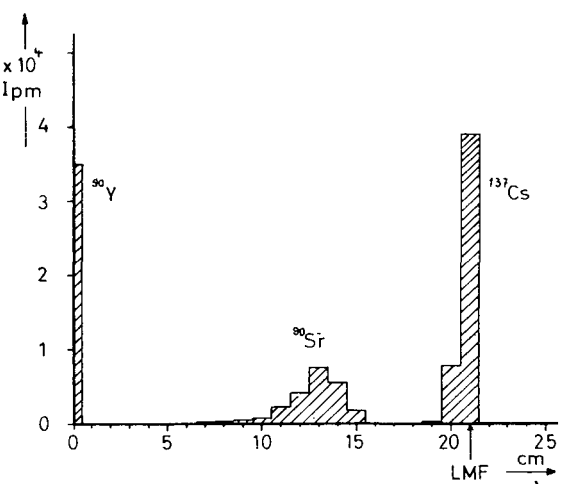

Fig. 3. Trennung eines Gemisches von ^{90}Sr, ^{90}Y und ^{137}Cs mit NH_4NO_3-Lösung (5 *N*, pH 3.5) als Laufmittel auf EH-Papier.

Wert von 0. Mit $NaHCO_3$-Lösung oder Natronlauge als Laufmittel liessen sich auf Whatman 3MM-Papier keine Trennungen erreichen, da die untersuchten Ionen mit der Front liefen bzw. keine scharfen Zonen bildeten.

Kationen. Die Mitfällung von Yttrium-Ionen an Eisen(III)-hydroxid zur Trennung von Strontium-Ionen legte den Gedanken von Strontium–Yttrium-Trennungen mit EH-Papier nahe. Zusätzlich wurde das Verhalten von Cäsium- und Cer-Ionen untersucht. Die Experimente wurden mit trägerfreiem ^{90}Sr, ^{90}Y, ^{137}Cs bzw. ^{144}Ce durchgeführt. Als Laufmittel dienten NH_4NO_3-Lösungen, die mit HNO_3 auf pH 3.5 eingestellt waren. Die R_F-Werte sind in Tabelle II zusammengestellt. Man

TABELLE II

R_F-WERTE VON ^{90}Sr, ^{90}Y, ^{137}Cs, ^{144}Ce AUF EH-PAPIER UND WHATMAN 3MM-PAPIER IN ABHÄNGIGKEIT VON DER KONZENTRATION DER NH_4NO_3-LÖSUNG (pH 3.5)

Laufmittel	R_F							
	EH-Papier				*Whatman 3MM*			
	^{90}Sr	^{90}Y	^{137}Cs	^{144}Ce	^{90}Sr	^{90}Y	^{137}Cs	^{144}Ce
0.01 *N* NH_4NO_3	0.15	0	1	0.05	0.7	0.05	0.8	0.95
0.1 *N* NH_4NO_3	0.25	0	0.9	0.05	0.95	0.05	0.95	0.95
1 *N* NH_4NO_3	0.34	0	0.9	0.05	0.95	0.18	0.9	0.95
5 *N* NH_4NO_3	0.65	0	1	0	—	—	—	—
Dest. Wasser pH 3.5	0.18	0	0.9	0	0.83	0.05	0.87	1

erkennt, dass die R_F-Werte auf Whatman 3MM-Papier, mit Ausnahme von ^{137}Cs, verschieden von denen auf EH-Papier sind. Die ^{90}Y-Zonen sind auf EH-Papier im Gegensatz zu Whatman 3MM-Papier sehr scharf ausgeprägt. Fig. 3 zeigt eine mit NH_4NO_3-Lösung (5 *N*, pH 3.5) durchgeführte Trennung von ^{90}Sr, ^{90}Y und ^{137}Cs.

Weitere Trennungen von Radionukliden auf EH-Papier werden untersucht und sollen zu gegebener Zeit mitgeteilt werden.

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Gas chromatographic separation of benzene and deuterobenzenes

It has recently been shown[1] that isotopically substituted hydrocarbons can be separated by partition chromatography. In order to evaluate the efficiency of gas chromatography for the identification of isotopic molecules the replacement of deuterium atoms in the benzene molecule has been followed.

By using a glass capillary column of about 250 m coated with a non-polar liquid phase, such as squalane, with a number of theoretical plates of about 350,000 (using *n*-heptane as reference) a complete separation of C_6H_6 and C_6D_6 and a partial separation of $C_6H_3D_3$ and $C_6H_2D_4$ was obtained, as shown in Fig. 1.

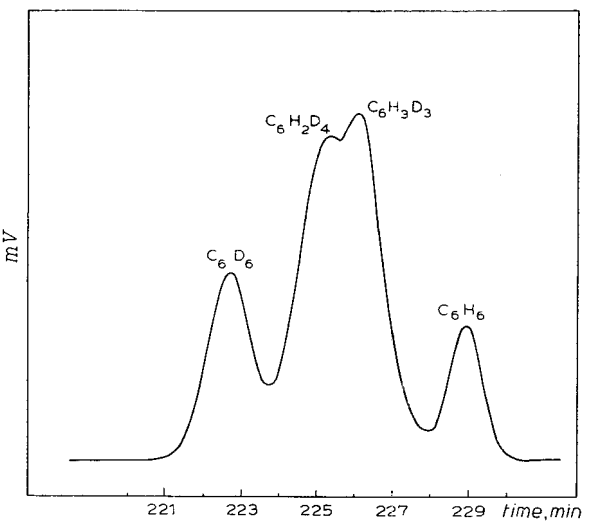

Fig. 1. Separation of benzene and deuterobenzenes. Column: glass capillary, 250 m long, 0.28 mm bore. Stationary phase: squalane. Carrier gas: nitrogen. Inlet pressure: 680 mm Hg. Temperature: 10°. Flow rate: 0.25 ml/min. Efficiency: 350,000 theoretical plates. Detector: flame ionization.

It seems that by increasing the number of theoretical plates and by choosing a suitable liquid phase, which plays a very important role also in the separation of isotopically substituted molecules[2], a mass difference of a few parts per hundred might be sufficient to achieve an effective separation.

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Thin-layer chromatography using partially acetylated cellulose as adsorbent

The separation of polycyclic aromatic hydrocarbons by chromatography on acetylated paper (and also on columns of partially acetylated cellulose) has been described by SPOTSWOOD[1,2], and the technique has since found extensive application. The method suffers from the disadvantage that different batches of acetylated paper, and of acetylated cellulose, have somewhat different characteristics. It has now been found that polycyclic aromatic hydrocarbons can also be satisfactorily separated by thin-layer chromatography using acetylated cellulose. No adhesive is required to prepare a satisfactory plate. The separated compounds, readily visible under ultraviolet light, can be scraped from the plates and identified by spectroscopy.

This method has the advantage that a large batch of acetylated cellulose can be prepared and used for the preparation of a great many plates over a prolonged period. Reproducible results can be obtained with a given batch of acetylated cellulose, and it may also be noted that the acetylated cellulose can be recovered and used again.

It is to be hoped that good quality acetylated cellulose will become commercially available; if distinguished by a batch number, this would facilitate the comparison of R_F values determined in different laboratories.

TABLE I

Compound	R_F
3:4-Benzopyrene	0.105
3:4-Benzofluoranthene	0.150
3:4; 9:10-Dibenzopyrene	0.217
11:12-Benzofluoranthene	0.230
Perylene	0.342
1:2; 4:5-Dibenzopyrene	0.497
1:2; 3:4-Dibenzopyrene	0.559

Table I lists the R_F values obtained for several polycyclic aromatic hydrocarbons, the solvent system being methanol–ether–water (4:4:1).

Experimental

Whatman's cellulose chromatography powder (200 g) was acetylated by SPOTSWOOD'S method[2] using a mixture of thiophen-free benzene (1700 ml) and acetic anhydride (800 ml) containing 92 % sulphuric acid (4 g) and 72 % perchloric acid (4 g).

A suspension of acetylated cellulose (35 g) in methanol or ethanol (60 ml) was applied to glass plates (20 cm × 20 cm) using a "Desaga" thin-layer spreading device, adjusted to give a 250 μ layer. The resulting plates were dried in air; compounds under test were applied in the usual way in 0.5 % solution; and the plates were developed by the ascending technique in a saturated chamber, the best results being obtained when the chromatography was carried out in an air-conditioned room (68°F). The solvent systems methanol–ether–water (4:4:1) and toluene–ethanol–water

(4:17:1) both proved satisfactory, but the former was found to be preferable as it gave more compact spots.

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[2] T. M. SPOTSWOOD, *J. Chromatog.*, 3 (1960) 101.

Received October 29th, 1962

Note

Similar results were described by T. WIELAND, G. LÜBEN AND H. DETERMAN, *Experientia*, 18 (1962) 430, on September 15th, 1962, and it is unlikely that these had reached Australia when this paper was sent off (October 24th, 1962).

THE EDITOR

Notes

A gas chromatographic trap designed to collect compounds which tend to form aerosols

A number of gas–liquid chromatographic trap designs have been reported in the literature[1–6], but no simple device has been described for the recovery of aerosol-forming compounds. Conventional cold traps are very inefficient for the collection of compounds such as the polynuclear aromatic hydrocarbons, sterols and high molecular weight paraffins. Yields are often as low as 60 %[5]. In this laboratory, for example, a 2 ft. × 5 mm O.D. glass coil cooled in liquid air gave recoveries of 50–60 % for many aromatic hydrocarbons.

We wish to describe a Pyrex glass trap which has been used successfully to collect samples eluted from 1/4 in. to 5/8 in. GLC columns at flow rates of from 40 to 200 ml/min with recoveries greater than 92 %.

This trap (Fig. 1) is designed to achieve a differential temperature between the outer wall and the inner wall of the condensing surface. The inner wall is kept at room temperature by directing a flow of air into the inner well while the outside wall is kept at the temperature of liquid air. The temperature differential between the two walls through which the exit gas must flow creates a turbulence, thus increasing the number of contacts of the aerosol particle with the cold wall and improving the trapping efficiency.

The exit line leading to the cold trap is 1/8 in. O.D. stainless steel tubing. This

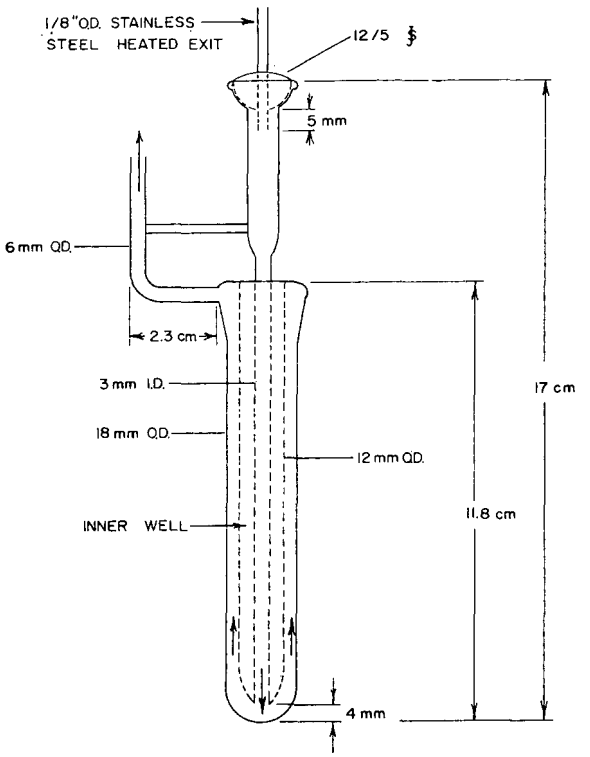

Fig. 1. Gas chromatographic collection trap.

line is heated up to the ball joint and is kept at the temperature of the detector, usually about 20° higher than the column temperature. A 5 mm extension of the 1/8 in. tubing beyond the male ball joint decreases the possibility of contamination of the joint with the previous eluate. A collected sample can be quantitatively recovered from the trap by rinsing with 2 to 3 ml of the appropriate solvent.

Acknowledgements

The authors wish to thank Dr. C. H. KEITH for his helpful suggestions in the design of this trap. The trap was fabricated by the Research Triangle Glass Blowing, Inc., Durham, N. C.

Research Department, Liggett and Myers Tobacco Company, Durham, N. C. (U.S.A.)

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Received August 20th, 1962

Chromatographic separation of 17-ketosteroids

Chromatographic separation of individual 17-ketosteroids has been performed mainly by the stepwise or gradient elution method, using an alumina[1–4] or a silica gel column[5].

Although these methods provide satisfactory separation of the common 17-ketosteroids in a relatively short time (a few hours), application of the gradient or changing of the eluants during chromatography is somewhat tedious.

On the other hand it has been shown that estrogens[6] and some Δ^4-3-ketosteroids[7] can be separated by a one-step elution method, using partially esterified Amberlite IRC-50 as the adsorbent and a mixture of alcohol and water as the eluant. This method has now been applied to the separation of 17-ketosteroids and it was possible to separate fourteen common 17-ketosteroids (Table I) by using three solvent systems. The mixture of steroids was first fractionated by using a mixture of methanol, ethanol and water (3:9:8 by vol., eluant A) as the eluant. The components that were eluted in the same fraction were next fractionated by using a mixture of methanol, ethanol and water (3:15:8 by vol., eluant B) or a mixture of isopropanol and water (15:8 by vol., eluant C) as eluants. The former eluant provided a good separation of monoketo-monohydroxy compounds from diketo compounds, and the latter eluant eluted dehydro-*epi*-androsterone, 11β-hydroxyandrosterone and 11-ketoandrosterone in the order described.

TABLE I

KEY TO STEROID DESIGNATION

No. in the figures	*Chemical name**	*Trivial name*
1	3-Hydroxyestra-1,3,5(10)-trien-17-one	Estrone
2	3α,11β-Dihydroxy-5β-androstan-17-one	11β-Hydroxyetiocholanolone
3	3α-Hydroxy-5β-androstane-11,17-dione	11-Ketoetiocholanolone
4	3α,11β-Dihydroxy-5α-androstan-17-one	11β-Hydroxyandrosterone
5	3α-Hydroxy-5α-androstane-11,17-dione	11-Ketoandrosterone
6	11β-Hydroxyandrost-4-ene-3,17-dione	11β-Hydroxyandrostenedione
7	Androst-4-ene-3,11,17-trione	Adrenosterone
8	3β-Hydroxyandrost-5-en-17-one	Dehydro-*epi*-androsterone
9	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone
10	3α-Hydroxy-5α-androstan-17-one	Androsterone
11	3β-Hydroxy-5α-androstan-17-one	*epi*-Androsterone
12	5β-Androstane-3,17-dione	Etiocholanedione
13	5α-Androstane-3,17-dione	Androstanedione
14	Androst-4-ene-3,17-dione	Androstenedione

* International Union of Pure and Applied Chemistry, *Nomenclature of Organic Chemistry, 1957*, Butterworths Sci. Publs., London, 1958.

Experimental

Materials. 11β-Hydroxyetiocholanolone and 11-ketoetiocholanolone were supplied by Dr. S. LIEBERMAN. Dehydro-*epi*-androsterone, androsterone, etiocholanolone, androstanedione, etiocholanedione, *epi*-androsterone and androstenedione were made available by Dr. A. KANBEGAWA and Miss M. KIMURA. 11-Ketoandrosterone was supplied by Dr. J. ENDO. Adrenosterone was prepared by chromic acid oxidation

of hydrocortisone, and 11β-hydroxyandrostenedione was prepared from hydrocortisone by sodium bismuthate oxidation. 11β-Hydroxyandrosterone was isolated from the urine of a patient with congenital adrenal hyperplasia. Estrone was a commercial product. The potassium hydroxide, dinitrobenzene and ethanol (99.5 %) used for the Zimmermann reaction were of analytical grade. Methanol, ethanol (99 %) and isopropanol were distilled before use.

Ion-exchange resin. Amberlite IRC-50 (A.G.) was pulverized and washed as described previously (200 to 300 mesh)[8]. It was partially esterified by refluxing for 40 h with aqueous acidic alcohol D, E or F (Table II). One liter of acidic alcohol was

TABLE II

COMPOSITION OF AQUEOUS ACIDIC ALCOHOL

Aqueous acidic alcohol	*Composition*
D	Methanol–ethanol–*N* HCl (1:3:2, by vol.)
E	Methanol–ethanol–2 *N* HCl (1:5:2, by vol.)
F	Isopropanol–2 *N* HCl (5:2, by vol.)

used for 70 to 100 ml of H form Amberlite IRC-50, and bumping of the mixture was prevented by adding two or three pieces of porcelain boiling stone (8 mm cube), the edges of which were removed by grinding[9].

Preparation of the chromatographic column. The partially esterified resin was transferred to a glass filter, washed with the eluant to be used for chromatography (Table III) and suspended by swirling in about two volumes of the same solvent.

TABLE III

CONDITIONS OF THE CHROMATOGRAPHIC SEPARATION

Figure	*Aqueous acidic alcohol with which Amberlite IRC-50 was boiled*	*Size of the column (cm)*	*Eluant*	*Temperature (°C)*
1	D	0.69 × 137	A	28 – 28.5
2	E	0.78 × 195	B	20 – 20.2
3	F	0.78 × 181	C	24 – 24.5

The suspension was poured into a jacketed chromatographic tube through a small funnel fitted with a ground glass joint and allowed to settle. After about 200 ml of the eluant have been passed through, the column is ready for use.

Chromatographic separation of synthetic mixtures. One ml of a solution of steroids in the eluant was applied to the column and elution was performed with the same eluant. The effluent was collected in fractions of 40 or 42 drops in test tubes, using a drop count type automatic fraction collector. Analysis of the steroids was performed by the Nathanson–Wilson modification of the Holtorff–Koch method[10] after evap-

oration of the eluant. The eluant was evaporated by placing the test tubes in a suitable rack and heating in a boiling water bath for about 1 h. The recovery of steroids with a Δ^4-3-keto-group was measured by ultraviolet absorption at 240 mμ and that of estrone at 280 mμ using a Beckman model DU quarz spectrophotometer after dilution of each fraction with aqueous ethanol (70 %) to a total volume of 3.5 ml.

Results and discussion

As shown in Figs. 1 to 3, fourteen 17-ketosteroids have been separated by using three solvent systems. They were first separated by using solvent A (Fig. 1). The fractions

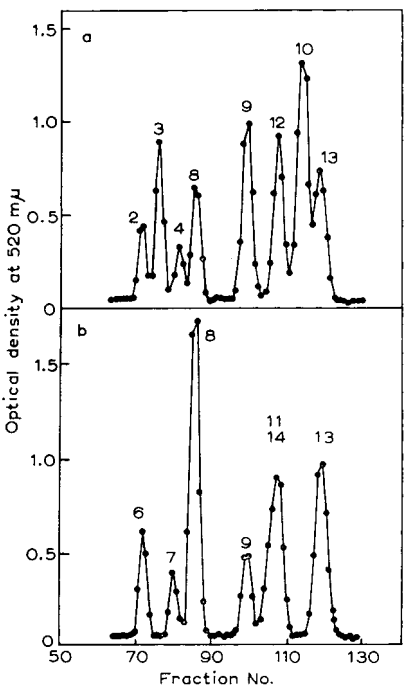

Fig. 1. Elution patterns of 17-ketosteroids. Elution patterns a and b were obtained in separate experiments performed under the same conditions. 11-Ketoandrosterone was eluted between 11β-hydroxyandrosterone and dehydro-*epi*-androsterone and estrone overlapped 11β-hydroxyetiocholanolone. The effluent was collected in fractions of 42 drops and the flow rate was 1.5 fractions per hour.

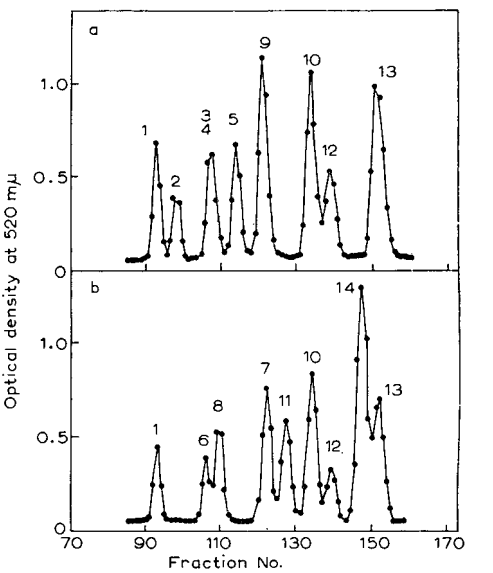

Fig. 2. Elution patterns of 17-ketosteroids. Elution patterns a and b were obtained in separate experiments performed under the same conditions. The effluent was collected in fractions of 40 drops and the flow rate was 2 fractions per hour.

containing two or more steroids were pooled and separated by using solvent B or C (Figs. 2 and 3). Good separation was obtained of the 5α- and 5β-isomers of the steroids, and since the chromatographic method described utilizes non-ionic adsorption of steroid molecules on partially esterified carboxylic acid type ion-exchange resin, the order of elution from the column is determined by both carbon number and degree of oxygenation of the molecule. The C_{18} steroid (estrone) is eluted faster than C_{19}

steroids (*e.g.* dehydro-*epi*-androsterone) if the number of oxygen atoms in the molecule is the same.

Increase of the alcohol concentration of the eluant exerted two effects on the elution volume of the steroids: (1) among steroids with the same number of oxygen atoms in the molecule, those with the greater number of hydroxyl group(s) were

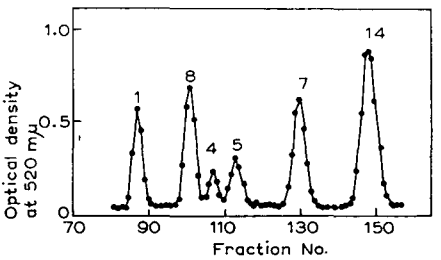

Fig. 3. Elution pattern of 17-ketosteroids. 11β-Hydroxyandrostenedione was eluted between dehydro-*epi*-androsterone and 11β-hydroxyandrosterone. The effluent was collected in fractions of 40 drops and the flow rate was 2 fractions per hour.

preferentially accelerated, and (2) less oxygenated steroids were preferentially accelerated. The elution volume was also sensitive to changes of column temperature. When the column temperature was elevated, less oxygenated steroids were preferentially accelerated and among steroids with the same number of oxygen atoms in the molecule, those with the greater number of keto-groups were preferentially accelerated.

The recovery of 17-ketosteroids with a Δ^4-3-keto-group and estrone, shown in Table IV, was satisfactory and the elution pattern was reproducible when the temper-

TABLE IV

PER CENT RECOVERY OF Δ^4-3-KETOSTEROIDS AND ESTRONE FROM THE CHROMATOGRAPHIC COLUMNS

Steroids	*Eluant*		
	A	*B*	*C*
11β-Hydroxyandrostenedione	93	96	99
Adrenosterone	93	95	100
Androstenedione	91	90	100
Estrone	93	96	95

ature of the column was kept constant. The optimum load was 100 to 500 μg/cm² for each component and as much as 5 mg/cm² could be added if there was an efficient separation of components.

Acknowledgements

The authors wish to express their appreciation to Prof. H. KIKKAWA and Prof. K. OKANO for their encouragement and interest in this work; to Dr. S. LIEBERMAN,

who kindly provided samples of 11β-hydroxyetiocholanolone and 11-ketoetiocholanolone; to Dr. A. KANBEGAWA and Miss M. KIMURA, who kindly provided samples of dehydro-*epi*-androsterone, androsterone, *epi*-androsterone, etiocholanolone, androstanedione, etiocholanedione and androstenedione; and to Dr. J. ENDO, who kindly provided a sample of 11-ketoandrosterone.

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J. Chromatog., 10 (1963) 400–404

Determination of ornithine, lysine, arginine, citrulline and histidine

Separation of the common amino acids in plant material with good resolution on a single sheet of filter paper cannot be accomplished with present procedures. MCFARREN[1] and MCFARREN AND MILLS[2] used a series of buffers and solvents, but these procedures have been found to be long and tedious. THOMPSON *et al.*[3] partially overcame this problem by separating the amino acids on columns of Dowex 50X4 resin into a basic fraction and an acidic and neutral fraction. This preliminary separation also purified the acids from extraneous materials which was necessary for high resolution. In

TABLE I

R_F VALUES OF AMINO ACIDS AND AMINES SEPARATED ON EDTA BUFFERED PAPER

Compound	R_F
Hydroxylysine	0.03
Ornithine	0.07
Lysine	0.12
Arginine	0.24
Citrulline	0.31
Histidine	0.40
Homocitrulline	0.52
Tyramine	0.65
Ethionine	0.76

order to obtain good separation of the basic amino acids it is necessary that the reagents and paper be free of metal ions. THOMPSON AND MORRIS[4] accomplished this by washing the papers and repurifying the reagents. In addition, 84 h was required to run the basic acids. More recently SIBALIC AND RADEJ[5] reported a rapid method using high temperature separation for lysine, arginine, and histidine.

The present procedure will not only separate the three acids listed above, but will also resolve ornithine, citrulline, hydroxylysine, homocitrulline, tyramine and ethionine (Table I). These separations can be accomplished in 12 h and without prior washing of the paper or purifying the reagents.

Materials

Filter paper. Whatman No. 52, size 18 1/4 × 22 1/2 in.

Buffer No. 1. 29.2 g ethylenediaminetetraacetic acid (EDTA) in deionized water. Add NaOH to dissolve the acid and adjust to pH 7.0 and make to 1 l.

Buffer No. 2. 3.944 g boric acid in deionized water. Add NaOH (about 1.45 g) and adjust to pH 9.3 and make to 1 l.

Phenol–cresol solvent. 190 ml 88 % phenol, Fisher liquified, Cat. No. A-931, 165 ml *m*-cresol, Eastman practical grade, Cat. No. P-369 and 45 ml pH 9.3 buffer.

Ninhydrin 1 % and triethylamine 0.25 % in 95 % ethanol.

Standard amino acids. (Nutritional Biochemicals Corporation, Cleveland, Ohio.) DL-Ornithine monohydrochloride, L-lysine, L-arginine, DL-citrulline and L-histidine. These were made to concentrations of 5, 10, 15 and 20 mg of amino nitrogen per 100 ml of 10 % isopropanol.

Procedure

Approximately 10 g of fresh leaves are extracted four times in a Lourdes mixer with 80 % ethanol; the extract is passed through 2 Dowex 50X4 columns[3] and the basic and acidic fractions recovered separately and taken to dryness. Each fraction is then taken up in 2 ml of 10 % isopropanol.

Sheets of Whatman No. 52 filter paper are dipped in EDTA buffer and hung on glass rods to drain for about 15 min. The damp papers are dipped in acetone, allowed to drain for 5 min, and then dried in an oven (Reco) at 65° for 1 h.

The samples are spotted on a Reco Model S-1250 sample applicator using a Hamilton syringe No. 701 with a guide and with the needle cut blunt to 3/4 in. The syringe is held in position by the holder on the applicator. The samples are spotted 1 in. apart with a set of standards on each paper. Two μl of each standard are applied to each spot in 0.4 μl increments, allowing each application to dry. The amount of sample applied may vary but it is necessary that all spots be the same size and the amount applied between dryings, that is 0.4 μl, be kept constant for both samples and standards.

The papers are placed in a chromatogram cabinet between two liners of Whatman No. 4 papers and with one sheet of paper in the bottom of the cabinet. These papers serve to increase the saturation of the cabinet. The liners are moistened with a mixture of 200 ml of pH 9.3 buffer and 8 ml of the phenol–cresol solvent. The remainder of the mixture is put into the trough holding the two liners. The troughs containing the spotted papers are filled with about 100 ml of the phenol–cresol solvent and allowed to run overnight or about 12 h. The temperature is maintained at 27° ± 2°.

After development the papers are removed from the cabinet and dried in an oven at 65° for 1 h. They are then dipped in ninhydrin and after the excess alcohol has dried placed in an oven at 65° for 30 min.

Following development of the color the papers are kept in the dark for 30 min and then read in subdued light on a Photovolt Model 501 A Densitometer using a 570 mμ filter or the spots may be cut out of the paper, placed in tubes of 50 % ethanol, and read on a colorimeter.

If the latter procedure is used, the concentration of the standards and samples spotted on the papers should be increased 10 times. Also the concentration of the ninhydrin should be 2 g per 100 ml.

Results and discussion

The use of this method has given excellent separation of basic amino acids in plants (Fig. 1). Whatman No. 52 paper was selected rather than Whatman No. 1 because the former has greater wet strength and better resolution. The wet strength is important for dipping the paper in buffer.

In preparing the EDTA buffer one lot of EDTA was found unsatisfactory because of impurities that reacted with ninhydrin. However, three other lots, all by different manufacturers, have been satisfactory. The concentration of the buffer has been studied using 0.01, 0.05, 0.1, and 0.15 *M* EDTA. At the two lowest concentrations citrulline and histidine did not separate while with 0.15 *M* EDTA arginine and citrulline remained unresolved. The pH of the buffer was also compared. There was good separation between pH 6.0 and pH 7.0 with slightly more compact spots using the buffer adjusted to the higher pH. The papers are dipped in acetone following dipping

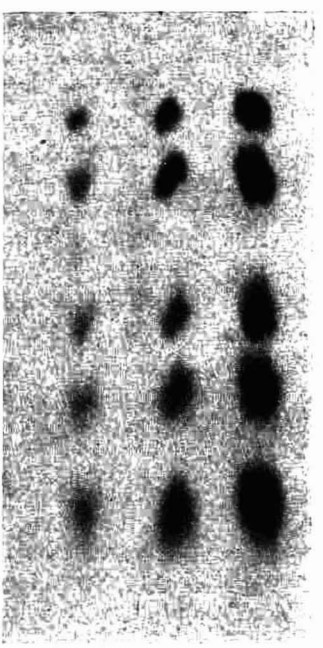

Fig. 1. One-dimensional paper chromatogram of ornithine, lysine, arginine, citrulline, and histidine.

in buffer to prevent an accumulation of salt on the surface which will interfere with the final reading.

The solvent used for developing the papers is essentially that of LEVY AND CHUNG[6]. Redistilling the phenol and cresol has not been found to improve the procedure. A high degree of saturation of the cabinet is important during the time the separations are being made. A lack of vapor can prevent good resolution, particularly of ornithine and lysine. This was the case when only a single narrow strip of paper was run. It is desirable to run at least two papers each time.

The development of color with ninhydrin causes the entire paper to have a light pink color. This color will darken slowly with time and the papers should be kept in subdued light. The ninhydrin color is sufficiently stable to obtain satisfactory determinations (Table II). Comparisons were made of developing the color in a ventilated

TABLE II

VARIATION OF DENSITY FOLLOWING TIME OF DEVELOPMENT WITH NINHYDRIN

Compound	*Densitometer**		*Spectrophotometer***	
	30 min	*2 h*	*30 min*	*24 h*
Ornithine	0.39	0.42	0.20	0.20
Lysine	0.39	0.39	0.22	0.20
Arginine	0.31	0.28	0.25	0.25
Citrulline	0.27	0.27	0.30	0.30
Histidine	0.20	0.22	0.25	0.23

* The density of the spots was read through the paper.
** The spots were eluted and the density of the solvent was determined.

oven as compared with using a closed box heated to 65° and filled with CO_2. The latter gave less color, especially with arginine, citrulline and histidine.

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BOOK REVIEWS

Biochemical Applications of Gas Chromatography, by H. P. BURCHFIELD AND E. E. STORRS, Academic Press, New York–London, 1962, 680 pages, price $22.00.

This book has been written with the aim of supplying the theoretical background and experimental details to anyone desiring to apply gas chromatography to biochemistry. In order to achieve this aim the authors have very carefully arranged the material, devoting about one fifth of the book to general principles, conventions and instrumentation and the rest to applications. Applications have been treated in a very general way, so that the determination of any material which may be of interest to analytical biochemists has been reported; a variety of fields including foods, essential oils, amino acids, carbohydrates, pesticides, clinical chemistry, etc., are covered.

The procedures are fully described, operating conditions given and in several cases critically discussed; other experimental methods, besides gas chromatography, are sometimes included so that enough information is presented to carry out a certain determination.

Literature references are extensively reported and experimental details are also taken from papers in journals that are not easily available. A minor remark should, however, be made regarding the presentation of coulometry as a means of sensing: the possibility of this technique has been fully investigated since 1957 (*Analytica Chimica Acta*, 1957).

The book is an exhaustive treatise and will be very valuable to any analyst engaged in a gas chromatographic determination; both the experienced research worker and the beginner will find in this text a good deal of information about any problem they may encounter.

A. LIBERTI (Naples)

J. Chromatog., 10 (1963) 408

Physical Aids to the Organic Chemist, by M. ST. C. FLETT, Elsevier Monographs, Elsevier Publishing Company, Amsterdam, 1962, viii + 388 pages, Dfl. 22.50, 45 s.

This is a book which is very timely, since the use of physical methods is assuming great importance in all branches of organic chemistry, and has led in recent years to developments in many fields, where new techniques of separation, structural determinations, and methods of analysis depending upon physical phenomena can contribute to the solution of complex problems. It was to be expected therefore, that this book would have chapters on chromatographic separations, gas–liquid chromatography, zone refining, electronic absorption spectroscopy, infra-red spectroscopy, electron spin resonance spectroscopy, nuclear spin resonance spectroscopy, mass spectrometry, X-ray crystallography.

The author clearly has a good appreciation of how much a chemist needs to know of the theory and practice of a technique to judge how applicable it is to a particular problem. For those who wish to have such a background, this is undoubted-

ly a most useful and reliable book, well arranged and sensibly presented. The selection of material is admirably suited to the non-specialist who may need to use the methods described—and there are few who will escape such a requirement if they aim at keeping abreast of the times. It will also be useful for the undergraduate working for his first degree, whether he intends later to specialize in organic or inorganic chemistry. At the end of each chapter there is a limited bibliography for those requiring to follow up the methods further.

While fully appreciating the significance of the techniques described and the diagnostic character of the evidence they produce, nevertheless the reviewer feels that such a book might well end with a warning to the rising generation born in an electronic age, that there is still much to be achieved by the use of purely chemical methods, and also that instruments are not infallible and need constant maintenance if their evidence is to be reliable.

F. H. POLLARD (Bristol)

Announcement

SYMPOSIUM ON THIN-LAYER CHROMATOGRAPHY

An International Symposium on Thin-Layer Chromatography will be held at the Istituto Superiore di Sanità, Rome, Italy, on May 2nd and 3rd, 1963. The lecturers will include Prof. E. STAHL, Ing. L. LABLER, Dr. E. DEMOLE, Dr. P. WOLLENWEBER, Dr. M. LEDERER, Prof. G. B. MARINI-BETTÒLO. One afternoon will be devoted to original papers.

Participants desiring to present a paper should submit a short summary to the organiser not later than April 15th.

Applications and inquiries should be addressed to:

Prof. G. B. MARINI-BETTÒLO,
Istituto Superiore di Sanità,
Viale Regina Elena 299,
Rome,
Italy

Bibliography of Paper Chromatography

1. REVIEWS AND BOOKS

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2. FUNDAMENTALS, THEORY AND GENERAL

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GAS CHROMATOGRAPHIC ANALYSIS OF ALIPHATIC NITRILES IN AQUEOUS ACIDIC SOLUTION

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INTRODUCTION

Gas chromatography has proved very successful in the analysis of a great variety of organic mixtures. For aqueous solutions, however, separation of the components has been found difficult because of excessive tailing of the water with the stationary phase[1].

Several authors have dealt with this problem in different ways. SUFFIS AND DEAN[2] used non-aqueous extraction prior to injection into the chromatograph and were thus able to separate alcohols from aqueous solutions. BODNER AND MAYEUX[3] analysed only low-boiling compounds which were eluted before the water. For higher boiling compounds HUNTER AND ORTEGREN[4] designed a special combustion tube for oxidizing the fractions emerging from the column. The mixture was absorbed by magnesium perchlorate and the dry carbon dioxide was measured in thermal conductivity cells. The water content in these experiments was up to 50 %. An ingenious method was devised by KUNG *et al.*[1] who injected the aqueous solution into a calcium carbide precolumn, thus converting all the water into acetylene. By this method they were able to obtain good results with mixtures of alcohols, aldehydes and esters.

In the present work, it was necessary to determine different nitriles in aqueous acidic solutions in the presence of KCl. One method used was the extraction of the nitriles with *o*-dichlorobenzene prior to injection into the column. Results obtained when using this procedure showed that extraction of the nitriles was not complete unless large volume ratios of solvent–aqueous phase were employed. The final stock solutions were too dilute, thus reducing the accuracy of the method.

In this work, a simple method is presented which proved successful in the case of nitriles, and could probably be generalized to some other aqueous solutions. The method involves addition of a five-fold excess of solvent to the aqueous solution, and subsequent dehydration of the mixture with anhydrous Na_2SO_4. The dry organic solution was then injected into the column. It is possible that in some mixtures preferential adsorption of some of the components might take place; this effect is, however, diminished when a good solvent for the organic components is used. It will be shown in the experimental results that no preferential adsorption takes place in the case of the nitriles used in this work.

EXPERIMENTAL

Apparatus

A gas chromatograph Aerograph Model A 350 with a hot wire detector and a 2-m 6 mm i.d. copper spiral column packed with diethylene glycol polyester adipate (LAC-2-R-466, Cambridge, Ind.) and 2 % H_3PO_4 on Chromosorb W (25:100, by weight) 30–60 mesh[5], were used.

Procedure

Mixtures of known composition of nitriles were dissolved in aqueous HCl saturated with respect to KCl. The total nitrile concentration was approximately 15 % w/w. 10.25 c.c. of the solution were added to a fivefold excess of *o*-dichlorobenzene* in which about 50 g of anhydrous Na_2SO_4 were present. The mixture was shaken for 5 min until the aqueous phase disappeared and the organic phase was perfectly clear. The solution was filtered and 10–15 μl injected into the chromatograph.

When monobasic nitriles were analysed, the temperature of the column was kept at 70°, while for the dibasic nitriles the temperature was 220°.

For mixtures of mono- and dibasic nitriles the column was programmed starting at 70°, and raised at the rate of 10° per minute up to 220°.

Elution took place in the following order: acrylo-, propio-, butyronitrile, *o*-dichlorobenzene, succino- and adiponitriles. No trace of water peak was detected when using this procedure.

RESULTS AND DISCUSSION

The results are presented in Table I and a characteristic chromatogram of a mixture of mono- and dibasic nitriles is shown in Fig. 1. The amounts were estimated by measuring the areas under the peaks and it was assumed that the concentration of any nitrile was proportional to its peak area.

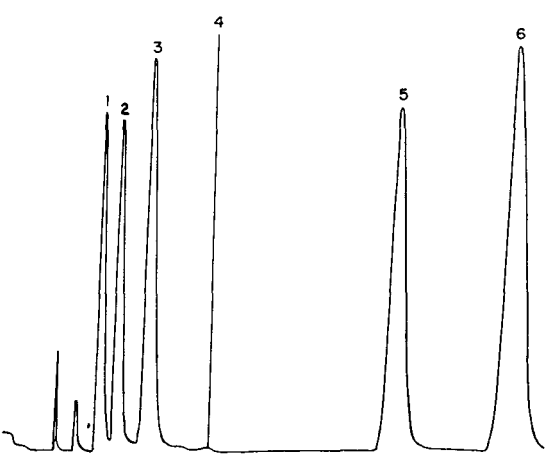

Fig. 1. Chromatogram of a mixture of mono- and dibasic nitriles. (1) Acrylonitrile; (2) propionitrile; (3) butyronitrile; (4) *o*-dichlorobenzene (solvent); (5) succinonitrile; (6) adiponitrile.

* When monobasic nitriles are not present, chloroform may be used as solvent.

TABLE I

No.	Acrylonitrile			Propionitrile			Butyronitrile			Succinonitrile			Adiponitrile		
	% weight taken	% area found	% deviation	% weight taken	% area found	% deviation	% weight taken	% area found	% deviation	% weight taken	% area found	% deviation	% weight taken	% area found	% deviation
1	36.5	35.6	−3.0	37.0	36.1	−3.0	27.0	28.3	+4.3						
2	31.6	29.6	−6.3	40.1	41.5	+3.7	28.3	29.0	+2.5						
3	44.2	41.3	−2.5	21.5	22.6	+5.1	34.3	36.1	+5.2						
4	55.2	55.6	+0.7	11.8	12.5	+5.9	33.0	31.9	−3.3						
5	60.1	59.4	−1.1	8.6	9.2	+6.9	31.3	31.4	+0.3						
6										48.4	47.8	−1.2	51.6	52.2	+1.2
7										36.1	34.8	−3.6	63.9	65.2	+2.0
8										31.0	30.2	−2.6	69.0	69.8	+1.2
9										31.3	29.0	−7.3	68.7	71.0	+3.3
10										14.5	15.0	−3.4	85.5	85.0	−0.6
11	25.0	24.3	−2.8	50.0	49.4	−1.2	25.0	26.2	+5.0	50.0	48.6	−2.8	50.0	50.7	+1.4
12	33.3	34.2	+2.7	33.3	33.7	+1.3	33.3	32.1	−3.4	50.0	52.1	+4.2	50.0	47.2	−5.6
13	33.3	32.5	−2.4	33.3	34.8	+4.2	33.3	32.6	−2.7	50.0	50.8	+1.6	50.0	48.9	−2.2
14	18.8	18.8	0	50.0	49.6	−0.8	31.3	32.5	−3.8	47.5	48.8	+2.7	52.5	51.1	−2.7
15	6.2	6.9	+10.4	62.5	61.0	−2.4	31.3	32.2	+3.2	50.0	49.5	−1.0	50.0	50.5	+1.0
16	10.0	9.8	−7.0	40.0	38.9	−2.8	50.0	51.9	+3.8	50.0	50.5	+1.0	50.0	49.5	−1.0
17	42.8	45.1	+5.3	28.6	28.3	+1.6	28.6	26.4	−7.5	50.0	48.8	−2.4	50.0	51.2	+2.4
18	33.3	33.3	0	33.3	32.3	−3.0	33.3	34.5	+3.6	50.0	50.5	+1.0	50.0	49.5	−1.0
19	50.0	51.8	+3.2	16.7	14.6	−12.8	33.3	33.9	+1.8	28.6	30.8	+9.4	71.4	69.2	−3.1

In experiments in which all the above-mentioned nitriles were analysed together (Nos. 11–19, Table I) the amounts of mono- and dibasic nitriles were calculated on the assumption that each class of nitriles constitutes a 100 % total. An attempt to treat the total area under all peaks as a total of 100 % resulted in poor accuracy in the determination of the separate constituents of the mixture.

It was shown in separate experiments that HCl remained with the Na_2SO_4 phase and did not interfere in the chromatographic analysis. From the results in Table I it can be seen that the individual nitriles can be determined quantitatively with an average error of between 2 and 4 %.

SUMMARY

A mixture of acrylo-, propio-, butyro-, succino- and adiponitriles in aqueous solution in the presence of HCl and KCl was analysed by gas chromatography. The method involved extraction with *o*-dichlorobenzene and drying over anhydrous Na_2SO_4 prior to injection into the chromatographic column. No adverse effect of the drying agent was detected. The same method of drying aqueous solutions for chromatographic analysis could be extended to other systems.

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CHROMATOGRAPHY OF HUMAN HEMOGLOBIN

FACTORS INFLUENCING CHROMATOGRAPHY AND DIFFERENTIATION OF SIMILAR HEMOGLOBINS

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Several chromatographic procedures that utilize Amberlite IRC-50 or carboxymethylcellulose have been described for the detection of heterogeneity and the separation of different hemoglobins[1-12]. The procedure described by ALLEN, SCHROEDER AND BALOG[6] has been used successfully in these laboratories for the past five years during which time a number of modifications and applications have resulted[9,11,13]. Several important factors which influence the chromatographic behaviour under these conditions have become apparent. The purpose of this paper is to review briefly the procedure of ALLEN, SCHROEDER AND BALOG and its modifications, to emphasize factors that influence the chromatography of hemoglobins, and to describe a method for differentiating very similar hemoglobin components by radioactive tracers.

ALLEN, SCHROEDER AND BALOG[6] employed sodium phosphate buffers of different ionic strength and pH as chromatographic developers. All chromatograms used one developer throughout but each developer was designed for a specific task. Thus, developer No. 1 was used for the separation of normal adult hemoglobin into two zones, designated A_I and A_{II} in order of elution whereas developer No. 4 was designed to resolve the A_I zone into its components on rechromatographing.

CLEGG AND SCHROEDER[9] in extending the method devised a developer which separated normal adult hemoglobin into 6 hemoglobin fractions in one chromatogram. This developer (No. 5) was similar to the weak developers of ALLEN, SCHROEDER AND BALOG and effected a resolution of the rapidly moving minor components (A_I components) at a temperature of 6°. When the front of the main component, A_{II}, approached the lower end of the chromatographic column, the remaining components were eluted rapidly by warming the column to room temperature. With this modified procedure, CLEGG AND SCHROEDER demonstrated the presence of a small zone A_{III} that moved more slowly than the main component, A_{II}. SCHNEK AND SCHROEDER[11] separated this A_{III} zone into two components and correlated each component from the entire chromatographic separation of normal adult hemoglobin with the components obtained by starch block electrophoresis.

The procedure of ALLEN *et al.*[6] and its modification[9,11] have also been applied to studies of the chromatographic behaviour of a number of abnormal hemoglobins

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including Hbs, C, D, H, and S[13,14]. The problem of identifying hemoglobins on the basis of their chromatographic behavior and the factors which influence this behavior will be considered in the present paper.

MATERIALS AND METHODS

Preparation of hemoglobin solutions

Oxyhemoglobin solutions were prepared by DRABKIN's method[15] as modified by ALLEN *et al.*[6] and CLEGG AND SCHROEDER[9]. Immediately after preparation, all hemoglobin solutions were dialyzed against a chromatographic developer at 4° and stored thus until used.

Ferrihemoglobin cyanide (cyanomethemoglobin) was obtained by dialyzing solutions of oxyhemoglobin against 0.01 *M* $K_3Fe(CN)_6$ in developer No. 4 for 8 h at 4°. Excess $K_3Fe(CN)_6$ was then removed by dialysis against one of the chromatographic developers.

Radioactive hemoglobins were prepared by the procedure of BORSOOK *et al.*[16] as modified by VINOGRAD AND HUTCHINSON[17]. L-Leucine uniformly labeled with ^{14}C was the radioactive amino acid in all instances.

The concentration of hemoglobin solutions was determined with a Beckman spectrophotometer, Model DU, at a wave length which depended upon the nature of the hemoglobin derivative. The concentration of hemoglobin in mg per ml was calculated by multiplying the optical density for a 1 cm length of solution by an appropriate factor shown in Table I[18,19].

TABLE I

FACTORS FOR ESTIMATION OF HEMOGLOBIN CONCENTRATION[18,19]

Wave length (mμ)	*Hemoglobin*	*Factor*
522	Carbonmonoxyhemoglobin	1.77
522	Ferrihemoglobin cyanide	1.77
540	Ferrihemoglobin cyanide	1.44
542	Oxyhemoglobin	1.14
542	Carbonmonoxyhemoglobin	1.14
563	Oxyhemoglobin	1.77
563	Ferrihemoglobin cyanide	1.77

Preparation of chromatographic columns

Amberlite IRC-50 resin was prepared, and columns were poured according to the procedure of ALLEN *et al.*[6].

Particles of mesh size 200–250 and 250–325 have been used in different columns without apparent influence on the chromatographic properties of hemoglobin. Columns 1 × 35 cm in dimension were used for analytical work and 1.5, 2.5, and 3.5 cm in diameter by 35 cm in length for preparative work. These were maintained at 6° either by circulating water through the jackets or by using in a cold room. Before each newly poured column was used or when a column was converted from one chromatographic developer to another, the appropriate buffer was passed through at 6° at a

rate of 3–12 ml per cm^2 of cross sectional area of resin per hour until 0.8–1.5 l per cm^2 area had passed.

Bio-Rex 70 (equivalent to Amberlite IRC-50) of appropriate mesh size may be obtained from Bio-Rad Laboratories (32nd and Griffin Ave., Richmond, Calif. U.S.A.). One lot with a particle range of 200–230 mesh (sized wet and in the hydrogen form but shipped in the sodium form) was used as supplied without further treatment. It was simply suspended in developer, the pH was adjusted to the original pH of developer (with phosphoric acid in this instance), and the column was poured and equilibrated.

The least mixing of effluent as it emerges from the column may be obtained with the chromatographic columns of SPACKMAN, STEIN AND MOORE[20]. These may be purchased from Scientific Glass Apparatus Co., Inc. (catalog No. SJ-1665-1-B, item No. JC-2800, 45 cm in length above disc). The effluent may be led from the column to a fraction collector through small-bore Teflon or polyethylene tubing.

Chromatographic developers

The chromatographic developers were prepared exactly as described by ALLEN *et al.*[6] from anhydrous Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$. Substitution of these reagents by other hydrates has given unreliable results. The composition and pH of the developers which were employed are listed in refs. 6, 9 and 11. In general, the flow of developer through the column has been controlled by the hydrostatic head that was produced by raising or lowering the reservoir. It is perhaps more convenient to use a constant volume pump such as that manufactured by the Milton Roy Company, Philadelphia, Pa. (CHMMl-B-29 HC Simplex Chromatographic Minipump).

Operation of column

Prior to each chromatogram, the column was carefully adjusted to a vertical position with a level. The top 1 to 3 cm of resin bed was then stirred, the suspended resin was allowed to settle, and the supernatant developer was removed. Chromatograms were started only if the surface of the freshly settled resin was even and horizontal. Small irregularities in the surface often resulted in the formation of distorted zones and therefore poor resolution of components.

When developer still remained above the surface of the column to a depth of 1 to 3 mm, the hemoglobin solution was pipetted slowly down the side of the glass tube onto the resin bed with a bent-tip pipet. This method of application resulted in the least disturbance of the top of the resin bed, but, in the event that the surface of the resin was disturbed, the effect could be minimized by restirring the top of the column into the overlying hemoglobin solution before the sample had run in. Instead of permitting the sample to drain into the column and then rinsing with developer as described by ALLEN *et al.*[6], the hemoglobin sample was carefully layered over with one or two ml of developer, and the whole was permitted to drain into the column before the main development was begun. A sharp interface between hemoglobin solution and developer could be obtained if the latter was added very slowly onto the wall of the tube several mm above the existing fluid surface. The overlayering has resulted in more even zones and more rapid application of samples. The sample did not exceed a concentration of 100 mg/ml and was applied in a volume of 0.5–2.0 ml/cm^2 of cross sectional area of resin.

The chromatograms were developed essentially as previously described[6,9,11]. The flow rate of developer was generally 5 or 6 ml/h at 6.0° ± 0.2° for the 1 × 35 cm columns and was often doubled for the elution of slowly moving hemoglobins. When the columns were warmed to 28°, the flow rate was maintained at 8–10 ml/h. One to 10 ml fractions were automatically collected.

When zones were to be rechromatographed, the effluent was collected in chilled vessels and then refrigerated. Prior to rechromatographing, pooled fractions were concentrated by ultracentrifugation according to a procedure of VINOGRAD AND HUTCHINSON[17] and SCHNEK AND SCHROEDER[11], and then dialyzed against fresh developer. Recently, fractions have been concentrated by ultrafiltration according to a method described by SMITH[21]. The latter procedure is more convenient than concentrating by ultracentrifugation.

Analogous procedures were used for the larger preparative columns. In preparative work, especially if only one hemoglobin is to be isolated, the desired hemoglobin frequently may be isolated in fairly concentrated solution by the following procedure. The desired hemoglobin is chromatographed normally at 6° until its leading edge approaches the lower end of the column and all less strongly adsorbed material has been eluted. If more strongly adsorbed hemoglobins are present, the resin that contains them is removed from the column. The portion of the column that remains is then warmed to 40° for 10 min after which the desired hemoglobin is washed from the column in a few minutes at the maximum flow rate of developer.

Spectrophotometry and measurement of radioactivity

The optical density of each fraction of the chromatogram was measured in a 1-cm cuvette either in a Beckman model DU spectrophotometer at 280 and 415 mμ or in a Beckman model B spectrophotometer at 415 mμ. In certain cases, optical density at 522, 542, or 563 mμ was also measured. The transfer of fractions to and from the spectrophotometer cuvette has been facilitated by the use of an "automatic transferator" (Gilson Medical Electronics, Middleton, Wis.).

Radioactivity was measured with one of two counting systems. Some radioactive countings were made with Nuclear-Chicago model ClOOB automatic sample changer equipped with a model D-47 gas flow geiger counter. Other countings were made with a Nuclear-Chicago model C115 low background automatic sample changer. "Micromil" windows were used in both detector systems. Volumes of 0.5–1.0 ml of hemoglobin solution were pipetted onto aluminum, stainless steel, or copper planchets. These samples were then evaporated to dryness with an infra red lamp. In general, less than 0.5 mg of protein was placed on these planchets so that correction for self absorption was unnecessary[17]. Corrections were not made for the absorption by the buffer salts or for the absolute counting efficiency of the system. However, the same kind and volume of developer and the same counting equipment were used for any one experiment. Therefore, the relative activity of all samples within any given experiment was comparable.

RESULTS AND DISCUSSION

Factors influencing the chromatography of hemoglobins

The chromatographic behavior of hemoglobins when studied by the procedure of

ALLEN, SCHROEDER AND BALOG is dependent upon the pH and ionic concentration of the chromatographic developers, the state of equilibrium of the IRC-50 resin with the developer, and the temperature during equilibration and chromatography[6,9,11]. Observations which will not be presented confirm the importance of these factors. In addition, it has now been noted that the amount of hemoglobin on the chromatogram influences the rate of movement during chromatography. This effect of load was apparent when differences were observed in two chromatograms that were identical except for the amount of hemoglobin applied. When 50 mg of sickle

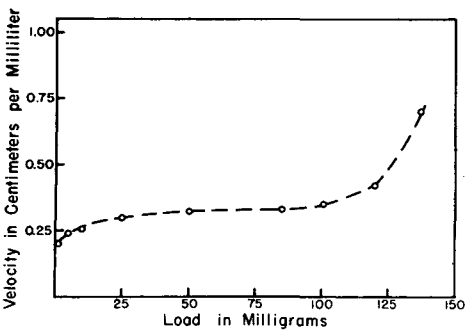

Fig. 1. The effect of the amount of hemoglobin S_{II} on the migration rate of the zone. Different quantities of hemoglobin were chromatographed with developer No. 1 on a 1 × 35 cm column.

cell hemoglobin (Hb S) was chromatographed with developer No. 1 on a 1 × 35 cm column, the peak of the main component S_{II} (see refs. 13 and 22) emerged at an effluent volume of 94 ml whereas with 10 mg of Hb S the peak emerged at 130 ml. The effect of the amount of hemoglobin S_{II} on the migration rate of the zone on the chromatographic column is depicted in Fig. 1. Similarly, the rate of movement of F_{II}, the main component in cord blood hemoglobin, is dependent upon the load on the column and this effect was confirmed by MATSUDA *et al.*[23]. Observations with other hemoglobins indicate that the influence of load on the rate of movement under the conditions described is a general phenomenon which must be considered when chromatographic movement is used to characterize an unknown hemoglobin.

Formation of double zones of single hemoglobin components

An apparent but probably false heterogeneity of hemoglobin is best illustrated in chromatograms of hemoglobin S. For example, each of more than twenty samples of hemoglobin S exhibited the formation of two zones in the region of the main component as depicted in Fig. 2. This heterogeneity of the main component is readily apparent as the zone moves down the column. If an excessively large amount of hemoglobin (200 mg on a 1 × 35 cm column) is chromatographed, the main zone appears to be homogeneous. The formation of double zones of component F_{II} has not been observed although both the amount of hemoglobin and conditions of development have been varied. The main components of hemoglobins A, C, D, H, as well as S and some minor components such as A_{IC} (ref. 6) have been observed to form double zones.

Since the initial report of this apparent heterogeneity[22], further experiments indicate that this phenomenon may be similar to the "double zoning" which occurs

with simpler compounds[24] and which has recently been reviewed by KELLER AND GIDDINGS[25]. This conclusion is based in the main on experiments with hemoglobin S_{II}. Although the formation of two zones of hemoglobin S_{II} was observed on a 1 × 35-cm column when 5–200 mg were chromatographed with developer No. 1, the detection of two peaks in the effluent fractions was generally found only when 40–100 mg were

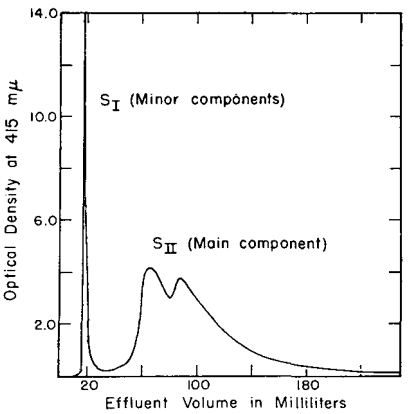

Fig. 2. Double zoning of hemoglobin S_{II}. A 50 mg sample of hemoglobin from a patient with sickle cell anemia was chromatographed on a 1 × 35 cm column of IRC-50 at 6° with developer No. 1. A slowly moving minor component, S_{III}, is not shown.

used. The more rapidly moving of the two zones proceeded down the column at an essentially constant velocity (depending upon the load), but the more slowly moving zone proceeded at a continually decreasing velocity. In a few experiments, the more rapidly moving zone appeared to divide into two new zones. Conversely, when small amounts were chromatographed, the demarcation between the more rapidly and the more slowly moving zones often disappeared before the front of the faster zone had been eluted from the column. A complete separation of the two zones has never been observed. These observations indicate a possible conversion of the main component from one chromatographic form to another (see ref. 25 for discussion of interconverting species).

When portions from each peak of the two zones of hemoglobin S_{II} were rechromatographed separately, each again produced two zones. Although a reversible change in solution is thus probable, the conversion which takes place during chromatography appears to be dependent upon interaction between hemoglobin and the resin and occurs principally during chromatographic development. For example, when the development of a chromatogram of hemoglobin S was interrupted for several hours and then continued, the behavior of the two zones was at least grossly similar to that of chromatograms in which the development had been continuous.

The N-terminal peptides from each zone of hemoglobin S_{II} as examined by the DNP-procedure[26] showed the normal ratio of α to β chain in both zones. Thus, dissociation into asymmetric subunits such as α_2, β_2 or individual α and β chains with gross separation of these subunits is not an explanation for this phenomenon. Dissociation into asymmetric subunits without gross separation of the subunits also does not seem to be involved. This was concluded from an experiment in which hemo-

globin S_{II} was applied to a column and allowed to form the two zones. Radioactive hemoglobin A_{II} was then applied in such a way that it formed double zones which passed through the region of the two S_{II} zones and were eluted from the column before the S_{II} component. Because no transfer of radioactivity to the hemoglobin S was detected, dissociation into subunits which are free to transfer between the hemoglobins[17] apparently did not occur. Because the ferrihemoglobin cyanide forms of S_{II} and A_{II} likewise produce two zones, the partial conversion of oxyhemoglobin to this form can be eliminated as an explanation of the effect.

Thus, although many possible causes for the double zone phenomenon may be eliminated, the basic cause has not yet been identified. Nevertheless these studies do indicate that the two zones are probably a single molecular species in primary structure which undergoes a reversible change from one chromatographic form to another.

Radioactive hemoglobins as chromatographic references

Differences in the chromatographic behavior of hemoglobins such as A, C, F, H, and S are so great under appropriate conditions of development that they may be easily differentiated from one another and identified simply by inspection of the column during development or by gross comparison of the elution diagrams. However, when two hemoglobins from different sources are very similar or identical in chromatographic behavior, proof of their identity or non-identity may be difficult to obtain. Small differences in chromatographic movement between an "unknown" and a reference hemoglobin cannot be detected with confidence by comparing two different chromatograms because of variations due to differences in load, temperature, and other factors which effect the chromatographic behavior of hemoglobins. Such factors obviously cannot influence the comparison if the hemoglobins are chromatographed together in a single or mixed chromatogram. Such a comparison is very sensitive if one of the two hemoglobins contains a radioactive label which does not significantly influence its chromatographic behavior. Thus, if a radioactive hemoglobin and a non-radioactive hemoglobin are mixed and chromatographed, chromatographic identity requires that the specific radioactivity be constant throughout the zone. If it is not, the two obviously are different, and the slope of the curve of specific activity through the zone, whether positive or negative, will tell the relative movement of the two.

This principle has been applied in several instances to determine whether or not a hemoglobin component was identical with an appropriately labelled known hemoglobin. For example, the chromatographic behavior of the original hemoglobin D[27,28] (hemoglobin $D_{\text{Los Angeles}}$) has been compared with that of hemoglobin S. This hemoglobin D is indistinguishable from hemoglobin S in its chromatographic movement when separate chromatograms of each hemoglobin are examined. This result was anticipated from the electrophoretic identity of these hemoglobins as reported by ITANO[27]. However, a detectable difference in the chromatographic movements of hemoglobins D and S became apparent when samples containing hemoglobin D were chromatographed with a small amount of radioactive hemoglobin S. Figs. 3, 4, and 5 illustrate three chromatograms which bear on this problem. Fig. 3 depicts the results from the chromatography of a mixture of radioactive hemoglobin S_{II} and non-radioactive hemoglobin S. The specific activity should be constant throughout the hemoglobin S_{II} zone of this chromatogram. However, these experiments were per-

formed before it was recognized that the ferrihemoglobin cyanide migrates more slowly than the oxyhemoglobin (see following section); the fact that the radioactive hemoglobin S_{II} was present partly as ferrihemoglobin S_{II} cyanide probably accounts for the small positive slope in the specific activity curve. Fig. 4 represents a chromatogram of a mixture of radioactive S_{II} and hemoglobin from an individual who is

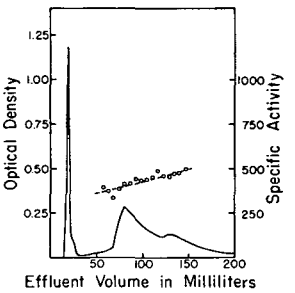

Fig. 3. Chromatogram of a mixture of 25 mg of hemoglobin S and 2.5 mg of radioactive hemoglobin S_{II} on a 1 × 35 cm column of IRC-50 at 6° with developer No. 1. — optical density at 542 mμ; —○—○— specific activity in counts per min per mg of hemoglobin.

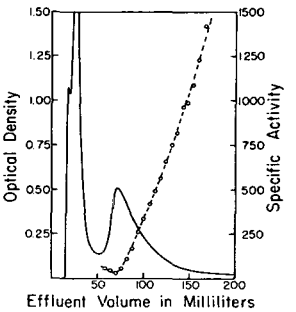

Fig. 4. Chromatogram of a mixture of 50 mg of hemoglobin from a patient heterozygous for hemoglobin A and D genes and 2.5 mg of radioactive hemoglobin S_{II}. Conditions and representation as in Fig. 3.

heterozygous for hemoglobin D and hemoglobin A genes[28]. Roughly equal amounts of hemoglobins D and A were present. The slope of the specific activity curve in this chromatogram is far different than that in Fig. 3 and indicates that hemoglobin D migrates more rapidly than S_{II}. Fig. 5 illustrates the chromatographic results from a mixture of radioactive hemoglobin S_{II} and hemoglobin from an individual with sickle cell-hemoglobin D disease (heterozygous for hemoglobin D and hemoglobin S genes[28]). The slope of the specific activity curve is intermediate between those shown in Figs. 3 and 4, and one may conclude that roughly equal amounts of hemoglobins S and D are present. The method clearly is a sensitive way to determine whether two hemoglobins are actually identical chromatographically. In this instance, other methods had showed that the two hemoglobins differed. When a radioactive reference was used, they were also shown to differ chromatographically.

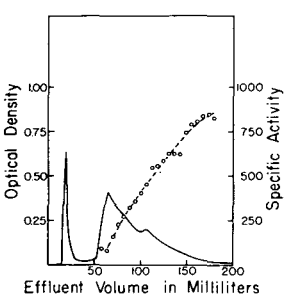

Fig. 5. Chromatogram of a mixture of 25 mg of hemoglobin from a patient with sickle cell-hemoglobin D disease and 2.5 mg of radioactive hemoglobin S_{II}. Conditions and representation as in Fig. 3.

Radioactive reference hemoglobins have been useful in showing the chromatographic similarity of the "adult" hemoglobin from an individual with thalassemia-hemoglobin H disease with that of normal adult hemoglobin[13], in investigating several minor components from hemoglobins A and S[14], and in comparing a gorilla hemoglobin with human adult hemoglobin[14].

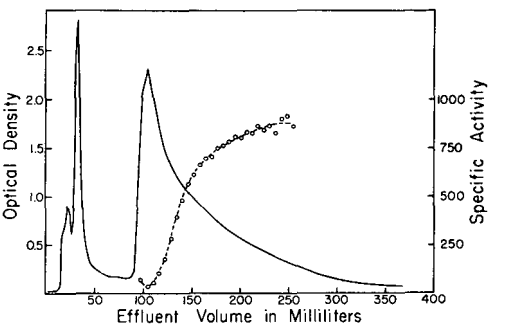

Fig. 6. Chromatogram of a mixture of 29 mg of umbilical cord blood oxyhemoglobin and 7 mg of radioactive ferrihemoglobin F_{II} cyanide on a 1 × 35 cm column of IRC-50 at 6° with developer No. 4. — optical density at 415 mμ; —○— —○— specific activity.

Differences in the chromatographic behavior of oxyhemoglobin and ferrihemoglobin cyanide

The introduction of cyanide ion into chromatographic developers in order to eliminate the formation of slowly moving extraneous zones due to the presence or formation of ferrihemoglobin (methemoglobin) during the chromatography of oxyhemoglobin was first used by ALLEN, SCHROEDER AND BALOG[6]. During the course of the present study, it became evident that the chromatographic movements of the ferrihemoglobin cyanide forms of hemoglobins A_{II}, S_{II}, and F_{II} are detectably different from their oxyhemoglobin forms. Although mixtures of these two forms of any one of these hemoglobins cannot be resolved into two separate zones and therefore appear to be the same in chromatographic behavior, small differences in their chromatographic movement can be demonstrated by radioactive tracers. Figs. 6 and 7 illustrate the results of two experiments with fetal hemoglobin. Fig. 6 depicts a chromatogram of a mixture of the oxyhemoglobin and ferrihemoglobin cyanide forms of hemoglobin F_{II}. Clearly, ferrihemoglobin F_{II} cyanide was not uniformly distributed throughout the

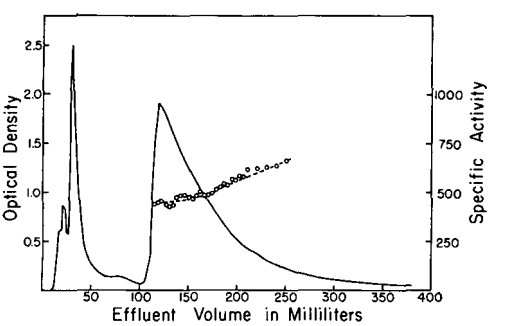

Fig. 7. Chromatogram of a mixture of 29 mg of umbilical cord blood ferrihemoglobin cyanide and 7 mg of radioactive ferrihemoglobin F_{II} cyanide. Conditions and representation as in Fig. 6.

F_{II} zone. Fig. 7 represents a control experiment in which the same mixture was first oxidized completely to ferrihemoglobin cyanide before chromatography. The distribution of the radioactivity is essentially uniform throughout the main portion of the F_{II} zone in this control. Although the migration rates of the oxyhemoglobin and ferrihemoglobin cyanide forms in the experiment shown in Fig. 6 may have been influenced by the difference in the load of the two forms, the experiments do indicate a definite difference in the chromatographic behavior of these two forms of fetal hemoglobin.

Analogous experiments have been made with hemoglobin A_{II} and Developer No. 2 and with hemoglobin S_{II} and Developer No. 1. In each of these experiments, equal amounts of the two forms were compared and positive slopes in the specific activity curves similar to Fig. 6 were observed. Probably the ferrihemoglobin cyanide form of each hemoglobin is more strongly adsorbed than its corresponding oxyhemoglobin form. However, because the difference in chromatographic behavior of the two forms is so small, the practice of adding potassium cyanide to the chromatographic developers in order to eliminate the extraneous zones of free ferrihemoglobin is certainly still useful.

Comments on the use of various developers

The chromatographic procedures which are under discussion here have been applied to many problems in the study of hemoglobin. They are based largely upon the premise that the chromatograms should be carried out under equilibrium conditions if at all possible because of the slowness with which the columns of IRC-50 come to equilibrium. As a result, gradient elution has never been used, and specific developers have been devised to solve specific problems. In conclusion, it seems desirable to indicate the way in which various developers may be most usefully applied.

Developer No. 1 is the strongest developer. It is useful for the separation of hemoglobins with grossly different behavior such as F, A, S, and C. It may be used to separate the minor components of hemoglobins S and C from the major component.

Developer No. 2 produces a more complete separation than developer No. 1 of F from A and also of the A_I components (the nomenclature of minor components is given in refs. 6 and 9) from A_{II}.

Developer No. 4 resolves umbilical cord hemoglobin into F_I and F_{II} while the hemoglobin A that is present is very strongly fixed. It is especially useful in the resolution of A_I and S_I into their components.

Developer No. 5 was devised for and is largely used for the separation of the minor components of hemoglobin A from the main component, A_{II}, in a single chromatogram.

Developer No. 6 is the weakest of the developers and is designed to retard rapidly moving components. It is especially useful in the isolation of hemoglobins γ_4, H, F_I, and A_{Ic}.

ACKNOWLEDGEMENTS

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SUMMARY

A procedure for the chromatographic separation of human hemoglobins on Amberlite IRC-50 is reviewed. Important factors which influence the chromatographic behavior of hemoglobins with this procedure include temperature, pH and ionic concentration of developers, state of equilibrium of the resin, amount of hemoglobin, and oxidation state of the heme in the hemoglobin applied.

A procedure for the comparison of hemoglobins with similar or identical chromatographic behaviors using ^{14}C-labeled hemoglobins is presented. Differences in the chromatographic behavior of hemoglobins S and D and of the ferrihemoglobin cyanide and oxyhemoglobin forms of hemoglobin F were demonstrated by this radioactive tracer technique.

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CHROMATOGRAPHY OF QUATERNARY NITROGEN COMPOUNDS ON BUFFERED CATION-EXCHANGE RESINS

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A procedure for the separation of betaines and other quaternary nitrogen compounds on columns of sulfonated polystyrene resin with acid eluents has been described by CHRISTIANSON *et al.*[1]. This method was especially satisfactory for isolating and characterizing many compounds from biological extracts because of easy removal of the eluting agent, dilute hydrochloric acid. However, certain compounds like amides or esters were decomposed by the acid, and others were not well separated in the acid system. Buffered columns as employed by MOORE, SPACKMAN AND STEIN[2] were investigated as a means of eliminating these disadvantages. An improvement in chromatography of amino acids on buffered columns has been made by HAMILTON[3] through the use of very fine cation-exchange resins of selected uniform particle size. Sharp resolution was maintained at accelerated flow rates obtained under pressure, thereby reducing time of chromatography.

This report describes the adaptation of these buffered column procedures to chromatographic separation of some betaines and other naturally occurring quaternary nitrogen compounds. These compounds were detected and determined by a modification of the procedure of WALL *et al.*[4] based on ultraviolet absorbance of their periodide derivatives. More rapid and better resolution of mixtures containing betaine, choline, carnitine, trigonelline, stachydrine, and others was achieved with this system than with columns in the hydrogen form[1]. For further characterization, the quaternary nitrogen compounds were isolated from the buffer effluent as their periodide derivatives, which were then converted to the original compound.

An application of the technique to the analysis of quaternary nitrogen compounds in extracts of whole corn is described.

METHODS

Buffers and ion-exchange columns

Sodium citrate buffers, pH 2.2, 3.25, and 4.25 containing 0.20 *N* sodium ion, and pH 5.28 containing 0.35 *N* sodium ion, were prepared as described by MOORE, SPACKMAN AND STEIN[2] except that BRIJ 35 detergent and phenol were omitted. The addition of detergent resulted in anomalous yields of periodide precipitates, and phenol interfered with measurements of the ultraviolet absorption of the effluent.

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A finely ground sulfonated polystyrene resin, Amberlite IR-120 (CG-120) type 2 (Rohm & Haas, Co., Philadelphia, Pa.)*, was separated into more uniform particle size fractions by the method of HAMILTON[3]. More recently preparations of the resin with selected particle sizes suitable for chromatography were purchased from Bio-Rad Laboratories, Berkeley, Calif. (Aminex-MS, fractions C and D.). The 0.9-cm diameter columns of these resins were packed following the procedure of MOORE *et al.*[2] employing fraction C for 15-cm and 50-cm height columns and D for 150-cm columns. After being washed with 0.2 *N* NaOH they were equilibrated with the pH 3.25 buffer.

Chromatography of known compounds

Mixtures of known quaternary nitrogen compounds containing 0.5–2.0 mg of each substance were dissolved in the pH 2.2 buffer and applied to 15-cm, 50-cm, and 150-cm long columns, 0.9 cm in diameter, jacketed for temperature control. The solutions also included 0.5–1.0 μmole of amines and amino acids related to the quaternary nitrogen compounds to enable a comparison of their behavior during chromatography. The compounds tested were high-purity products obtained from commercial sources or synthesized as described previously[4]. Initial elution usually was with the pH 3.25 buffer at 30° followed by buffers of higher pH with operation at elevated temperatures to remove compounds held more tenaciously. The columns were operated under pressures of nitrogen that gave the flow rates, 12–15 ml/h, recommended by MOORE *et al.*[2].

Corn extract

The corn extract subjected to chromatography was obtained by 80 % ethanol extraction of ground dent corn, a composite of commercial varieties. Details of preparation of sample, extraction procedure, and removal of contaminants, including proteins and lipids from the extract, are described elsewhere[5]. The final aqueous extract was analyzed for total nitrogen by the Kjeldahl method. A portion was taken to dryness and made up to volume with pH 2.2 buffer solution so that a 2-ml aliquot contained a suitable sample for chromatography.

Determination of quaternary nitrogen compounds

Column effluent fractions were analyzed for quaternary nitrogen compounds by measurement of the ultraviolet absorbance of their periodide derivatives. The reagent used for the preparation of the periodides in the buffered solutions consisted of 15.7 g iodine and 20.0 g potassium iodide dissolved in 100 ml of 3 *N* sulfuric acid. The sulfuric acid was added to reduce the pH of the buffered effluent fractions to a value giving optimum yields of periodide precipitates. Because iodine precipitated from the reagent upon standing, fresh reagent was prepared daily. To 0.5-ml aliquots of effluent fractions or standard solutions containing 10–100 μg of quaternary nitrogen compound in appropriate buffers, 0.2 ml reagent was added and the periodide obtained as described by WALL *et al.*[4]. The absorbance of the periodide dissolved in 5.0 or 10.0 ml ethylene dichloride was measured. Standard curves or equations relating optical density and periodide yields were used to determine the amounts of quaternary nitrogen compounds in unknown samples[4]. Values of correction factors for solubility

* Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

losses and of absorbances of periodides prepared from buffered solutions of quaternary nitrogen compounds are listed in Table I.

Initial survey of the effluent fractions was made by analyzing every third tube. Each fraction in the vicinity of a periodide-yielding tube was then reanalyzed care-

TABLE I

FACTORS FOR ESTIMATING QUATERNARY NITROGEN COMPOUNDS FROM PERIODIDE ABSORPTION*

Quaternary nitrogen compound	*Original pH of elution buffer*	*Periodide solubility (μg original compound in 0.7 ml reaction solution)*	*μg of quaternary nitrogen compound per unit absorbance of periodide at 365 mμ in 5 ml of ethylene dichloride*
Betaine·HCl	3.25	13.2	25.1
γ-Butyrobetaine·HCl	3.25	0.7	39.3
Carnitine·HCl	3.25	23.2	36.3
Choline chloride	3.25	0.9	27.0
N-Methylnicotinamide	5.28	1.1	40.7
Stachydrine·HCl	3.25	2.9	40.2
Trigonelline·HCl	3.25	2.0	31.2

* Values should be redetermined to allow for any variation in experimental conditions.

fully by using 0.1 to 0.5-ml aliquots made up to 0.5 ml by addition of the appropriate buffer where necessary. When larger aliquots were required, the volume of reagent was increased proportionally as was the correction factor for solubility losses.

Other analytical procedures

The amino acids and amines in effluent fractions were determined by the colorimetric ninhydrin method of MOORE AND STEIN[6]. The aromatic compounds were detected in the effluent fractions by measuring absorbance at 260 mμ with the Beckman D.U. spectrophotometer.

Identification of quaternary nitrogen compounds

The quaternary nitrogen compounds were isolated from the pooled remainders of the effluent buffer solution fractions constituting the peak by forming their water-insoluble periodide derivatives as described for the analytical procedure but on a larger scale. As little as 200 μg of the quaternary nitrogen compound can be converted to the chloride salt in essentially quantitative yields by the method of STANEK[7]. For this conversion the periodide was reacted with 0.5 g powdered copper to reduce the complexed iodine. Then 0.2 g copper chloride and 2 ml water were added to the mixture which was heated 1 h in a boiling water bath. The resulting cuprous iodide was removed by centrifugation. Excess copper was removed by addition of H_2S and filtration. The identity of the quaternary nitrogen compound was verified by comparison with known compounds on paper chromatograms. The solvents and techniques employed to separate and detect the compounds on papers have been summarized by BLOCK *et al.*[8].

RESULTS

Chromatography of known substances

Betaine, trigonelline and choline were well resolved in that sequence on a 15-cm column with 120 ml pH 3.25 sodium citrate buffer at 30° (Fig. 1).

A more complex synthetic mixture required a 50-cm column for resolution with an elution schedule of 300 ml pH 3.25 buffer at 30° followed by 400 ml pH 5.28 buffer

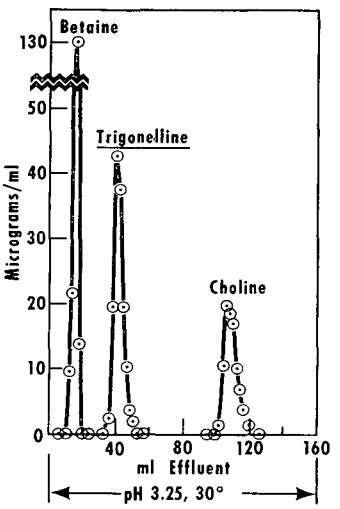

Fig. 1. Separation of betaine, trigonelline, and choline on Aminex-MS, 15-cm column, pH 3.25 sodium citrate buffer, 0.20 *N* sodium ion.

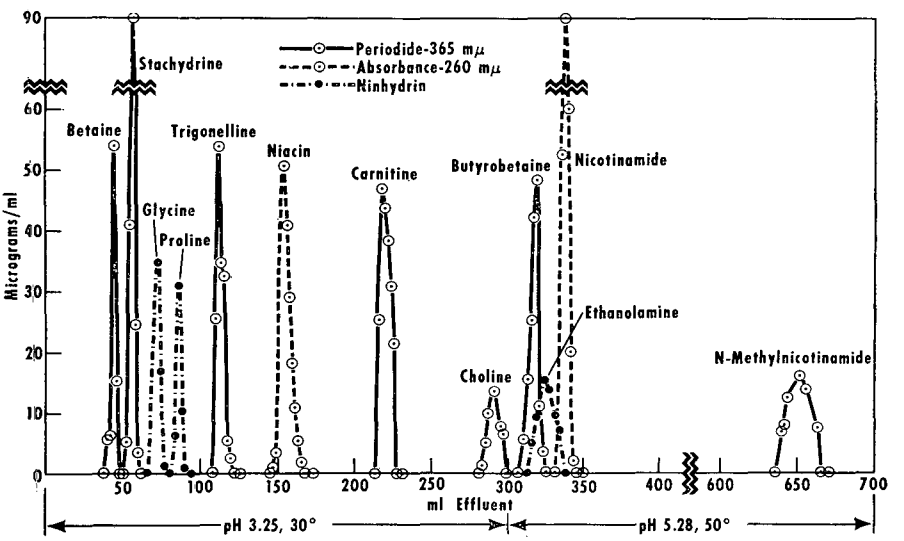

Fig. 2. Separation of a synthetic mixture of quaternary nitrogen compounds and their related amino acids or amines on Aminex-MS, 50-cm column, pH 3.25 sodium citrate buffer, 0.20 *N* sodium ion and pH 5.28 sodium citrate buffer, 0.35 *N* sodium ion.

at 50°. The elution positions of the quaternary nitrogen compounds and their related amino acids or amines from this column are shown in Fig. 2.

Better separation of earlier eluting quaternary nitrogen compounds was obtained by use of a 150-cm column with 250 ml pH 3.25 buffer. However, the more basic

TABLE II

RECOVERIES OF QUATERNARY NITROGEN COMPOUNDS AFTER ION-EXCHANGE CHROMATOGRAPHY

Substance	*Number of chromatographic determinations*	*Samples applied (mg)*	*Average recovery (%)*	*Average deviation (%)*
Betaine·HCl	3	0.61, 0.61, 1.51	100.8	±2.5
Stachydrine·HCl	3	0.62, 1.22, 1.22	100.3	±3.1
Trigonelline·HCl	3	0.62, 1.22, 1.22	102.2	±1.7
Carnitine·HCl	4	1.20, 1.20, 1.20, 1.51	95.7	±2.5
γ-Butyrobetaine·HCl	2	0.99, 1.14	100.6	±4.3
Choline chloride	2	0.57, 0.63	103.1	±1.5
N-Methylnicotinamide	1	1.50	97.0	

compounds required large volumes of pH 5.28 buffer for elution. Buffer eluent schedules and/or column temperatures can be varied to facilitate separation of specific compounds. For example, γ-butyrobetaine will precede choline on the 50-cm column if, after 200 ml pH 3.25 buffer, elution is continued with pH 4.25 buffer. From a 15-cm column initially equilibrated with pH 5.28 buffer, N-methylnicotinamide emerged after an elution with 200 ml pH 5.28 buffer at 50° in a sharp peak well separated from the other compounds. The more neutral quaternary nitrogen compounds were eluted early with poor resolution similar to the behavior of acidic and neutral amino acids in this system[2].

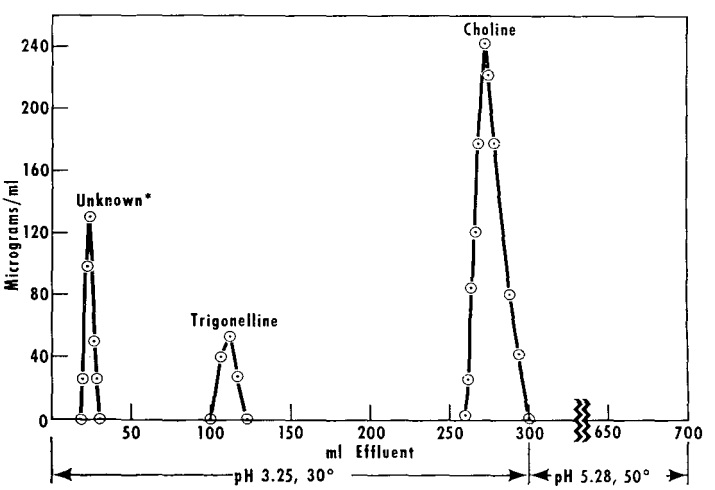

Fig. 3. Chromatographic separation of quaternary nitrogen compounds from whole corn grain on Aminex-MS, 50-cm column, pH 3.25 sodium citrate buffer, 0.20 *N* sodium ion and pH 5.28 sodium citrate buffer, 0.35 *N* sodium ion. (*Microgram unknown based on betaine periodide absorbance).

The recoveries of the compounds separated on a 50-cm column tabulated in Table II were essentially quantitative. The measured recovery of carnitine was low because the high solubility of its periodide may introduce errors in the analysis.

Chromatography of corn extract

An extract equivalent to 30 g corn was chromatographed as illustrated in Fig. 3. Two substances yielding periodide precipitates were identified as trigonelline and choline by position of elution and paper chromatography. These compounds have been chromatographically isolated and identified previously[1] as constituents of corn.

An unknown substance yielding a periodide derivative was also detected in the initial fractions. Work is in progress to characterize this compound.

DISCUSSION

Chromatography on buffered columns offers the same advantages in the separation of quaternary nitrogen compounds as those described for the separation of amino acids by MOORE AND STEIN[9]. As indicated by the recovery values in Table II, the destruction of amides such as N-methylnicotinamide was hardly significant when the buffer procedure was used, whereas the use of hydrochloric acid eluents caused 69% degradation of this substance[1].

The use of the buffered column resulted in improved separation of certain compounds not well resolved by the acid system, since their differences in charge were emphasized at the pH's of the buffers. For example, betaine and choline were well separated with the buffered columns while with acid columns these substances were eluted in close proximity[1] because of the diminished carboxyl ionization. In the buffered systems the differences in acidity between α- and ω-carboxyls of the betaines resulted in butyrobetaine and carnitine being eluted after trigonelline, similar to the elution of γ-aminobutyric acid after α-aminobutyric acid as reported by SPACKMAN, STEIN AND MOORE[10].

Most of the quaternary nitrogen compounds were eluted before their amine analogues by buffer as indicated by the pairs: glycine and betaine, trigonelline and nicotinic acid, and choline and ethanolamine (Fig. 2). The greater charge on the quaternary nitrogen compounds at the pH of the buffers makes them more polar than the amines or amino acids. Thus quaternization increases the affinity for the polar eluent more than it does adsorption to the aromatic resin. An exception to this is N-methylnicotinamide, which is eluted much after nicotinamide. The quaternary compounds were usually eluted after their related amines on columns wholly in the hydrogen form[1].

The fine mesh-sized resin enabled satisfactory resolution at accelerated flow rates. Thus a typical analysis on the 50-cm column can be completed in 2 days in contrast to the 6 days needed for chromatography on a 200–400 mesh resin. This improvement in rate permits use of the chromatographic method for more routine analysis of plant and other biological extracts. The finer mesh resin of selected size can also be employed for chromatography with acid eluents at accelerated rates where the elution pattern obtained with that system is desired.

ACKNOWLEDGEMENT

The authors wish to thank CECIL H. VANETTEN for preparation of the laboratory-fractionated resin.

SUMMARY

Quaternary nitroge ncompounds and their amine analogues were separated by chromatography on columns of a uniformly sized, pulverized, sulfonated polystyrene resin using buffers for elution. Substances of biological interest thus separated and determined by the ultraviolet absorption of their periodide derivatives include betaine, stachydrine, choline, trigonelline, N-methylnicotinamide, and carnitine. Better separation of these compounds is obtained with buffered eluents than with hydrochloric acid because of greater differences in degrees of ionization in the buffer system. Corn grain extracts were rapidly analyzed by the procedure.

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ION-EXCHANGE CHROMATOGRAPHY OF SULFUR AMINO ACIDS AND THE SEPARATION OF DIASTEREOISOMERS*

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Cysteine (CySH) and glutathione (GSH) have been shown to participate in several reactions involving conjugation with other organic compounds. Identification of nitrogenous compounds excreted in the urine has been accelerated by chromatographic techniques, most recently by ion-exchange column chromatography as refined in the automatic recording apparatus of SPACKMAN, STEIN AND MOORE[1].

These authors have described the behaviour under ion-exchange chromatography of methionine sulfone, methionine sulfoxides, cysteic acid, S-carboxymethylcysteine, homocystine, felinine, cystathionine, and GSSG[1,2]. HAMILTON AND ANDERSON have noted the chromatographic behavior of DL + *meso*-lanthionine in a similar system[3]. FRIMPTER AND BASS recorded the position of S-aminoethylcysteine, L-2-thiolhistidine, S-methyl-cysteine, S-ethyl-cysteine, S-*n*-butyl-L-cysteine, S-carboxyl-L-cysteine, L-ethionine, D-penicillamine, L-djenkolic acid, and the optical isomers of the disulfide of cysteine and homocysteine, all in the system of SPACKMAN, STEIN AND MOORE[4]. The partial separation of *meso*-cystine from racemic cystine was noted by HIRS, MOORE AND STEIN[5].

The present study was undertaken to confirm the presence in urine of compounds demonstrated by other methods[6], and to describe the chromatographic location of compounds which might be found under certain circumstances in biological fluids or in hydrolysates of synthetic peptides. The chromatographic separation of diastereoisomers of several sulfur compounds is of particular interest because it affords a means of resolving them. In general, the compounds were prepared in the laboratory in Japan, and the chromatographic studies were performed in the laboratory in the United States.

METHODS

Compounds

The compounds investigated are numbered according to their order of elution from the resin column. Compound 5 was purchased from the California Corp. for Biochemical Research, Los Angeles. Compounds 4 ("isovalthine"), 7, 9 ("isobuteine"), 10, 12, 13 were synthesized according to methods previously described[6,7]. Compounds 2, 3, 6 and 8 were synthesized according to the methods described by several authors[8–11]

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** Senior Research Fellow, New York Heart Association.

respectively. Compound I was prepared by reaction of reduced glutathione with (±)-α-bromoisovaleric acid in triethanolamine solution. Although Compound I was not pure, it yielded isovalthine, glutamic acid, and glycine after hydrolysis in 2 *N* HCl. Its chromatographic behavior on paper will be reported elsewhere[12]. Compound II was synthesized as usual by reaction of L-cysteine with β-bromo-α,α-dimethylpropionic acid in liquid ammonia. Elemental analysis of the Compound II was correct and its R_F values on paper have been reported in a previous paper[7]. β-Bromo-α,α-dimethylpropionic acid was prepared according to reported methods[13,14].

Chromatography

Ion-exchange chromatography was carried out essentially according to the method recommended by SPACKMAN, STEIN AND MOORE for analysis of physiological fluids, that is, at 30°, pH 3.23 with change to 50° and pH 4.25 (0.2 *N* sodium citrate buffers) after 350 effluent ml.

RESULTS

A composite tracing showing the location of the various compounds examined is shown in Fig. 1. Ninhydrin color at 570 mμ, relative to leucine, is listed in Table I.

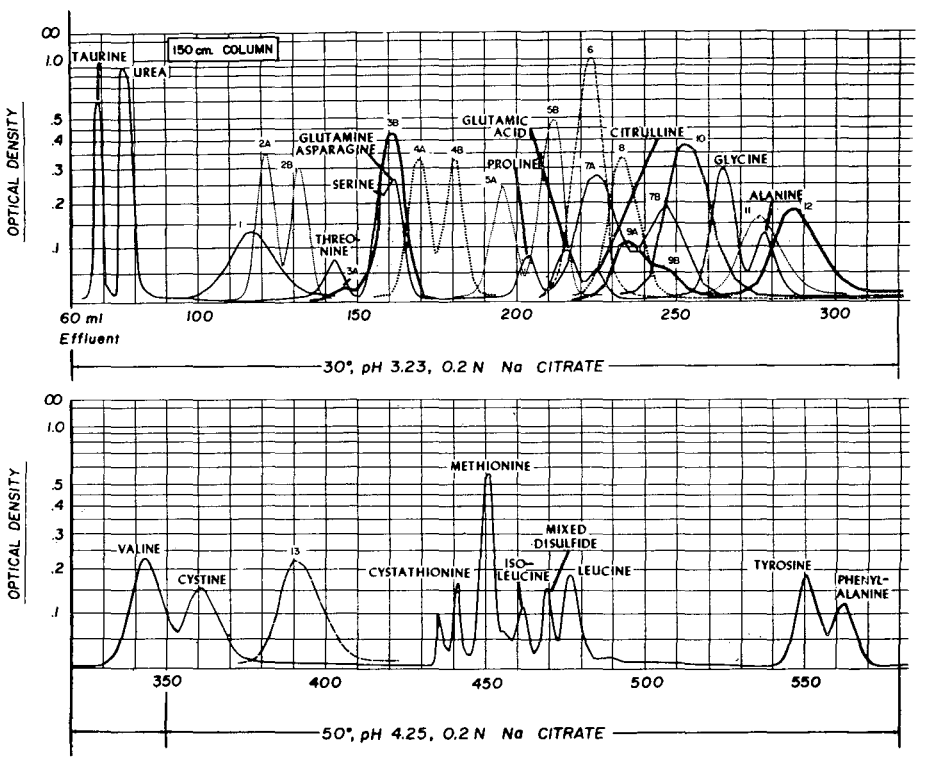

Fig. 1. Composite tracing of ion-exchange chromatogram. The substances which might be found in biological fluids are traced in solid black lines. The numbered peaks correspond to those in Table I.

The chromatographic runs were generally performed once, so that the color value listed is only approximate.

TABLE I

Figure location	*Formula*	*Per cent of leucine color (570 mμ)*
1	GS–CH(COOH)$CH(CH_3)_2$	impure
2 (A + B)	CyS–$CH(CH_3)$COOH	91
3 (A + B)	CyS–$C(CH_3)_2$COOH	impure
4 (A + B)	CyS–CH(COOH)$CH(CH_3)_2$ (isovalthine)	87
5 (A + B)	CyS–$CH_2CH(NH_2)$COOH (lanthionine)	102
6	CyS–CH_2CH_2COOH	100
7 (A + B)	CyS–CH(COOH)$CH_2CH_2CH_3$	92
8	CyS–$CH(CH_3)CH_2$COOH	76
9 (A + B)	CyS–$CH_2CH(CH_3)$COOH (isobuteine)	—
10	CyS–$C(CH_3)_2CH_2$COOH	95
11	CyS–$CH_2C(CH_3)_2$COOH	82
12	CyS–$CH(CH_3)CH(CH_3)$COOH	—
13	Cys–$CH_2CH(CH_3)CH_2$COOH	79

DISCUSSION

In general, the three factors that appear to contribute to delay elution of the CySH conjugates are: increased distance of the carboxyl group from the S, the presence of side chains, and increased molecular weight. The early appearance of Compound 1 is consequent to the presence of 3 carboxyl groups.

Compounds 1, 6, 8, 10, 11, 12 and 13 yielded single peaks suggestive of a "pure" amino acid. The formulas of Compounds 6, 10 and 11 reveal no opportunity for diastereoisomerism. The breadth of peaks from Compounds 1, 12 and 13, whose formulas suggest opportunity for diastereoisomerism, lead to the conjecture that they may represent poorly separated isomers.

Compounds 2, 4, 5, 7 and 9 revealed double, sharp peaks characteristic of diastereoisomers. Lanthionine, 5, probably is separated into *meso*- and DL-forms, which were stated to be present by the manufacturer. Inspection of formulas of the other compounds yielding 2 peaks, *i.e.*, 2, 4, 5, 7 and 9, reveals that all have two potentially asymmetrical carbon configurations. In all of these, one asymmetrical carbon would be at the alpha carbon of CySH and the other at the carbon atom adjacent to the carboxyl of the conjugated acid. Compound 3 produced two peaks which were not typical of those shown by diastereoisomers, and the compound does not contain two asymmetrical carbon atoms. It is concluded, therefore, that the smaller of the two peaks must represent some ninhydrin-positive contaminant.

All compounds yielding paired peaks (except lanthionine) were synthesized by reaction of L-CySH with the appropriate (±)-bromo acid. This suggests the presence of L- and L-*allo*-amino acids.

No definite statement can be made as to which isomer constitutes which peak, since the column effluent was not analyzed. Sufficient amounts were not obtained for optical rotation determinations. Although the resolution of isovalthine diastereo-

isomers was somewhat improved by operation at 50°, increasing the load on the column tended to cause loss of resolution. Chromatography of isovalthine isomers on a 165 × 2 cm "preparative" column of amberlite IR-120 at 50° with pH 3.23, 0.2 *N* sodium citrate buffer did not produce adequate resolution. *meso*-Cystine is eluted prior to DL-cystine in this system[1,4,5]. When cystathionine is chromatographed in this technique, but the shift to pH 4.25 buffer is not made and elution is continued with pH 3.23 buffer[15], *allo*-cystathionine is eluted prior to LL-cystathionine at 515 ml. *allo*-Isoleucine precedes isoleucine[1,2]. L-*allo*-Isovalthine prepared by reaction of L-cysteine with freshly prepared (—)-α-bromoisovaleric acid in triethanolamine solution is eluted prior to L-isovalthine prepared by reaction of L-cysteine with fresh (+)-α-bromoisovaleric acid*. Because the *allo*- or *meso*-form of these four compounds precedes the "racemic" or L-(+) form, it is assumed that this is also the case with the remaining isomers. This may offer a practical method for the separation of chromatographic amounts of diastereoisomers.

A small peak in the area of isovalthine (Compound 4) was observed upon ion-exchange chromatography of the urine of a three-week old cretin. However, the amount present was too small to permit chemical confirmation. It may be noted, however, that the possible presence of some of the compounds in biological fluids would be obscured by usually occurring compounds, *e.g.*, Number 11 by alanine.

SUMMARY

The technique of SPACKMAN, STEIN AND MOORE[1] was used to chromatograph some sulfur-containing amino acids. The appearance of double peaks indicated the probable separation of diastereoisomers.

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* The detailed method of resolution of (±)-α-bromoisovaleric acid to each antipode by using ephedorine will be published.

ELECTROPHORESIS OF LIPOPROTEINS USING PRE-STAINED SERUM

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McDONALD AND BERMES[1], in 1955, proposed a pre-staining technique for the determination of serum lipoproteins by paper electrophoresis, in which a saturated solution of SBB or AcSBB** in 95 % ethanol was used. The acceptance of this method grew rapidly and later modifications were introduced by WILCOX *et al.*[2] and by KANABROCKI *et al.*[3], in attempts to simplify the original method. However, the use of ethanol in these methods was said to present some possible limitations since the lipoprotein complex may be sensitive to even small amounts of this solvent[4].

Thus, SOLINAS *et al.*[4] proposed a method in which diacetin was used as solvent for SBB. Later, LARKEY AND BELKO[5] used a mixture of petroleum ether–ethyl alcohol (1:4) and ZAKELJ AND GROS[6] proposed the use of a mixture of dioxane and ethylene glycol. Further, other systems have been tried out with greater or lesser success. Among these, a system containing ethyl acetate and propylene glycol was successfully used by McDONALD *et al.*[7,8], in the chromatographic separation of lipoproteins.

In an attempt to overcome some of the difficulties found in this type of determination, solvents other than ethyl alcohol and diacetin were re-investigated by McDONALD AND RIBEIRO[9]. This investigation showed that propylene and ethylene glycol possessed some inherent advantages as compared to ethyl alcohol and other solvents.

Due to the interest shown in these pre-staining methods, we decided to undertake a more detailed study of the use of propylene and ethylene glycol as solvents for SBB in the pre-staining of lipoproteins.

EXPERIMENTAL

Sudan Black B solutions

Preliminary experiments showed that propylene glycol solutions of SBB always contained more dissolved dye than those prepared with ethylene glycol, as shown by spectrophotometric measurements.

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** The following abbreviations will be used: SBB = Sudan Black B, AcSBB = Acetylated Sudan Black B.

Using the technique previously described by MCDONALD AND RIBEIRO[9] it was also verified that AcSBB always gave somewhat sharper differentiation between the lipoprotein zones. Therefore, all subsequent experiments were performed using a saturated solution of AcSBB in propylene glycol. The saturated solution was prepared by heating 10 ml of propylene glycol (Eastman Kodak Co., Rochester, N.Y.) to 100–110° and 0.1 g of AcSBB, prepared according to CASSELMAN[10] from SBB (Hartman-Leddon Co., Philadelphia, Pa., certification No. Czb-5), was added to the hot solvent, with thorough stirring for 5 min. The solution was filtered hot on Whatman No. 2 filter paper, cooled and re-filtered on the same kind of paper.

Care must be taken not to exceed 110° to avoid the formation of a useless gelatinous mixture. The solution thus prepared was found to be stable for at least one month, when kept in a tightly-closed container in the dark.

Paper electrophoresis

The paper electrophoresis apparatus used for these experiments was of the horizontal type (Buchler Instruments, Inc., New York City) and all separations were performed using a veronal–Na veronal buffer having a pH of 8.6 and an ionic strength of 0.05. Of the several types of paper used, we found that Whatman No. 3MM and Macherey and Nagel No. 2214ff were the most suitable for this type of experiment. However, it seemed that Macherey and Nagel No. 2214ff offered some slight advantages since the bands appeared somewhat more clearly separated when this paper was used.

The ionograms were scanned with the aid of the Photovolt Transmission Densitometer Model 525 (Photovolt Corp., New York City) and the measurements made at 595 mμ. Quantitation of the ionograms was made by planimetry.

RESULTS AND DISCUSSION

Amount of dye solution to be added to the serum

Since excess solvent may cause alterations in the lipoprotein patterns the amount of dye solution to be added to the serum should be determined carefully. This determination was carried out by adding slowly, with constant, gentle stirring, increasing amounts of the saturated solution of AcSBB to the same volume of identical serum samples (0.5 ml) placed in small centrifuge tubes.

The serum–dye mixture was allowed to stand at room temperature for 45 min and then centrifuged for 15 min. Aliquots of 20 μl of the supernatant were used for the electrophoretic separations using a potential gradient of 8 V/cm for 2 h. After fractionation, the strips were dried at 50° in the dark and scanned. Table I shows the results of these experiments.

The results illustrated in Table I show that from No. 1 to No. 3 there is a linear increase of the areas of the fractions with the amount of dye solution added to the serum. This is evident from the fact that the values of the β/α-ratio remain constant. However, this does not hold for the values in strip No. 4.

Since the α- and β-lipoprotein zones are decreased in strip No. 4 and the chylomicrons are increased, it is possible that some denaturation occurred at this point due to excess solvent added to the serum. These facts are visualized better in Fig. 1 and suggest that pre-staining should be carried out by adding less than 0.1 ml of

TABLE I

AREA OF THE LIPOPROTEIN ZONES AFTER ELECTROPHORESIS OF SERUM PRE-STAINED WITH INCREASING AMOUNTS OF DYE SOLUTION

Strip No.	*Amount of dye solution**	*Area (cm²)*				*β/α-ratio*
		Chylo-microns	*β-Lipo-proteins*	*α-Lipo-proteins*	*Total area*	
1	0.025	1.05	2.50	0.95	4.50	2.7
2	0.050	2.25	4.50	1.65	8.40	2.7
3	0.075	3.25	6.85	2.55	12.65	2.7
4	0.100	5.05	6.25	2.45	13.75	2.5

* ml of dye solution added to 0.5 ml of serum.

dye solution to 0.5 ml of serum. The point where 1 volume of dye solution is added to 10 volumes of serum was selected as the most convenient proportion for pre-staining lipoproteins.

Time of incubation

The time of incubation of the serum sample with the dye solution was determined by incubating aliquots of 0.5 ml of the same serum sample with 0.05 ml of the saturated solution of AcSBB for different periods of time. After the incubation period was over, all tubes were centrifuged for 15 min and samples of 20 μl used for the electrophoretic fractionation. Separations were carried out for 2 h using a potential gradient of 10 V/cm.

After electrophoresis, the strips were dried in the dark at room temperature and scanned. The results obtained are presented in Table II.

The serum and dye solution mixture was also incubated at 37° for 30 and 40 min, respectively. However, the results failed to show any improvement when the incubation mixture is kept at 37°, as proposed by LARKEY AND BELKO[5]. Since increasing

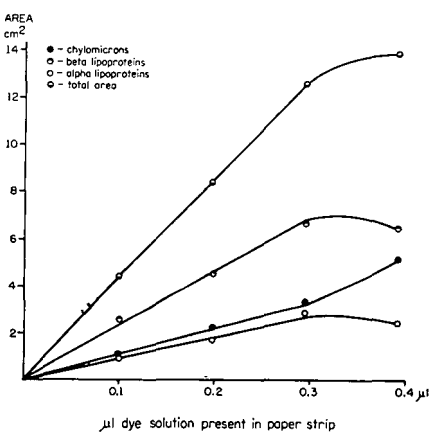

Fig. 1. Effect of the amount of dye solution added to the serum on the color intensity of the lipid zones.

TABLE II

AREA OF LIPOPROTEIN ZONES AFTER ELECTROPHORESIS OF SERUM INCUBATED WITH DYE SOLUTION FOR DIFFERENT PERIODS OF TIME

Strip No.	Time of incubation* (min)	Area (cm^2)		β/α ratio
		α-Lipoprotein	β-Lipoprotein	
1	5	0.50	2.61	5.22
2	10	0.55	2.60	5.00
3	25	0.50	2.70	5.40
4	35	0.84	3.30	3.93
5	45	0.83	3.28	3.95
6	60	0.85	3.31	3.89

* All tubes incubated at 25°.

the temperature may also alter the protein–lipid complex because of its sensitivity to heat, 25° was selected as the most suitable temperature for accomplishing pre-staining of serum lipoproteins.

Amount of stained serum added to the paper

After establishing the previously discussed conditions it became necessary to check the dependence of the amount of pre-stained serum in the strip with Beer's law. This dependence was studied with a serum sample pre-stained by adding 0.05 ml of the saturated solution of AcSBB to 0.5 ml of serum. After 45 min at room temperature the tubes were centrifuged and aliquots of 5 μl to 50 μl were used for the electrophoretic separations. These were carried out on Whatman No. 3MM filter paper, using a potential gradient of 7.7 V/cm for 2.5 h.

The strips were then dried, scanned, and the areas found for α- and β-lipoproteins were plotted on a graph against the volume of pre-stained serum applied to the paper strip, as shown in Fig. 2.

Fig. 2 clearly shows that there is a linear increase of the area between the points

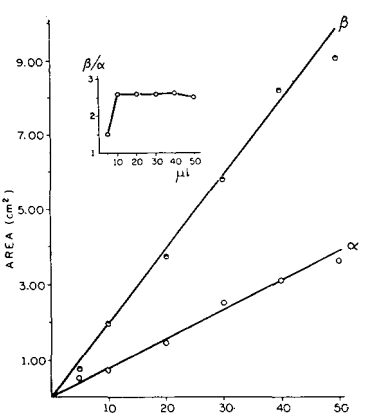

Fig. 2. Variation of the area of lipoprotein fractions with the volume of pre-stained serum applied to the paper strip.

where 10 μl and 40 μl of pre-stained serum were applied to the paper strips. The β/α-ratio was practically constant throughout the range where linearity holds. Since the linear increase follows a straight line passing through the origin, the β/α-ratio is given by the ratio between the slopes of the lines representative of the β- and α-lipoproteins, respectively, and therefore there is an agreement with Beer's law.

We have selected 20 μl as the optimal volume to be used in further experiments since this point falls within the linearity range and yet is sufficiently distant from the limits of this range.

Stability of the color

The color of the ionograms of serum lipoproteins pre-stained with either SBB or AcSBB is sensitive to light[3,11]. In order to evaluate the effect of light on the stained lipoproteins, several samples of the same serum were pre-stained using the conditions selected in the preceding experiments. Electrophoresis was carried out applying 20 μl of each sample to Macherey and Nagel No. 2214ff filter paper strips under a potential gradient of 8 V/cm for 2 h. The strips were dried at 50° in the dark and immediately scanned. They were then separated into two groups of 3 strips each. One set was left exposed to the ordinary light (daylight and artificial light) of the laboratory. Both sets were scanned periodically.

In all strips the areas of the individual fractions were measured and the results, expressed as per cent of original readings, show immediately the amount of change which occurred. The results are in Tables III and IV.

TABLE III

PER CENT OF INITIAL AREA FOR LIPOPROTEINS IN THE STRIPS KEPT IN THE DARK

Fraction	*Hours kept in the dark*					
	0	*1.5*	*3*	*17.5*	*25.5*	*50*
α-Lipoproteins	100	102.4	102.4	98.4	101.2	98.4
β-Lipoproteins	100	101.0	101.8	96.6	99.2	98.4
Chylomicrons	100	98.6	103.5	102.1	96.5	96.5
Total area	100	100.7	102.5	98.8	98.9	97.8

TABLE IV

PER CENT OF INITIAL AREA FOR LIPOPROTEINS IN THE STRIPS EXPOSED TO LIGHT

Fraction	*Hours exposed to light*				
	0	*1*	*2*	*17*	*25*
α-Lipoproteins	100	94.6	79.2	72.1	69.5
β-Lipoproteins	100	99.2	89.6	80.2	64.5
Chylomicrons	100	87.7	79.4	81.1	74.1
Total area	100	94.7	83.8	78.2	68.5

The results of these experiments indicate that the color of stained lipoproteins is quite sensitive to light. However, this color seemed stable for about 50 h, when the strips were kept in the dark. There is also evidence that light is not the sole cause for

fading of SBB bound to, or dissolved in, human serum lipoproteins. MCDONALD AND BANASZAK[11] showed that there is a difference in the rate of fading of SBB bound to high-density and low-density lipoproteins when they are treated with 1 % hydrogen peroxide. The fading of SBB bound to the low-density lipoproteins occurs at a much faster rate than that bound to high-density lipoproteins. However, it was also observed that the addition of serum proteins inhibits the fading of SBB which is bound to ultracentrifugally-prepared lipoproteins.

These findings seem to suggest that fading of color in ionograms of lipoproteins pre-stained with AcSBB or SBB as described, is of a complex nature and that an oxidative reaction (probably catalyzed by light) plays an important role in the process. However, since pre-staining for electrophoresis is accomplished in the whole serum, fading is less of a problem due to the presence of serum proteins, and especially when the strips are protected from light, than that observed with ultracentrifugally-prepared lipoproteins.

Proposed technique

The results so far discussed show that a simple technique may be proposed for the determination of serum lipoproteins by paper electrophoresis, using pre-stained serum. Pre-staining can be achieved with a saturated solution of AcSBB in propylene glycol by adding 1 volume of the dye solution to 10 volumes of serum. After 45 min at room temperature the mixture is centrifuged and the supernatant used for the electrophoretic separations.

The pre-stained serum (20 μl) is added as a thin streak across the width of Macherey and Nagel No. 2214ff paper strips (2.5 cm × 33 cm in our apparatus) midway between the ends. A potential gradient of 8 V/cm is applied for 2 h, at 25°; it developed a current of 1.5 mA/strip at the end of the run, under our experimental conditions. Separations are carried out using veronal buffer with a pH of 8.6 and an ionic strength of 0.05. After completion of the run (carried out in the dark) the strips are removed and dried immediately in a horizontal position, at 50°, in the dark, and scanned. Duplicate runs of individual samples are always made.

Reproducibility and error of the method

Seven samples of the same serum were individually pre-stained and run in two separate sets, on two consecutive days. The electrophoresis was performed with the recommendations described in the preceding section, using Macherey and Nagel No. 2214ff filter paper strips. The usual 8 V/cm potential gradient was used for a period of 2 h.

When the electrophoretic separation was completed, the strips were dried in the

TABLE V

COEFFICIENT OF VARIATION FOR THE VALUES OF THE DIFFERENT LIPOPROTEIN FRACTIONS, EXPRESSED AS PERCENTAGES

Fraction	*Variation (%)*
α-Lipoproteins	± 3.0
β-Lipoproteins	± 2.6
Chylomicrons	± 3.0
β/α-ratio	± 5.4

dark, and scanned. After planimetry of the areas, the results were expressed as percentages of the total area and the standard deviations calculated. From this data the coefficient of variation for each fraction was calculated. This permitted a computation of the error of the method (as percentages) as shown in Table V.

The data in Table V show that the results obtained by the method described are quite reproducible and that they are within $\pm$ 3.0 % for each individual fraction. The method, which is simple to perform, time-saving and inexpensive, can therefore be considered to have a good precision and reproducibility.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. ADOLF RADMACHER of the Macherey, Nagel and Co., Düren, Germany, for kindly providing the samples of filter paper Macherey and Nagel No. 2214ff used in this investigation. The project, in its earlier phases, was supported in part by a grant-in-aid from the Chicago Heart Association.

SUMMARY

A simple method for the paper electrophoretic determination of serum lipoproteins, pre-stained with a saturated solution of AcSBB in propylene glycol, is described. Pre-staining is carried out by adding 1 volume of the dye solution to 10 volumes of serum. Electrophoresis is performed on Macherey and Nagel No. 2214ff filter paper in veronal buffer, pH 8.6, ionic strength of 0.05. A potential gradient of 8 V/cm is used for a period of 2 h.

The conditions for pre-staining are discussed and the influence of light and other factors are taken into consideration. The method is quite simple, inexpensive, and time-saving, showing a reproducibility of results which is within $\pm$ 3.0 % for each individual fraction.

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MICRO STARCH GEL ELECTROPHORESIS

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Because of its high resolving power, starch gel electrophoresis according to SMITHIES[1,2] is a widely used method for the separation of proteins. The fact, however, that it takes several hours to run and requires several microliters of sample may be a disadvantage. In the course of a study of newborn-mouse serum, we therefore tried to combine the resolving power of the starch gel method with the speed and the small sample volume of micro agar electrophoresis[3]. The resulting micro starch gel electrophoresis has some characteristics of its own as compared with the original methods.

EXPERIMENTAL

Materials

All reagents were analytical grade and solutions were made in distilled water.

Hydrolysed starch was purchased from the Connaught Medical Laboratories, Toronto, Canada.

Serum was obtained from healthy mice, strain C_{57} Black.

Methods

1. Preparation of the gel

To 50 ml 0.024 *M* boric acid and 0.0096 *M* NaOH, pH 8.4 at 20°, 1.25 times the amount of starch as prescribed for horizontal starch gel electrophoresis is added, and the mixture is heated in a conical flask just short of boiling with constant swirling over a naked flame. Removal of air bubbles under reduced pressure must be very rapid; to get reproducible results with this small volume, standard conditions are even more important than in the original starch gel method.

The gel is now poured on 12 cover slips (24 × 50 × 0.1 mm) as are used for histological slides. These have previously been glued on a glass plate of 25 × 30 × 0.3 cm, using very little petroleum jelly. On the four corners of this plate square pieces of glass, 1 mm thick, are laid. Another glass plate, of the same size as the first, is used as a cover. Over the latter glass plate a sheet of wet, thin cellophane is smoothly stretched. The cover plate, its cellophane covered side facing downwards, is obliquely lowered

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on the gel, as indicated in Fig. 1. Firm pressure is applied to remove superfluous gel, and a weight of about 2 kg is placed upon the cover plate.

After 15 min the cover plate is carefully removed, leaving the cellophane which is subsequently drawn off the gel. The histological cover slips with the overlying gel

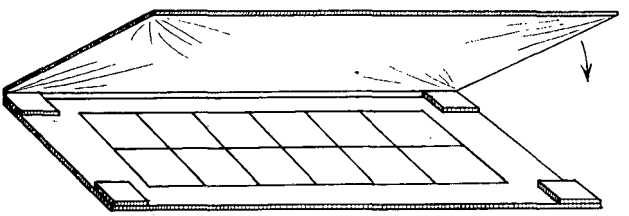

Fig. 1. Diagram of glass plates and cover slips used for the preparation of the gels, as described in the text.

are cut out and removed from the supporting glass plate. These cover slips, each now covered by a 1 mm thick gel layer, may be used immediately or stored in a humid atmosphere for up to 24 h.

2. Introduction of the sample

Two or more rectangular fragments of a razor blade, mounted in a metal block, are used to imprint slits into the gel. The cutting edges and surfaces of these fragments should be absolutely clean and smooth, to prevent irregularities of the slits and of the protein zones.

Undiluted serum is drawn into a thin glass capillary, diameter 0.2 mm. Inserting this capillary into, and subsequently moving it along the slit, will result in the application of the right amount of sample, about 0.2 μl for a 7 mm slit. This procedure requires some experience, it is not feasible to correct excess sample application by blotting since this will adversely affect the electrophoresis results.

3. Electrophoresis

The apparatus used was designed for micro agar electrophoresis by Dr. J. OORT from the Pathological Laboratory of the University of Leyden, to whom we are grateful for permission to copy and to publish this design. For illustrations of the apparatus see Fig. 2. The midcompartment is filled, to a level of 1 cm above the platform, with 1.5 % agar, dissolved by heating in 0.3 *M* boric acid and 0.2 *M* NaOH pH 8.4 at 20°. After cooling, a slot, 3.5 cm wide and with vertical walls, is cut in the agar. The gel bearing cover slips will be placed over this slot. The electrode compartments are filled with the same buffer used for the preparation of the agar. If cathode and anode are interchanged after every run, the electrode buffer must be changed after 10 and the agar after 50 runs.

Pentane is poured into the midcompartment up to 0.5 cm above the agar. A continuous air current, produced by a vacuum pump, gives a nearly constant temperature of 10° when 2 strips are placed in the apparatus. It is recommended to cool the system some minutes before the first run of a series, when the pentane is still warm. 300 V from a stabilized source is applied to the electrodes; the potential in the starch gel is 85 V/cm. The current is 0.25 mA per strip when the apparatus is in thermal equilibrium.

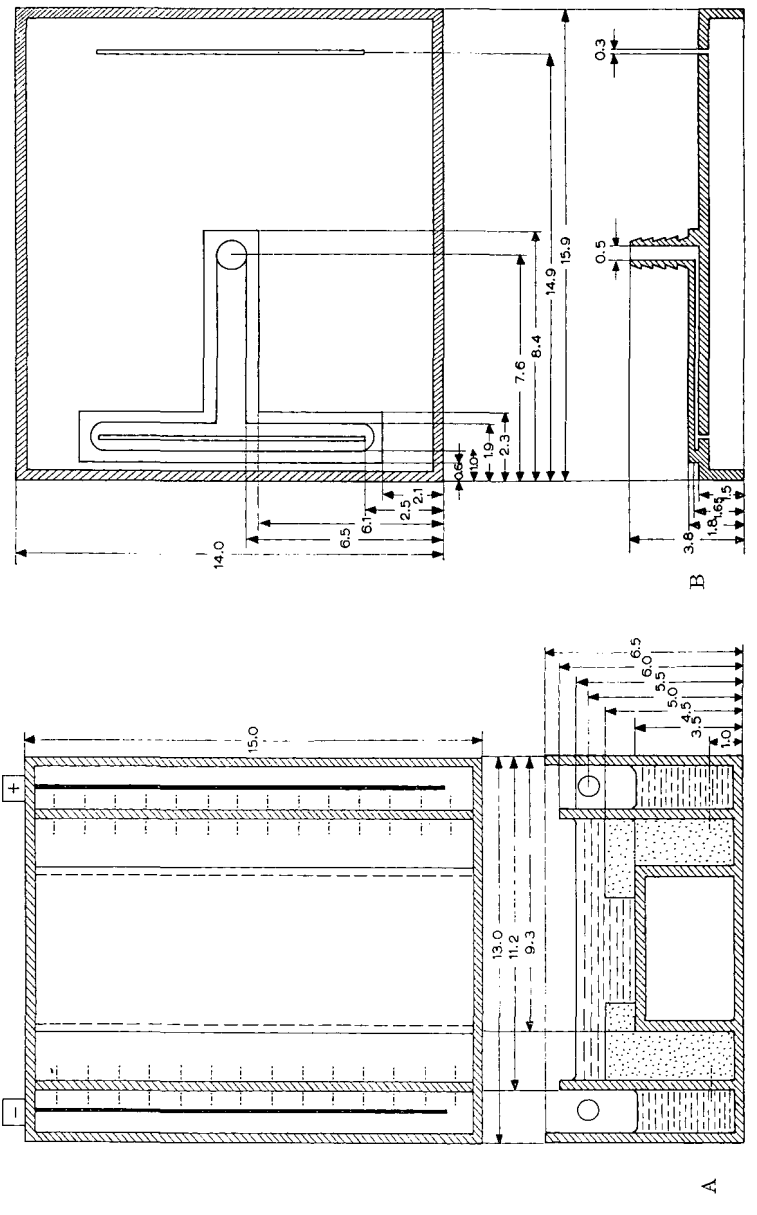

Fig. 2. (A) Electrophoresis apparatus without cover shown from above and in transverse section. Measurements in centimeters. Material is Perspex, 3 mm thick. Mid- and side-compartments are shown filled with agar overlaid with pentane, and electrode buffer respectively. Holes between compartments have a diameter of 1 mm. (B) Cover of the electrophoresis apparatus; *cf.* Fig. 2 (A). Views shown are from above, in longitudinal and in transverse section.

In 15 min a good separation of serum proteins will be achieved. Longer duration of electrophoresis results in less sharply delineated but more widely separated zones, which may be of advantage for scanning of electropherograms.

4. Staining and preserving of the strips

After electrophoresis, the gel strips are removed from the cover glasses and transferred to a saturated solution of Amido Black 10B in methanol–water–acetic acid (50:50:10, v/v).

Staining and washing can be done very conveniently in 5 histological staining trays, the first being filled with the dye solution and the others with the solvent. After staining for 30 sec the gels remain in each of the other trays for 1 min, so that the whole process is completed within 5 min.

The small size of the strips renders it possible to keep a great number of electropherograms in a container filled with solvent. It is, however, also feasible to dry them between cellophane and filter paper under slight pressure. Drying without these precautions leads to curling and opacity of the gels.

RESULTS AND DISCUSSION

The resolution, using this micro starch gel electrophoresis, is equal to that reached by the original vertical macro method. From normal human serum it is possible to obtain 18 protein fractions. The electropherograms also show the same pattern, but the relative distances of the protein zones are slightly different in both methods, as is shown in Fig. 3. This may be due to the different distances travelled by the proteins through the gel, the supposed sieving effect being smaller in the case of micro starch gel electrophoresis.

When applied to sera (*e.g.* rat serum) which contain a large amount of amylase, the micromethod has the advantage that there is practically no breakdown of the starch gel during the short time of electrophoresis and the protein zones are as sharp as with other sera.

When the thickness of the strips is kept constant, the gel concentration required for optimal results seems to be proportional to the potential. 300 Volts, as applied in the described method, requires 1.25 times the concentration used by SMITHIES[1]. A lower concentration gives very diffuse bands and a higher concentration undesirably reduces the mobility of the proteins. It is useless to increase the potential, since the increased heat production will cause an irregular distribution of the protein bands in the different gel layers, as is evident when comparing the two sides of the strips. Unless the strips are to be made translucent by drying, the latter phenomenon may not be very disturbing. In this case one may use histological object slides, which are easier to handle, instead of cover slips. Their greater thickness causes insufficient cooling of the under side of the gel. Consequently, the mobility there will be greater than in upper layers.

The discontinuous buffer system of POULIK[4], as well as the combination of this system with 7 *M* urea[5], can also be used with this micro method. The urea gel takes an hour to harden, and electrophoresis also requires double the time mentioned above. Melting of the agar at the heated contact points with the starch gel can be prevented by using starch gel instead. With this method essentially the same results were ob-

tained with the lens protein, α-crystalline, as were published by BLOEMENDAL *et al.*[6], using the macro method.

In preliminary experiments, the electropherograms obtained by the method described here were found to be suitable for the application of histochemical staining techniques. It has also been found that the strips may be scanned automatically with a Chromoscan from Joyce Instruments, Newcastle-upon-Tyne, England. This apparatus, recording reflected light from the strips, is specially adapted for starch gel electrophoresis. A very narrow split is of course required here.

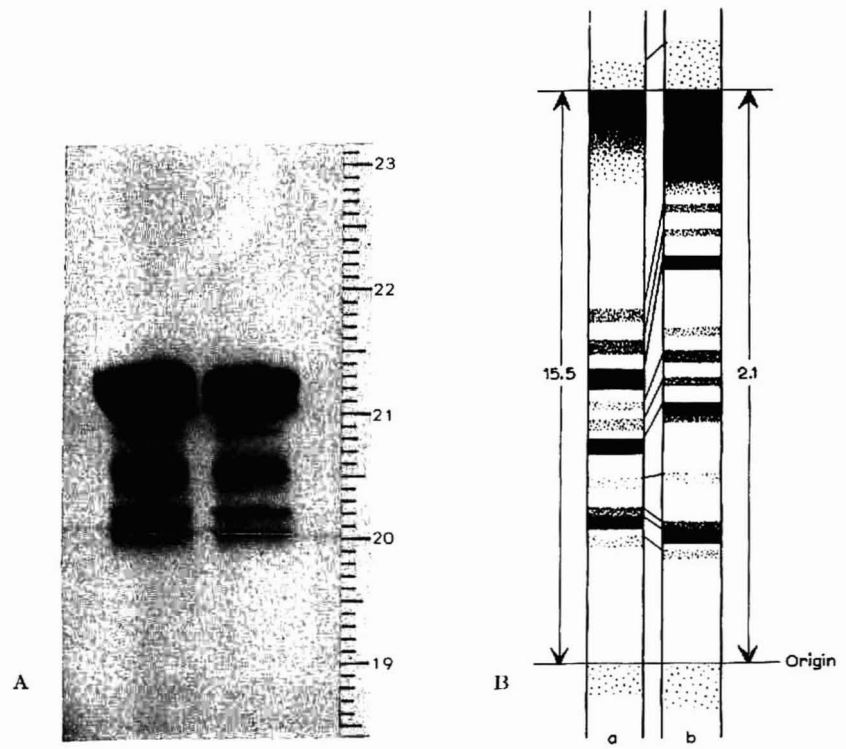

Fig. 3. (A) Electropherogram of mouse serum after micro starch gel electrophoresis. Both slits contained ± 0.2 μl of C_{57}BL male serum. Duration of electrophoresis is 15 min. Staining: Amido Black 10B. Ruling in centimeters. (B) Comparison of electropherograms obtained with (a) macro and (b) micro starch gel electrophoresis. For both electropherograms identical mouse serum was used.

SUMMARY

A micro method for starch gel electrophoresis is described, which has the same resolving power as the original method, but has moreover the following advantages:

(1) It is more rapid, since preparing and hardening of the gel takes 20 min; for serum the electrophoresis takes 15 min, and staining plus washing takes 5 min only.

(2) It requires a very small sample only (0.2 μl for serum).

(3) The gels are small and easy to handle and to store.

ADDENDUM

After the completion of this manuscript, our attention was drawn to the micro electrophoresis method described by MOURAY *et al.* (H. MOURAY, J. MORETTI AND J.-M. FINE, *Bull. Soc. Chim. Biol.*, 43 (1961) 993). This method is intermediary between SMITHIES' macro method and the micro method described above. MOURAY *et al.*, using object glasses, require a migration time of 1 1/2–2 hours and a sample volume of 5–10 μl.

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DETERMINATION OF ADENINE NUCLEOTIDES BY PAPER ELECTROPHORESIS

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INTRODUCTION

When studying the metabolism of phosphorus in red blood cells we had to choose a method which would enable us to combine high specificity with the possibility of studying the incorporation of ^{32}P into individual adenine nucleotides. Paper electrophoresis has been given preference to chromatographical methods of separation, as the latter are less rapid. Paper electrophoresis was first used for the separation of individual nucleotides by MARKHAM AND SMITH[1], who applied high voltage as did a number of subsequent authors[2–5]. This method is quick and yields good results, but because of the risk of working with high voltages and because of complications arising from the necessity of cooling the electropherograms to prevent hydrolysis, we decided in favour of low voltage electrophoresis. Several authors have applied low voltage electrophoresis for the qualitative analysis of nucleotide mixtures. Buffers ranging from pH 2.5 to 11.1 were used[6–18]. The theoretical advantages of using pH 3.5 and pH 5.0 buffers when separating various nucleotides with a different base were pointed out by SMITH[19].

We had, however, to find a most suitable electrolyte for the separation of individual adenine nucleotides differing in the content of phosphoric acid residues. The following factors had to be investigated experimentally:

1. the most suitable buffers,
2. the method of deproteinisation and the most suitable extraction agent,
3. preparation of the sample,
4. elution from the electropherogram,
5. spectrophotometric determination.

EXPERIMENTAL

Conditions of electrophoresis

We examined many buffers mentioned in the literature and the results are summed up in Fig. 1. The best separation was obtained by using citrate buffer. From Fig. 2 it can be seen that the separation of individual adenine nucleotides depends on the pH of the citrate buffer, the best results being obtained in the region of pH 4.0–5.0. Fig. 3 shows the dependence of the resolution of adenine nucleotides on the ionic strength. In the range examined separation was improved by lowering the ionic strength.

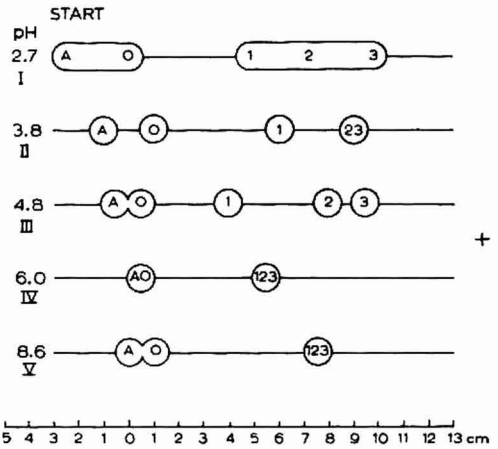

Fig. 1. Electrophoretic separation of adenine nucleotides in different buffers. I. Butyric acid–sodium hydroxide; II. acetic acid–sodium acetate; III. citric acid–sodium citrate; IV. pyridine–acetic acid; V. barbituric acid–sodium barbiturate. A = adenine; O = adenosine; 1 = AMP; 2 = ADP; 3 = ATP.

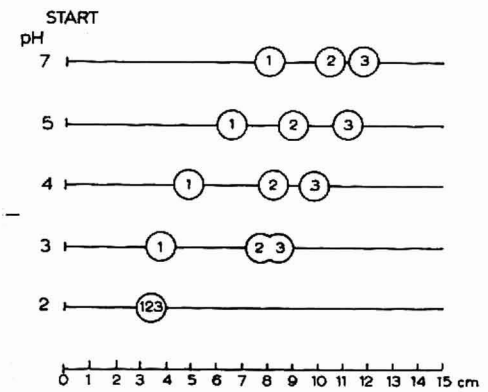

Fig. 2. Electrophoretic separation of adenine nucleotides in citrate buffers of different pH.

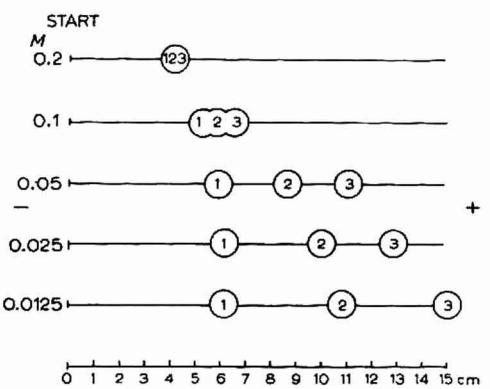

Fig. 3. Electrophoretic separation of adenine nucleotides in citrate buffers of different ionic strength (pH = 4.8).

We chose as optimum conditions: pH 4.8, 0.05 *M* citrate buffer, 3½ hours at room temperature. The separation of an adenine nucleotide mixture under these conditions is shown in Fig. 4. As citrate made chemical detection of inorganic phosphate

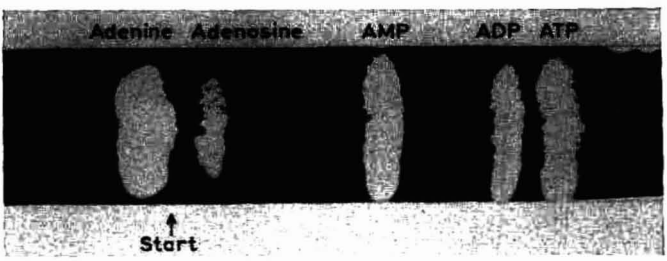

Fig. 4. Photoprint of an electropherogram of adenine nucleotides.

impossible, we used ^{32}P ortho- and pyrophosphate and examined them autoradiographically. It was found that both components migrated much faster than adenosine triphosphate (ATP) and did not interfere with the determination of the specific activity of this nucleotide (Fig. 5).

Preparation of the sample

Both ATP and inorganic phosphate are present in the acid-soluble phosphorus fraction. From the data in Table I it can be seen that extraction with perchloric acid gives a better recovery (by 50 %) than that carried out with trichloroacetic acid, which acid is still used by many authors.

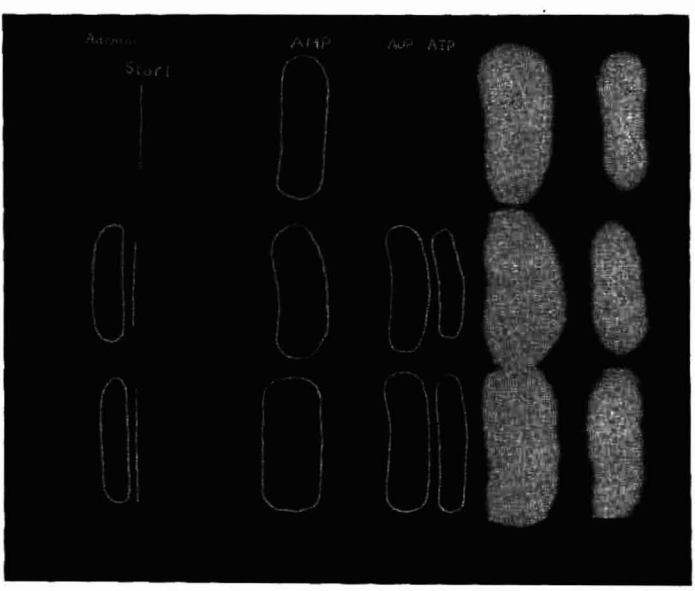

Fig. 5. Autoradiogram of an electropherogram of adenine nucleotides and radioactive ortho- and pyrophosphate. The spots of adenine nucleotides were outlined on the autoradiogram after the electropherogram.

TABLE I

DETERMINATION OF ADENINE NUCLEOTIDES IN HUMAN ERYTHROCYTES BY PAPER ELECTROPHORESIS

Extraction	*ATP (mg%)*	*ADP (mg%)*	*AMP*
Trichloroacetic acid	19.1 ± 5.5	14.7 ± 6.48	—
Perchloric acid	42.6 ± 14.5	10.7 ± 9.5	—

During the application and drying of the sample the concentration of the acid used for the extraction rises to an undesirable degree, causing hydrolysis of a part of the ATP and interfering with the results of the analysis. The excess of the acid must therefore be removed before applying the sample to the origin. Perchloric acid can be precipitated as insoluble potassium perchlorate. Fig. 6 shows that the result of the analysis is influenced by treatment of the sample before its application to the chromatogram.

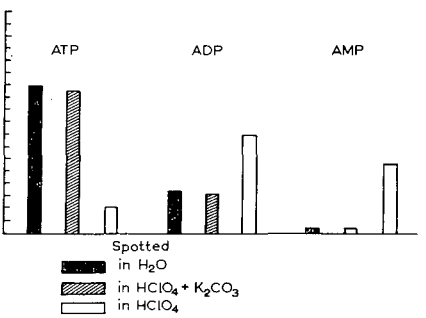

Fig. 6. Electrophoretic analysis of a sample of ATP "Fluka".

Elution

It is not necessary to carry out the elution in a special elution device. The outlined fraction may be cut out and dipped in a test-tube containing the eluent; 4 hours are sufficient for this type of elution.

Determination

The absorption maxima of the purine bases in dilute hydrochloric acid are situated around 257 mμ[20]. We measured the eluates at their maxima, *i.e.* at 257 mμ, and at 290 mμ[21]. The concentration of the nucleotides was calculated from the difference between the extinctions at these wavelengths. In this way better results could be obtained, because contamination caused by the elution of non-specific admixtures from the paper is eliminated.

The recoveries were checked with a series of samples. The results are summed up in Table II. It is obvious that in the range studied the recoveries (approximately 90 %) and the scatter of the values (approximately 3 %) remain practically unchanged. Recoveries of adenosine monophosphate (AMP) added to samples of bloodcells are given in Table III.

TABLE II

MEAN ERROR OF RECOVERY EXPERIMENTS

No.	*100 mg% solution of AMP*		*200 mg% solution of AMP*	
1	86.0		171.6	
2	88.6		171.6	
3	90.0		172.5	
4	90.0		172.8	
5	91.2		173.3	
6	91.2		173.3	
7	91.2	$\bar{x} = 91.4 \pm 2.59$ mg%	178.5	$\bar{x} = 179.7 \pm 5.9$ mg%
8	91.2		180.9	
9	91.5	*i.e.* $\sigma = 2.84$%	182.0	*i.e.* $\sigma = 3.28$%
10	92.4		183.5	
11	92.4		183.5	
12	92.4		183.5	
13	92.4		183.5	
14	92.4		183.5	
15	95.0		183.5	
16	95.0		183.5	
17			184.5	
18			189.5	

TABLE III

RECOVERY OF AMP ADDED TO SAMPLES OF BLOOD CELLS

AMP was added to water (control) and blood to give the final concentration of 150 and 300 μg in 0.1 ml.

Added AMP		*Recovered AMP*			
		Blood		*Control*	
μg	%	μg	%	μg	%
150	100	114	74	132	86
300	100	250.5	83.5	265.12	88.37

RECOMMENDED PROCEDURE

On the basis of the experiments concerning the conditions for electrophoretic determination of ATP the following method for the determination in erythrocytes was elaborated.

Preparation of haemolysate

1 ml of packed erythrocytes was added to 0.8 ml of ice-cold water with a little saponin.

Deproteinisation and extraction

To 1 ml erythrocytes haemolysed in 0.8 ml H_2O with saponin 0.2 ml of 40 % perchloric acid was added immediately after haemolysis.

After centrifugation the supernatant was transferred to a test-tube graduated to

TABLE IV

COMPARISON OF THE RESULTS OBTAINED WITH VARIOUS METHODS FOR DETERMINING ADENINE NUCLEOTIDES

Author	*ATP (μmole/ 100 ml)*	*ADP (μmole/ 100 ml)*	*AMP (μmole/ 100 ml)*	*Sample**	*Method*
PIRWITZ, *et al.*[22]	31–81[52]			B	Colorimetric
OVERGAARD-HANSEN AND JØRGENSEN[23]	51–57	8.3–9.3	0	B	Enzymic
HUGHES JONES[24,25]	63–116	12–25	3–5	RBC	Column chromatography with ion exchangers
	81	21	5	RBC	Column chromatography with ion exchangers
MILLS AND SUMMERS[26]	56.5	7.0	0.7	B	Column chromatography with ion exchangers
MANDEL AND CHAMBON[27]	82.4	14.0	2.1	RBC	Column chromatography with ion exchangers
BISHOP, *et al.*[28]	92.05	10.16	1.32	RBC	Column chromatography with ion exchangers
CARTIER, *et al.*[21]	68	31.5	10	RBC	Paper chromatography
GERLACH, *et al.*[29]	68	30	10	RBC	Paper chromatography
VOGEL[30]	45.8**	17.5**	3.7**	RBC	Paper chromatography
PRANKERD AND ALTMANN[31]	28–88	19–40		RBC	Paper chromatography
BARTLETT, *et al.*[32]	100	26	3	RBC	Paper chromatography
OVERGAARD-HANSEN, *et al.*[33]	116	24	7.5	RBC	Paper chromatography
STRÁNSKÝ, present paper	84.2	25.1	—	RBC	Paper electrophoresis
	42.6**	10.7**	—	RBC	Paper electrophoresis

* B= blood; RBC= red blood cells.
** mg%.

3 ml. The precipitate was washed twice with ice-cold 4% perchloric acid and all the supernatants were pooled in the 3 ml test-tube. A small quantity of potassium hydroxide, calculated to neutralize the perchloric acid used in the extraction and washing, was then added and subsequently the volume was made up to 3 ml.

Electrophoresis

We used a citrate buffer (pH 4.8, 0.05 *M*) and applied a voltage of 200 V for 3½ hours. On the origin 0.1 ml extract was placed. Electrophoresis was carried out in a self-constructed apparatus with horizontally placed electropherograms, which permitted simultaneous analysis of up to 18 samples.

Detection

The detection was carried out in monochromatic short wave U.V.-light, in a self-constructed detection device. The spots were outlined and cut out.

Elution

The individual fractions that had been cut out were eluted with 5 ml 0.1 *N* hydrochloric acid for at least 4 hours.

Determination

The eluates were measured in U.V.-light at wavelengths of 290 and 257 mμ. The difference in absorption at these wavelengths multiplied by a factor gave the concentration of the individual fractions. The results obtained with a series of blood samples are shown in Table I. From Table IV it can be seen that these values correspond to those reported by many authors who used other specific methods for the determination of adenine nucleotides, especially chromatography on ion exchangers.

SUMMARY

1. Low voltage paper electrophoresis in 0.05 *M* citrate buffer of pH 4.8, at a voltage of 200 V, for 3½ hours, is a suitable method for determining adenine nucleotides in erythrocytes.

2. Before applying the sample to the paper it is necessary to remove the excess of deproteinisation agent.

3. By means of the method described 42.6 ± 14.5 mg% ATP and 10.7 ± 9.5 mg % ADP were determined in the erythrocytes.

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PAPER CHROMATOGRAPHIC DETERMINATION OF AROMATIC α-KETO ACIDS

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INTRODUCTION

Several methods concerning paper chromatography of α-keto acids have been published[1], but most of these methods have been worked out only for aliphatic α-keto acids.

Mixtures of free aromatic α-keto acids are inadequately separated by paper chromatography, each acid showing several spots on the chromatogram due to keto–enol tautomerism[2,3] and destructive oxidation by atmospheric oxygen. Paper chromatography of 2,4-dinitrophenylhydrazones of α-keto acids was introduced by CAVALLINI, FRONTALI AND TOSCHI[4], and several modifications of this method for the determination of aliphatic α-keto acids have been published. Multiple spots are, however, often obtained on paper chromatograms of α-keto acid 2,4-dinitrophenylhydrazones owing to the separation of *cis trans* isomers[5,6], and so a quantitative determination of aromatic α-keto acids by these methods was not possible.

3-Alkylquinoxalinols formed by condensation of α-keto acids with *o*-phenylenediamine are stable compounds easy to separate by paper chromatography in alkaline solvent systems, as shown by HOCKENHULL AND FLOODGATE[7]. This principle was adapted by TAYLOR AND SMITH[8] for the determination of aliphatic α-keto acids in blood, using 1,2-diamino-4-nitrobenzene as a 3-alkylquinoxalinol-forming reagent. Owing to the instability of the free aromatic α-keto acids in solution, and to the inertness of these acids in forming quinoxalinols as compared with the aliphatic α-keto acids, the method of TAYLOR AND SMITH is not generally applicable to aromatic α-keto acids.

In the present paper a method for the quantitative determination of phenylpyruvic acid, *o*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and (3-indolyl)-pyruvic acid is described. Decomposition of the free acids by oxidation is avoided by using NaHS as an antioxidant, and the free acids are converted to quinoxalinols by condensation with *o*-phenylenediamine. After paper chromatography the amount of each quinoxalinol is determined by U.V.-spectrophotometry. The validity of the procedure, which may be of importance for the determination of aromatic α-keto acids in urine from patients with abnormal metabolism of phenylalanine, tyrosine and tryptophan[13–16], is indicated by tests with solutions of different α-keto acids of known concentration.

TABLE I

DATA FOR 3-ALKYLQUINOXALINOLS SYNTHETIZED FROM α-KETO ACIDS AND *o*-PHENYLENEDIAMINE

α-Keto acids	*3-Alkylquinoxalinols*	*M.p. (°C)*	*Analysis of the quinoxalinols*					
			% H		*% C*		*% N*	
			Exptl.	*Theor.*	*Exptl.*	*Theor.*	*Exptl.*	*Theor.*
PPA	3-Benzylquinoxalinol (BQ)	202.5	5.26	5.14	76.7	76.8	12.1	11.9
*o*HPPA	3-(*o*-Hydroxybenzyl)-quinoxalinol (*o*HBQ)	230.0	4.60	4.80	71.9	71.5	11.2	11.2
*p*HPPA	3-(*p*-Hydroxybenzyl)-quinoxalinol (*p*HBQ)	246.0	5.06	4.80	71.3	71.5	11.1	11.2
2,5DHPPA	3-(2,5-Dihydroxybenzyl)-quinoxalinol (2,5 DHBQ)	259–260	4.63	4.52	65.2	67.2	9.5	10.5
IPA	3-((3-Indolyl)methyl)-quinoxalinol (IMQ)	232	4.62	4.78	73.4	74.3	15.1	15.3
α-Ketoglutaric acid	3-(β-Propionic acid)-quinoxalinol (βPQ)	273.5						
Pyruvic acid	3-Methylquinoxalinol (MQ)	246.5						

EXPERIMENTAL*

Preparation of reference substances

Aromatic α-keto acids. Phenylpyruvic acid was prepared by hydrolysis of α-acetaminocinnamic acid in 1 *N* HCl[9]; *o*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and 2,5-dihydroxyphenylpyruvic acid were prepared by alkaline hydrolysis of the corresponding azlactone-derivatives[10,11], and (3-indolyl)-pyruvic acid was prepared from tryptophan by the method of SHAW *et al.*[12].

3-Alkylquinoxalinols. A mixture of 10 mmoles α-keto acid dissolved in 20 ml 96 % ethanol and 1.2 g *o*-phenylenediamine dissolved in 20 ml 50 % acetic acid in a stoppered tube is placed in a boiling water bath for 1 h. *o*-Hydroxyphenylpyruvic acid and 2,5-dihydroxyphenylpyruvic acid in their lactone form do not react with *o*-phenylenediamine by this procedure. Opening of the lactone-ring is accomplished by boiling the ethanolic solutions with 15 mmoles NaOH for 5 min under N_2 and neutralizing with 15 mmoles HCl. The precipitated quinoxalinols are filtered off after cooling, washed with 50 % ethanol and crystallized once from 70 % ethanol. The melting points and elementary composition of the 3-alkylquinoxalinols thus prepared are given in Table I.

Reagents for the paper chromatographic analysis

o-Phenylenediamine solution, 5 % (w/v), in water, is prepared just before use (Merck, p.a.).

Sodium hydrogen sulphide solution is prepared by mixing equal parts of 1 *M* aqueous Na_2S solution and 1 *N* HCl (Merck, p.a.). Aromatic α-keto acids in solution

TABLE II

SEPARATION AND DETECTION OF 3-ALKYLQUINOXALINOLS BY PAPER CHROMATOGRAPHY

Solvent systems: S_1 = Methyl ethyl ketone–aq. 0.1 *N* NaOH (20:1, v/v). Used in an atmosphere equilibrated with 5 *N* aqueous NH_3.
S_2 = Methanol–chloroform–aq. 0.1 *N* NaOH (5:5:1, v/v).
S_3 = Ethanol–aq. 0.1 *N* NaOH (10:1, v/v).
S_4 = Ethanol–chloroform–aq. 0.1 *N* NaOH (5:5:1, v/v).
Paper: Whatman No. 20, sprayed with 0.1 *N* NaOH and dried immediately before use (descending technique).
Detection: By U.V. light (main emission about 350 mμ).

Quinoxalinols[a]	$R_F \times 100$				Colour (fluorescence)
	S_1	S_2	S_3	S_4	
BQ	78	78	72	80	light blue
*o*HBQ	60	67	70	62	grey-blue
*p*HBQ	63	44	62	52	dark blue
IMQ	80	69	68	75	yellow
MQ	38	55	61	50	light blue
βPQ	0	8	42	4	light blue

[a] For abbreviations see Table I.

* Abbreviations used: PPA = phenylpyruvic acid; *o*HPPA = *o*-hydroxyphenylpyruvic acid; *p*HPPA = *p*-hydroxyphenylpyruvic acid; 2,5DHPPA = 2,5-dihydroxyphenylpyruvic acid; IPA = (3-indolyl)-pyruvic acid. The abbreviations BQ, *o*HBQ, *p*HBQ, 2,5DHBQ, IMQ, MQ, and βPQ are used for the quinoxalinols, see Table I.

are protected against oxidation by addition of about 1 part of this reagent to 4 parts of the α-keto acid solution.

The solvent systems suitable for one- or two-dimensional paper chromatography of six 3-alkylquinoxalinols are given in Table II.

The chromatography paper, Whatman No. 20 (with a very slow flow rate) is sprayed immediately before use with 0.1 *N* NaOH and dried in a current of air.

Method

The paper chromatographic separation and quantitative determination of PPA, *o*HPPA, *p*HPPA and IPA* as their corresponding 3-alkylquinoxalinol derivatives is carried out as follows:

(1) 10 ml of an aqueous solution or urine specimen containing 0.1–2.0 μmoles of each aromatic α-keto acid and about 1 mmole NaHS is used for each determination.

(2) Immediately before the addition of *o*-phenylenediamine, 6 *N* HCl is added, bringing the H^+ concentration to about 0.15 *M*. 0.5 ml *o*-phenylenediamine solution is now added, and the mixture is heated to 90° for 45 min on a water bath. The reaction should be carried out in a hood because of the formation of H_2S.

(3) After cooling, the precipitated sulphur and impurities are removed by centrifugating, and the supernatant transferred to a glass-stoppered tube for extraction. The precipitate is washed twice with 1 ml methanol–ethyl acetate mixture (1:1) and the washings are added to the supernatant.

(4) The supernatant is extracted 3 times with ethyl acetate (5 ml, 2 ml and 2 ml), and the combined extracts are washed first with 5 ml 1 *M* aqueous sodium hydrogen carbonate solution and then with 3 ml water.

(5) The ethyl acetate phase, containing the quinoxalinols, is evaporated to dryness under reduced pressure in a tube (100 mm × 30 mm ø), the bottom of which is shaped as a reservoir (15 mm × 7 mm ø). The whole of the remainder is transferred to the reservoir by repeated washing of the inner wall of the tube with ethyl acetate, followed by evaporation. The dry residue in the reservoir is dissolved in 200 μl methanol, containing about 3 % ammonia, and an aliquot is transferred to the chromatography paper.

(6) The quinoxalinol mixture is separated by descending paper chromatography (see Table II).

(7) The chromatograms are dried in an air current at room temperature, and the quinoxalinols are made visible in ultra-violet light. The quinoxalinol spots are marked in pencil, cut out, weighed and eluted by rotating gently for 20–30 min in a tube with 5 ml 0.1 *N* NaOH. A paper blank is treated in the same way as the samples. The optical densities of the eluates are measured in a Beckman spectrophotometer at the following wavelengths, corresponding to the light absorption maxima of the quinoxalinols: BQ* and *p*HBQ: 350 mμ; *o*HBQ: 358 mμ; IMQ: 348 mμ.

RESULTS AND DISCUSSION

Paper chromatography of 3-alkylquinoxalinols

Alkaline solvent systems are the most suitable for paper chromatography of the 3-

* See footnote on p. 465.

alkylquinoxalinols, because of the poor solubility of these compounds in acid and neutral aqueous solutions. Several solvent systems were tested, but the best separations of the four quinoxalinols BQ, *o*HBQ, *p*HBQ and IMQ were obtained with the solvent systems given in Table II. The R_F values of MQ and βPQ are also given in Table II, because pyruvic acid and α-keto-glutaric acid are commonly found in blood and urine. Two-dimensional chromatography must be carried out if these six quinoxalinols are to be separated simultaneously.

Chromatography paper with a slow flow rate has proved to be most suitable for the solvent systems given in Table II. The spots tend to streak on chromatograms developed on papers having a faster flow rate. It appears that the treatment of the chromatography paper by spraying with 0.1 *N* NaOH and drying in an air current immediately before use gives the best separations and the most compact spots.

The smallest quantity of a quinoxalinol that can be detected on the paper by U.V.-light after two-dimensional chromatography is about 0.02 μmole when pure quinoxalinols are separated. Fluorescing impurities on the chromatograms may raise the detection limit to about 0.05 μmole, if the quinoxalinols are isolated from urine before chromatography.

Pure 3-alkylquinoxalinols eluted from chromatography paper with 5 ml 0.1 *N* NaOH after one- or two-dimensional chromatography obey Beer's law in the concentration range 0.05–1.00 μmole quinoxalinol per 5.0 ml eluate (E/μmole, see Table III experiment B). The recoveries of the quinoxalinols by chromatography and elution from the paper are also set out in Table III (*cf.* experiments A and B).

Extraction of the 3-alkylquinoxalinols from aqueous solutions

Ethyl acetate is commonly used when extracting aromatic compounds from aqueous solutions. Extraction from urine is often lengthy owing to the formation of a relatively stable emulsion. This is easily avoided by adding 1 ml 10 *N* NaOH to the urine specimen after the quinoxalinol formation (described in point (2) of the method) and heating on a boiling water bath for 5 min. After this alkaline treatment the mixture is neutralized and the quinoxalinols are isolated by extraction and chromatographed as described below.

The quinoxalinol yield by the extraction method is reproducible and independent of the quinoxalinol concentration in the range 0.1–5.0 μmoles per 10 ml aqueous solution (E/μmole, see Table III experiment C).

Quinoxalinol formation

Attempts to use 1,2-diamino-4-nitro-benzene for the formation of 3-alkyl-nitroquinoxalinols with aromatic α-keto acids present at these low concentrations were not successful. On the other hand, the nitroquinoxalinols could be synthetized as reference substances from PPA and *p*HPPA, but not from IPA.

1,2-Diamino-4-methyl-benzene and *o*-phenylenediamine react so readily with the aromatic α-keto acids present at low concentrations, that these compounds could be used as the quinoxalinol-forming reagents in the method here described. Preliminary investigations into the quinoxalinol yield per μmole α-keto acid did not show that either of these reagents offered any advantage over the other and therefore only *o*-phenylenediamine was used in the further development of this method.

It appears from point (2) of the method that the quantity of *o*-phenylenediamine

TABLE III

RECOVERIES OF QUINOXALINOLS AT THE SUCCESSIVE STEPS OF THE PAPER CHROMATOGRAPHIC PROCEDURE

E indicates the extinctions of the quinoxalinols in 5 ml 0.1 *N* NaOH solution (1 cm light path). μmole indicates the quantity of the quinoxalinols or the corresponding α-keto acids. The numbers in parentheses indicate the recoveries in relation to the pure quinoxalinol solution.

Expt.	*Quinoxalinols*	*E*/μmole			
		BQ	*oHBQ*	*pHBQ*	*IMQ*
A	Pure quinoxalinols	1.80 (100 %)	1.60 (100 %)	2.06 (100 %)	1.86 (100 %)
B	Pure quinoxalinols separated by two-dimensional paper chromatography and eluted from the paper (points (6) and (7) in the method)	1.75 (97 %)	1.55 (97 %)	1.87 (91 %)	1.80 (97 %)
C	Pure quinoxalinols extracted from water with ethyl acetate, separated by two-dimensional paper chromatography and eluted (points (4), (5), (6) and (7) in the method)	1.54 (86 %)	1.38 (86 %)	1.69 (82 %)	1.49 (80 %)
D	Quinoxalinols formed from α-keto acids in aqueous solutions and determined by the method (points (1)–(7)) (results from Table IV)	1.03 (57 %)	1.12 (70 %)	1.18 (57 %)	0.86 (46 %)
E	Quinoxalinols formed from α-keto acids in urine and determined by method (points (1)–(7)) (results from Table IV)	1.03 (57 %)	1.16 (72 %)	0.99 (48 %)	0.57 (31 %)

that must be added to the α-keto acid solutions is 10^2 to 10^3 times greater than is stoichiometrically required for the formation of quinoxalinol. Fig. 2 shows the results of an experiment demonstrating how the quinoxalinol yield per μmole of the four aromatic α-keto acids depends on the quantity of the *o*-phenylenediamine added.

The quinoxalinol yield per μmole α-keto acid depends on the concentration of H^+ during condensation. The optimal concentration range is 0.1 to 0.2 *M* H^+ for quinoxalinol formation with *o*HPPA and IPA and 0.1 to 0.5 *M* H^+ with PPA and *p*HPPA.

The reaction temperature of 90° during the formation of the quinoxalinols was arbitrarily chosen in order to maintain the temperature at a constant high level. Increasing the reaction time to more than the recommended 45 min will not increase the quinoxalinol yield per μmole α-keto acid.

A comparison between the results of experiments C, D and E in Table III gives the yield of the quinoxalinols formed from the corresponding α-keto acids, using the method described in the concentration range 0.167 to 2.0 μmoles α-keto acid per 10ml solution. In spite of their low values the yields are reproducible with reasonable accuracy (see Table IV).

Protection of the aromatic α-keto acids against oxidation

Various inorganic reducing compounds (I^-, HSO_3^-, HS^- and HSe^-) were added to the aqueous solutions of the aromatic α-keto acids to prevent their destruction by atmospheric oxygen, but only hydrogen sulphide was found suitable for this purpose.

To demonstrate the stability of PPA, *o*HPPA, *p*HPPA and IPA in aqueous hydrogen sulphide solutions, 0.25 μmole and 1.00 μmole of these acids were added to

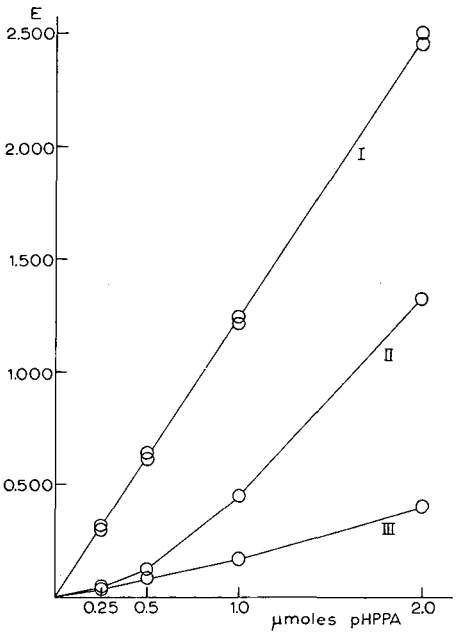

Fig. 1. Calibration curves of *p*HPPA determined as *p*HBQ by paper chromatography. *E* indicates the extinction of *p*HBQ eluted from the chromatography paper by 5 ml 0.1 *N* NaOH. μmole refers to the quantity of *p*HPPA. Curves I, II and III correspond to solutions I, II and III, see text.

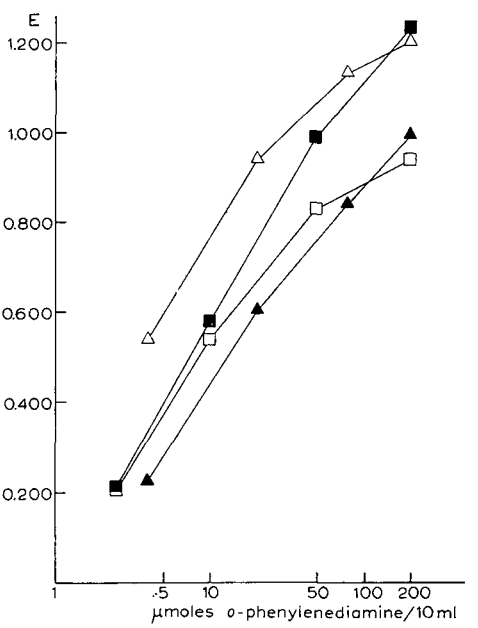

Fig. 2. Relationship between the quantity of quinoxalinol formed and the concentration of *o*-phenylenediamine. *E* indicates the extinction of the quinoxalinols derived from 1 μmole of the corresponding α-keto acids in solutions with different concentrations of *o*-phenylenediamine. ▲—▲, PPA; △—△, *o*HPPA; ■—■, *p*HPPA; □—□, IPA.

10 ml 0.1 *M* NaHS solutions and stored at 0° in a loosely corked tube. The quantities of the α-keto acids were determined by the procedure described in specimens taken during 7 days. The loss of α-keto acid, which gradually increased with time, was about 10 % for PPA, 0 % for *o*HPPA, about 12 % for *p*HPPA and about 16 % for IPA after 7 days.

The antioxidative effect of H_2S during formation of the quinoxalinol is demonstrated by the experiment illustrated in Fig. 1. Different amounts of *p*HPPA were added to three series of aqueous solutions I, II and III. I consisted of 0.1 *M* NaHS solution, II of water almost saturated with N_2 and III of water equilibrated with the atmosphere. The concentration of H^+ was brought to 0.15 *N*, *o*-phenylenediamine was added and the formation and determination of the quinoxalinols was carreid out as described in the method by one-dimensional paper chromatography.

TABLE IV

REPRODUCIBILITY OF THE DETERMINATION OF FOUR AROMATIC α-KETO ACIDS BY ONE- OR TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

Determinations with the same group number are carried out simultaneously. In the determinations numbered 1, 2, 3, 4, 5 and 6 the α-keto acids are added to water, and in determinations numbered 7 and 8 to urine. In determinations 1, 2, 3 and 4 one-dimensional chromatography with solvent system S_1 is used, and in determinations 5, 6, 7 and 8 two-dimensional chromatography with solvent systems S_1 and S_2.

E/μmole is the ratio of the extinction of the 3-alkylquinoxalinols extracted with 5 ml 0.1 *N* NaOH and the quantity of the corresponding α-keto acid added. Each value in columns A represents a duplicate determination. Columns B show the arithmetical mean values of determinations from the same group.

Determination group No.	*Quantity of α-keto acid (μmoles)*	*E*/μmole							
		PPA		*oHPPA*		*pHPPA*		*IPA*	
		A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
1	0.250	1.09	1.05	1.28	1.24	1.27	1.24	0.91	0.91
	1.00	1.01		1.20		1.21		0.92	
2	0.250	1.07	1.06	1.24	1.20	1.27	1.29	0.89	0.89
	1.00	1.05		1.15		1.30		0.90	
3	0.250	1.00		1.16		1.18		0.92	
	0.500	1.00	1.01	1.16	1.15	1.18	1.18	0.95	0.94
	1.00	1.03		1.14		1.18		0.96	
4	0.125	1.03		1.15		1.27		1.08	
	0.250	1.02		1.22		1.24		0.92	
	0.500	0.97	1.01	1.22	1.21	1.27	1.24	0.93	0.95
	1.00	1.02		1.25		1.22		0.91	
	2.00	1.01		1.22		1.24		0.91	
5	0.167	1.04	1.03	1.09	1.12	1.17	1.17	0.89	0.87
	1.667	1.02		1.14		1.16		0.84	
6	0.167	0.99	1.03	1.10	1.12	1.18	1.19	0.86	0.85
	1.667	1.07		1.15		1.19		0.84	
7	0.167	1.07	1.04	1.16	1.15	0.98	0.96	0.57	0.55
	1.667	1.02		1.13		0.93		0.53	
8	0.167	1.08	1.03	1.22	1.16	1.03	1.02	0.61	0.58
	1.667	0.97		1.09		1.01		0.55	

Accuracy of the method

In order to test the accuracy of the method described, aliquots from methanolic stock solutions of PPA, *o*HPPA, *p*HPPA and IPA (10 μmoles/ml) were added to 10 ml samples of water or urine, each containing NaHS in a concentration of 0.1 *M*. The method was carried out on these solutions as described, and the extinction of the eluates was determined. The results expressed as E/μmole are given in Table IV, each value in column A representing the average of a duplicate determination. It appears that the E/μmole values from determinations carried out simultaneously (same group number in Table IV) are independent of the amount of α-keto acid, and that the average values of E/μmole for such experiments can be reproduced with reasonable accuracy, when the paper chromatographic technique used and the solutions to which the α-keto acids are added are identical.

The difference between two single determinations of E/μmole carried out on the same quantity of different α-keto acids does not vary significantly from one keto acid to another or from determination to determination carried out in the same manner. A common standard error on single determinations of E/μmole is therefore calculated for determinations of the four α-keto acids added in the same quantity. The standard errors on single determinations of E/μmole in experiment 1,2, 3 and 4 (Table IV) are 0.04/μmole when the quantity of α-keto acid is 0.25 μmole and 0.02/μmole when the quantity is 1.00 μmole. In experiments No. 5 and 6 the standard errors on single determinations of E/μmole are 0.04/μmole and 0.02/μmole and in experiments No. 7 and 8 they are 0.06/μmole and 0.03/μmole, when the quantities of α-keto acids are 0.167 μmole and 1.667 μmoles respectively.

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SUMMARY

A paper chromatographic method is described for the quantitative determination of phenylpyruvic acid, *o*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and (3-indolyl)-pyruvic acid in aqueous solutions or urine. The α-keto acids, protected against oxidation by sodium hydrogen sulphide, react with *o*-phenylenediamine to form 3-alkylquinoxalinols. The 3-alkylquinoxalinols are separated by paper chromatography, eluted from the paper, and the quantities determined by U.V.-spectrophotometry. The successive steps of the method are discussed, and the accuracy of the method is demonstrated by tests with solutions of α-keto acids of known concentration.

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STRUCTURAL AND CHROMATOGRAPHIC CORRELATIONS OF SOME FLAVONOID COMPOUNDS

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INTRODUCTION

The contributions of correlations between structure, chromatographic behaviour, and reactivity to chromogenic spray reagents towards predicting the structures of those flavonoid compounds which are derived from plant sources, was shown previously by ROUX AND MAIHS[1], and ROUX, MAIHS AND PAULUS[2]. Such work is now extended by similar comparison of wider groups of flavonoid analogues of established absolute configuration.

EXPERIMENTAL AND RESULTS

Origin of substances

(+)-Leuco-fisetinidin [(+)-7,3′,4′-trihydroxyflavan-3,4-diol] from the heartwood of *Acacia mearnsii* (formerly *A. mollissima*[3]) was hydrogenated with palladium catalyst under conditions established by WEINGES[4] to (—)-fisetinidol [(—)-7,3′,4′-trihydroxyflavan-3-ol][5]. (+)-Fustin [(+)-3,7,3′,4′-tetrahydroxyflavan-4-one] also from the heartwood of *A. mearnsii*[6] was converted to (—)-butin [(—)-7,3′,4′-trihydroxyflavan-4-one][7] by hydrogenolysis using conditions similar to those described by PEW[8]. The flavonone was reduced to (+)-7,3′,4′-trihydroxyflavan-4-ol by catalytic hydrogenation with platinum[7], and to (—)-7,3′,4′-trihydroxyflavan by catalytic hydrogenation with palladium[9]. Fisetin was isolated from the heartwood of *Rhus glabra*[6], and butein was synthesised.

(+)-Leuco-robinetinidin [(+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol] was isolated from the heartwood of *Robinia pseudacacia*[4,10], and (—)-robinetinidol [(—)-7,3′,4′,5′-tetrahydroxyflavan-3-ol] from the bark of *A. mearnsii*[11]. (+)-Dihydrorobinetin [(+)-3,7,3′,4′,5′-pentahydroxyflavan-4-one] was obtained from *R. pseudacacia* heartwood in partly racemized form[12], and resolved into the optically pure (+)-form by chromatographic methods[7]. This flavanonol was converted successively to (—)-robtin [(—)-7,3′,4′,5′-tetrahydroxyflavanone], (+)-7,3′,4′,5′-tetrahydroxyflavan-4-ol, and (—)-7,3′,4′,5′-tetrahydroxyflavan by the same conversions as outlined for (+)-fustin[7,9]. Robinetin was isolated from *R. pseudacacia*[13], while robtein was synthesised[7].

2,4,4′-Trihydroxychalcone and (±)-liquiritigenin [(±)-7,4′-dihydroxyflavan-4-one] were synthesised[14], and the latter converted to (±)-7,4′-dihydroxyflavan-4-ol by catalytic hydrogenation with platinum[15]. 7,4-Dihydroxyflavonol was also synthesised[15].

Chromatographic methods

The pure substances were applied within the concentration range 10–15 μg on Whatman No. 1 chromatographic paper and the chromatograms mounted on stainless steel frames. Chromatograms were developed with 2 % acetic acid by upward migration to a point 12 in. (30.5 cm) from the starting line over about 6 h. After drying they were sprayed with the reagents described before[1], and also with those described below. R_F values (Table I) were calculated to an average of three values in the presence of (+)-catechin (R_F 0.35) and (+)-gallocatechin (R_F 0.32) as reference compounds.

Other chromatograms were prepared as above but developed in water-saturated butan-1-ol (*n*-butanol) and in butan-1-ol–acetic acid–water (6:1:2, v/v) by upward migration to points about 10 in. (25.4 cm) from the starting lines. R_F values in each solvent systems were calculated as above (Table II).

Spray reagents

(*a*) *Quinonechloroimides.* 2,6-Dichloroquinonechloroimide (2 %) in absolute ethanol was sprayed evenly and after allowing for the evaporation of the alcohol the chromatograms were fumed with ammonia. Blue or mauve colorations were developed by flavans, flavan-3-ols, flavan-4-ols, flavan-3,4-diols, flavanones and flavanonols of the "resorcinol series" with catechol B nuclei. Those with pyrogallol B nuclei gave spots with pale yellow centres and blue edges, changing slowly to brown on aging. In all cases the colorations were developed rapidly with flavan-4-ols and flavan-3,4-diols, and usually, although not in all instances, more slowly by the other types of flavonoids. (+)-Catechin (catechol grouping) (blue) and (+)-gallocatechin (pyrogallol grouping) (blue with yellow centre) also behave consistently.

Those flavonoids examined with a single hydroxyl group on the B nucleus (4′-hydroxyl) gave weaker colours, exceptions being the flavan-4-ol (mauve) and the flavan-3,4-diol (7,4′-dihydroxyflavan-3,4-diol)[15,16] (mauve).

2,6-Dibromoquinonechloroimide and unsubstituted quinonechloroimide give similar colours, but the 2,6-dichloro- and 2,6-dibromo-derivatives are preferred as the colours developed are more intense and the shades of blue and violet more characteristic than with the unsubstituted quinonechloroimide.

(*b*) *Erlich's reagent. p*-Dimethylaminobenzaldehyde (2 %) in 2 *N* hydrochloric acid[17] shows an immediate pink in the cold (15–20°) with (+)-catechin and (+)-gallocatechin, but not with flavonoids containing resorcinol A nuclei. However, amongst the latter group, flavans develop an intense purple after 10–15 min and flavan-3-ols the same colour after 15–20 min. Thereafter flavan-4-ols develop a blue-purple and flavan-3,4-diols a pink, presumably due to the presence of hydrochloric acid in the reagent. These chromatograms were not heated.

(*c*) *p-Toluenesulphonic acid.* With catechol- and phloroglucinol-containing flavan-4-ols this reagent gives an intense blue-purple fading to a light pink at 80°. A stable violet is given by 7,4′-dihydroxyflavan-4-ol.

DISCUSSION

Conclusions which may be drawn from the chromatographic behaviour of the wider range of flavonoid nuclei studied above, reaffirm the earlier deductions by ROUX, MAIHS AND PAULUS[2] regarding the effect of the position of hydroxylation on R_F in

aqueous medium. These flavonoid nuclei contain three types of hydroxyl groups: (a) phenolic, occupying the 5,7,3′,4′ and 5′-positions, (b) benzylic in the 4-position, and (c) aliphatic in the 3-position. In 2 % acetic acid ("adsorptive system") substitution of phenolic hydroxyls at 3′ and 5′ cause small reductions in R_F (compare the three groups of analogues in Table I), whereas substitution in the 5-position has been shown to cause large reduction[2]. The *p*-hydroxybenzyl hydroxyl at C-4 causes small increases in R_F (compare flavans with flavan-4-ols, ΔR_F +0.01 to +0.03, and flavan-3-ols with flavan-3,4-diols, ΔR_F +0.04 to +0.07, in Table I), whereas the truly aliphatic 3-hydroxyl is responsible for large increases (compare flavans with flavan-3-ols, ΔR_F +0.16 and flavan-4-ols with flavan-3,4-diols, ΔR_F +0.17 to +0.22, in Table I). The effect of the 4-hydroxyl groups on R_F in 2 % acetic acid is, therefore,

TABLE I

R_F VALUES OF STEREOCHEMICALLY RELATED FLAVONOID ANALOGUES IN 2 % ACETIC ACID

Flavonoid type	R_F*		
	Pattern of phenolic hydroxylation		
	7,3′,4′,5′ ($R = R' = OH$)	7,3′,4′ ($R = OH$, $R' = H$)	7,4′** ($R = R' = H$)
(—)-Flavan (I)	0.26	0.32	
(—)-Flavan-3-ol (II)	0.42	0.48	
(+)-Flavan-4-ol (III)	0.27	0.35	0.44
(+)-Flavan-3,4-diol (IV)	0.49	0.52	
(—)-Flavanone (V)	0.19	0.22	0.29
(+)-Flavanonol (VI)	0.35	0.38	

* R_F values are in relation to (+)-catechin (0.35) and (+)-gallocatechin (0.32).
** These compounds were racemates, the R_F of the flavan-3,4-diol indicated, being of the forward running enantiomer.

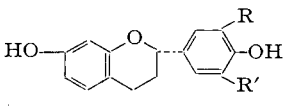
TABLE II

R_F VALUES OF STEREOCHEMICALLY INTERRELATED FLAVONOIDS AND THEIR FLAVONOL AND CHALCONE ANALOGUES IN WATER-SATURATED BUTAN-1-OL AND BUTAN-1-OL–ACETIC ACID–WATER (6:1:2, v/v)

Flavonoid type	R_F*		
	Pattern of phenolic hydroxylation		
	7,3,′4′,5′ ($R = R' = OH$)	7,3′,4′ ($R = OH$, $R' = H$)	7,4′** ($R = R' = H$)
(—)-Flavan (I)	0.77 (0.80)	0.88 (0.88)	
(—)-Flavan-3-ol (II)	0.59 (0.69)	0.76 (0.79)	
(+)-Flavan-4-ol (III)	0.50 (0.60)	0.72 (0.78)	0.92 (0.91)
(+)-Flavan-3,4-diol (IV)	0.37 (0.47)	0.61 (0.65)	
(—)-Flavanone (V)	0.74 (0.81)	0.84 (0.86)	0.89 (0.91)
(+)-Flavanonol (VI)	0.59 (0.69)	0.76 (0.79)	
Flavonol	0.42 (0.45)	0.70 (0.71)	0.90 (0.88)
Chalcone	0.50 (0.61)	0.82 (0.84)	0.90 (0.91)

* R_F values for water-saturated butan-1-ol are indicated first, and those for butan-1-ol–acetic acid–water are in parentheses. R_F values are in relation to (+)-catechin 0.55 (0.65) and (+)-gallocatechin 0.35 (0.45).
** The flavan-4-ol and flavanone of this group are racemates.

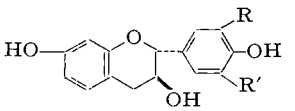
consistent with their properties which are intermediate between the most weakly dissociated phenolic hydroxyls (acidic due to partial dissociation in water) and neutral aliphatic hydroxyls (undissociated). Apart from the nature of the hydroxyl groups, their relative position on the C_{15} skeleton must also exert a large influence on chromatographic behaviour, as shown for the phenolic hydroxyls[2].



(I)

R=OH, R′=H. (—)-7,3′,4′-trihydroxyflavan

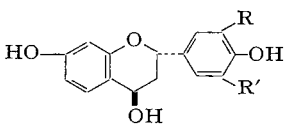
R=R′=OH. (—)-7,3′,4′,5′-tetrahydroxyflavan



(II)

R=OH, R′=H. (—)-fisetinidol

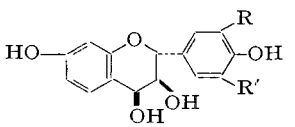
R=R′=OH. (—)-robinetinidol



(III)

R=OH, R′=H. (+)-7,3′,4′-trihydroxyflavan-4-ol

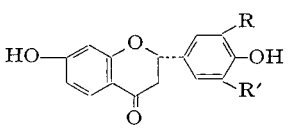
R=R′=OH. (+)-7,3′,4′,5′-tetrahydroxyflavan-4-ol



(IV)

R=OH, R′=H. (+)-leuco-fisetinidin

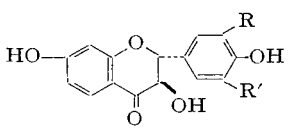
R=R′=OH. (+)-leuco-robinetinidin



(V)

R=OH, R′=H. (—)-butin

R=R′=OH. (—)-robtin



(VI)

R=OH, R′=H. (+)-fustin

R=R′=OH. (+)-dihydrorobinetin

Perhaps the most interesting of the comparisons in Table I are the large differences in R_F between flavans and flavanones of the same (2S) absolute configuration[7,9]. The flavanones in each instance show a lower R_F than the flavan analogue (ΔR_F —0.07 to —0.10) in 2% acetic acid, and the same conclusion may be reached[2] by comparison of the flavanonols (2R:3R configuration) with the stereochemically related flavan-3-ols (2R:3S configuration) (ΔR_F —0.07 to —0.10). This reduction of R_F due to a carbonyl group at C-4 is difficult to explain, the proton-accepting carbonyl group, capable of forming strong hydrogen bonds with water, should, for example, not reduce the solubility of the flavonoid nucleus appreciably. Furthermore, modern stereochemical concepts suggest that flavans and flavanones have similar conformations of the heterocyclic ring system[18], and differences in their affinity for cellulose, related in this instance also to their solubility in water[2], are therefore unlikely to be attributable to differences in molecular shape.

By comparison, in water-saturated butan-1-ol (Table II), there exist remarkable agreements between the R_F values of flavans and flavanones (ΔR_F 0.03–0.04) and of flavan-3-ols and flavanonols (ΔR_F 0.00) for each group of analogues. However, flavan-3-ols show consistently higher R_F values than flavan-4-ols (ΔR_F 0.04–0.09). Similar comparison of the R_F values of flavans with flavan-3-ols, flavan-4-ols, and flavan-3,4-diols within each group shows that individual reductions of R_F introduced by the 3- and 4-hydroxyls are almost additive in the flavan-3,4-diols. Flavonols have lower R_F values than the corresponding flavanonols (dihydroflavonols) (ΔR_F —0.06 to —0.17) due to affinity effects or low solubility[2] resulting from the planar nature of the flavonol molecule. For the same reason chalcones which are isomeric with flavanones, have lower R_F values, the additional hydroxyl in chalcones (2-position) being strongly bonded with the carbonyl group. Apart from these anomalies, increase of the degree of hydroxylation of the flavan and flavanone groups always leads to reduction in R_F, the interval in water-saturated butan-1-ol being somewhat less regular than in the butan-1-ol–acetic acid–water (6:1:2, v/v) mixture (Table II).

R_F values of the 7,4′-dihydroxy analogues are apparently too high to permit accurate differentiation of similar effects, the comparative values in Table II showing that the magnitude of the above R_F differences decrease with decrease in the degree of phenolic hydroxylation, in both "partitioning" systems.

The spray reagent 2,6-dichloroquinonechloroimide has been used for the chromatographic location of polyhydric phenols[19], and flavan-3,4-diols[20], while unsubstituted quinonechloroimide was used for estimation of benzyl alcohol groups in lignins although the reaction was not specific[21]. For the flavonoids examined, the reaction is similarly not specific, but with those containing *p*-hydroxybenzyl alcohol groups, namely flavan-4-ols and flavan-3,4-diols, the blue develops rapidly and is intense. This reagent and *p*-toluenesulphonic acid are particularly useful for locating flavan-4-ols and flavan-3,4-diols[15] of the resorcinol-phenol group, which possess no phenolic *ortho*-hydroxy groups. These give no reaction with ferric alum, and their reactions with ammoniacal silver nitrate and bisdiazotized benzidine are exceptionally weak.

Erlich's reagent, known to give reactions with free resorcinol nuclei[17,22] may be used to differentiate between flavan-3-ols of the "resorcinol" and "phloroglucinol" series, although this differentiation is achieved almost more effectively with bisdiazotized benzidine and vanillin–*p*-toluenesulphonic acid sprays[1,23]. The slow development of mauves by flavans and flavan-3-ols of the "resorcinol series" with Erlich's reagent may be due to hydrolytic fission of the heterocyclic ring to give "free" resorcinol nuclei.

ACKNOWLEDGEMENT

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SUMMARY

The chromatographic behaviour of flavan, flavan-3-ol, flavan-4-ol, flavan-3,4-diol, flavanone, flavanonol, flavonol and chalcone analogues of robinetinidin and fisetinidin chlorides in 2 % acetic acid, water-saturated butan-1-ol, and in butan-1-ol–acetic

acid–water (6:1:2, v/v), is discussed. Similarly, the diagnostic value of quinonechloroimides, Erlich's reagent, and *p*-toluenesulphonic acid as spray reagents for flavonoids is examined.

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DIE ANWENDUNG PAPIERCHROMATOGRAPHISCHER METHODEN ZUM TOXIKOLOGISCHEN NACHWEIS VON ARZNEIMITTELN

III. ÜBER DAS PAPIERCHROMATOGRAPHISCHE VERHALTEN EINIGER BASISCHER ARZNEIMITTEL IM ZUSAMMENHANG MIT IHRER KONSTITUTION

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EINLEITUNG

Beim Studium einiger neuen Identifikationsmethoden für Antihistaminika und Ataraktika mit Hilfe der Papierchromatographie wurde im Umkehrphasensystem Petroleum/Äthanol–Wasser–Ammoniak gearbeitet. Es wurde festgestellt, dass die R_F-Werte dieser Arzneimittel im hohen Masse von der Zusammensetzung der durchfliessenden Phase abhängig sind, wobei die R_F-Werte der Präparate mit tertiärem Stickstoff mit zunehmendem Äthanolgehalt in der mobilen Phase ansteigen, während bei quartären Basen unter gleichen Bedingungen ein R_F-Wert-Abstieg festgestellt wurde[1].

Es war nun interessant, inwieweit diese Gesetzmässigkeit auch für andere basische Verbindungen gültig ist, und ob dieses System allgemein für synthetische basische Arzneimittel verwendbar ist, denn viele von ihnen können nicht in sauren, für Alkaloide benutzten, alkoholischen Systemen wegen ihrer hohen R_F-Wert identifiziert oder aus Gemischen von einander getrennt werden.

Zu diesem Zwecke wurden 30 Arzneimittel aus der Gruppe der Spasmolytika, Analgetika, Antiparkinsonpräparate, Psychotherapeutika und anderer ausgesucht, die in der Therapie häufig verwendet werden und deren Identifikation für die Toxikologie von praktischer Bedeutung ist.

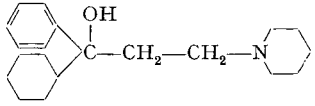
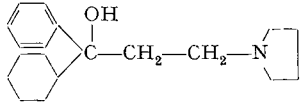
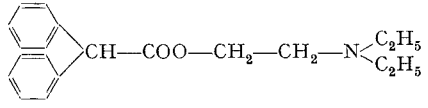
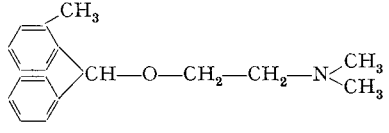
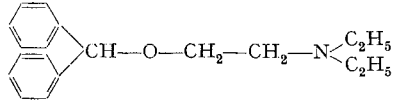
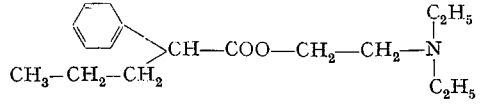
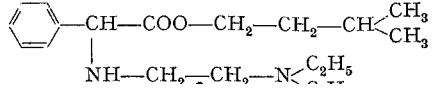
Von den angeführten Arzneimitteln besitzen 20 Verbindungen tertiären (oder sekundären) und 10 Präparate quartären Stickstoff im Molekül. In der ersten Spalte der Tabelle I sind die Namen derjenigen Präparate angeführt, die getestet wurden, und in der dritten Spalte ihre Synonyma. Die in Kursivdruck angeführten Namen wurden von der Weltgesundheitsorganisation als nicht wortgeschützte vorgeschlagen[2].

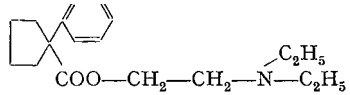
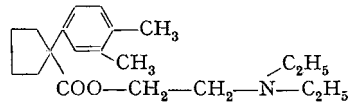
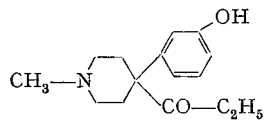
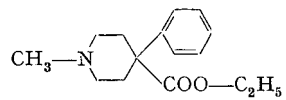
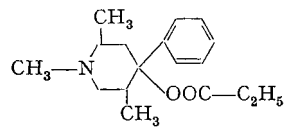
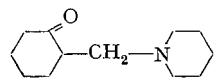
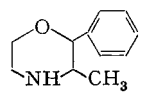
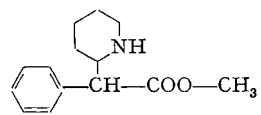
Mit Absicht wurden einige strukturell ähnliche Verbindungen ausgesucht, um die Trennmöglichkeit dieses Systems und die Bedingungen des besten Aufteilens für Stoffe ähnlicher Konstitution oder mit einem annähernd gleichen R_F-Wert feststellen zu können.

* Direktor: Prof. Dr. K. KÁCL.

TABELLE I

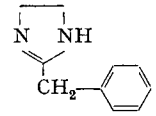
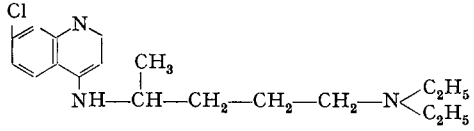
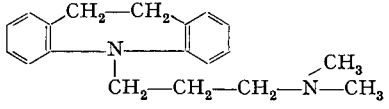
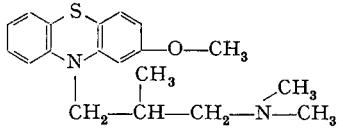
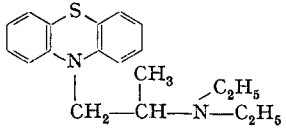
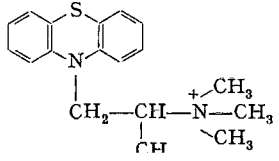
R_F-WERTE VON 30 BASISCHEN ARZNEIMITTELN

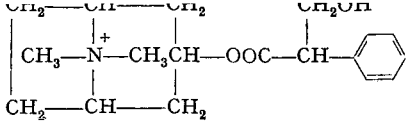
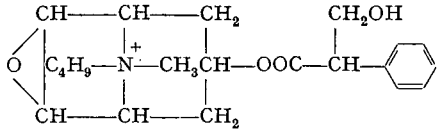
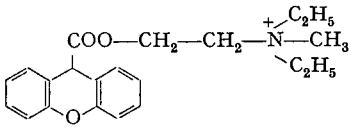
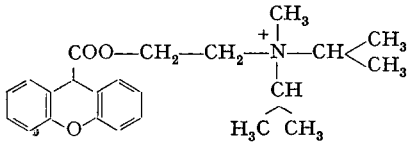
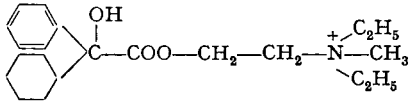
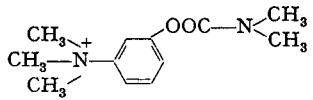
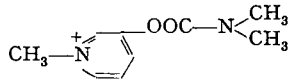
Bezeichnung	*Strukturformel*	*Synonyma*	*R_F-Werte in den mobilen Phasen*						
			B	*A*	*1*	*2*	*3*	*4*	*5*
Artan		*Trihexyphenidylhydrochlorid*, Paralest, Triesifenidile, Pargitan, Pipanol, Peragit, Parkopan, Win 511			0.05	0.09	0.16	0.29	0.47
Kemadrin		*Procyclidinehydrochlorid*, Metanin, Prociclidina, Lilly 14045			0.08	0.14	0.24	0.40	0.55
Trasentin		*Adiphenin*, Diphacil, Sentiv, Patrovina, Solvamin AB, Spasmolytin, Vagospasmyl			0.08	0.14	0.26	0.44	0.59
Disipal		*Orphenadrine*, Mephenamine, Brocadisipal, B.S.5930			0.24	0.41	0.57	0.66	0.74
Antiparkin		Etanautine, Nautamine, Rigidyl, S-45, PKM-Tabletten			0.12	0.21	0.37	0.52	0.64
Prospasmin		Episton, Propivane, Tropiston			0.14	0.27	0.44	0.56	0.70
Avacan		Acamylophenin, Novospasmina			0.24	0.41	0.58	0.68	0.76

Parpanit		*Caramiphen*, Panparnit, Pentaphen			0.13	0.23	0.40	0.54	0.63
Netrin		*Dimethylcaramiphen*, Metcaraphen, 3012G			0.09	0.16	0.28	0.45	0.59
Cliradon		*Cetobemidon*, Cymidon, K4710, *Ketobemidon*, Ketogan, Ciba 7115, Ketogin, Hoechst 10720, A21 Lundbeck, Win 1539	0.88	0.88	0.88	0.88	0.86	0.85	0.84
Dolsin		*Pethidin*, Adolan, Antidol, Demerol, Dolantin, Dolin, Meperidin, Narcofor, Lidol, Penthenal, Piperosal	0.23	0.40	0.61	0.75	0.79	0.81	0.81
Promedol		*Trimeperidinehydrochlorid*	0.13	0.27	0.49	0.68	0.76	0.81	0.81
Spiractin		Na 66	0.34	0.40	0.50	0.63	0.67	0.71	0.72
Fenmetrazin		*Phenmetrazine*, Preludin, Oxazimédrine, Anorex, A 66, Fenmetrazina cloridrato, Delgacerol	0.82	0.82	0.82	0.82	0.82	0.82	0.82
Ritalin		*Methylphenidatehydrochlorid*, Centedrin, Rilatin, C 4311, Metilfenidato Cloruro	0.33	0.48	0.64	0.75	0.80	0.80	0.80

(Fortsetzung S. 482)

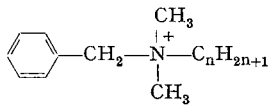
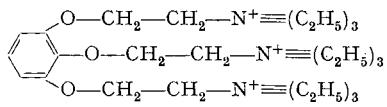
TABELLE I (*Fortsetzung*)

Bezeichnung	*Strukturformel*	*Synonyma*	*R_F-Werte in den mobilen Phasen*						
			B	*A*	*1*	*2*	*3*	*4*	*5*
Divascol		*Tolazolin*, Artonil, Benzolin, Diladon, Pridazol, Priscol, Prodil, Tolpal, Vasodilatin, Vasimid, Benim, Cyclocol	0.80	0.80	0.80	0.80	0.78	0.77	0.72
Resochin		*Chloroquine*, Aralen, Imagon, Avlochlor, Gontochin, Tanakan, Tresochin, Trochin, Iroquine, Sanoquin, W 7618	0.36	0.60	0.79	0.87	0.88	0.89	0.89
Tofranil		*Imipramine*, Imidobenzyle, G 22355			0.13	0.23	0.40	0.54	0.67
Nozinan		*Levopromazine*, Neozine, Methotrimeprazine, 7044 R.P.			0.11	0.18	0.34	0.49	0.60
Rodipal		*Profenaminehydrochlorid*, Dibutil, Ethopropazin, Parcidol, Parfezin, Parsitan, Isothiazin, 3356 R.P.			0.05	0.09	0.16	0.28	0.43
Padisal		Multergan, Multezin, Thiazinamium, Thiazinamon, Thiozinamin, 3554 R.P.			0.84	0.82	0.80	0.76	0.64

Eumydrin		*Atropinmethylnitrat*, Europen, Harvatrate, Methylon, Metropine	0.80	0.77	0.75	0.66	0.44
Buscopan		Bulamin, Buscapina, Buscopin, SKF 1637, Verbindung 1637	0.84	0.81	0.80	0.75	0.63
Banthin		*Methanthelin-Bromid*, Asabine, Avagal, Doladene, Metantyl, Pepulsan, Ulcuwas, Ulkophob, Vagamin, Vaganthin, Asatylon	0.84	0.83	0.82	0.77	0.68
Pro-Banthin		*Propanthelinbromid*, Pervagal, Neo-Metantyl, SC 3171	0.85	0.84	0.83	0.79	0.73
Antrenyl		*Oxyphenoniumbromid*, Ba-5473, Oxyfenon	0.87	0.86	0.84	0.83	0.76
Syntostigmin		*Neostigmin*, Eustigmin, Myastigmin, Neo-Eserin, Proserin, Prostigmin, Stiglin, Vasostigmine	0.81	0.77	0.74	0.66	0.45
Mestinon		*Pyridostigmin*, Ro 1-5130	0.78	0.74	0.69	0.57	0.34

(Fortsetzung S. 484)

TABELLE I (*Fortsetzung*)

Bezeichnung	*Strukturformel*	*Synonyma*	*R_F-Werte in den mobilen Phasen*						
			B	*A*	*1*	*2*	*3*	*4*	*5*
Zephirol					0.89	0.89	0.89	0.89	0.86
Flaxedil		*Gallamin*, Benzcurin, Tricuran, Parexyl, Relaxan, Remyolan, Syntubin, Retensin, Sincurarin, 3697 R.P.			0.77	0.71	0.64	(0.28)	(0.13)

EXPERIMENTELLER TEIL

Die Testlösungen wurden in Konzentrationen von 2 mg/ml durch Lösen der Salze der Verbindungen in Äthanol, bzw. in wässerigem Äthanol hergestellt.

Es wurden fünf Grundfliessmittelgemische (die mobilen Phasen 1–5) und zwei Ergänzungsfliessmittelgemische (die durchfliessenden Phasen A und B) aus Äthanol, Wasser und konz. Ammoniak in verschiedenen Verhältnissen vorbereitet (Tabelle II). Zur Herstellung wurde absolutes Äthanol benutzt.

TABELLE II

ZUSAMMENSETZUNG DER MOBILEN PHASEN

Mobile Phasen	*Volumenteile*		
	Äthanol	*Wasser*	*Ammoniak*
B	35	63	2
A	45	53	2
1	55	43	2
2	65	33	2
3	75	23	2
4	85	13	2
5	95	3	2

Die Testlösungen wurden auf das chromatographische Papier Whatman No. 1 in Mengen von 20–40 μg aufgetragen und die Papierbögen mit Petroleum (Fraktion 195–220°) imprägniert. Das Petroleum wurde mit Petroläther im Verhältnis 2:3 verdünnt. Die überschüssige Petroleumlösung wurde zwischen zwei Stücken Filterpapier abgepresst. Die auf diese Weise vorbereiteten Chromatogramme wurden in mit den zugehörigen durchfliessenden Phasen gesättigten Kammern absteigend auf einer Laufstrecke von 30 cm entwickelt. Die chromatographischen Gefässe wurden zum Schutz gegen Temperaturveränderungen mit Polystyrenplatten umhüllt. Es wurde bei einer konstanten Temperatur von 17 $\pm$ 0.5° gearbeitet. Die Stoffe, deren R_F-Wert von der Temperatur beeinflusst waren, wurden noch in einer weiteren Versuchserie bei einer Temperatur von 12–13° getestet.

Die Entwicklungsdauer entspricht derjenigen der ersten Mitteilung. Die Chromatogramme wurden durch einen warmen Luftstrom getrocknet und die Flecke der Basen durch Besprühen mit Jodoplatinat sichtbar gemacht. Die angeführten R_F-Werte (Tabelle I) stellen Mittelwerte aus 9–20 Versuchen dar.

ERGEBNISSE UND DISKUSSION

1. R_F-Werte

(*a*) *Einfluss der Zusammensetzung der mobilen Phasen.* Die durchschnittlichen R_F-Werte wurden in ein Diagramm in Abhängigkeit von den einzelnen mobilen Phasen eingetragen. Auf Grund des unterschiedlichen Verlaufes der auf diese Weise gewonnenen Kurven konnten die Verbindungen in zwei Hauptgruppen eingeteilt werden.

Die Verbindungen mit tertiärem Stickstoff im Molekül haben mit zunehmendem Äthanolgehalt in der durchfliessenden Phase ansteigende Kurven (Fig. 1 und Fig. 2).

Die Präparate mit quartärem Stickstoff sind durch einen absteigenden Kurvenverlauf charakterisiert; die R_F-Werte sinken also in diesem Falle mit zunehmendem Äthanolgehalt (Fig. 3).

Vergleicht man die R_F-Werte der tertiären Basen, so ist zu ersehen, dass sie zwei Untergruppen aufweisen. In die erste können die Verbindungen eingereiht werden, die in diesem System durch einen niedrigen R_F-Wert gekennzeichnet sind (in der

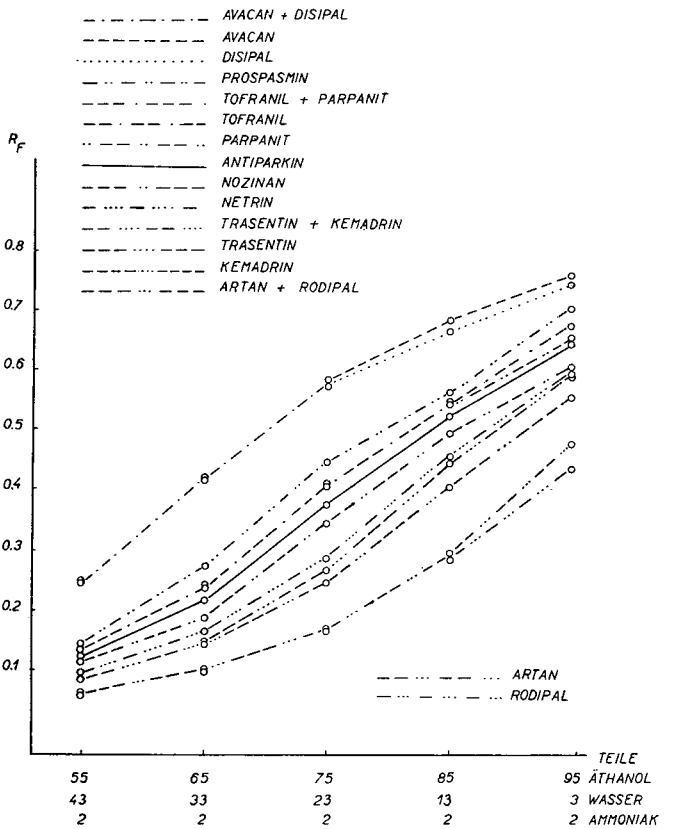


Fig. 1. Einfluss der Zusammensetzung der mobilen Phasen auf die R_F-Wert-Verschiebung von Arzneimitteln der ersten Untergruppe.

mobilen Phase 3 haben sie einen allgemein niedrigeren R_F-Wert als 0.60): Rodipal, Artan, Kemadrin, Trasentin, Netrin, Nozinan, Antiparkin, Parpanit, Tofranil, Prospasmin, Disipal und Avacan. Die Verbindungen dieser Untergruppe, die die niedrigsten R_F-Werte besitzen, wiesen einen konkaven Kurvenverlauf auf (Artan, Rodipal, Kemadrin). Für die Präparate mit dem höchsten R_F-Wert, welche einen Übergang zwischen der ersten und zweiten Untergruppen darstellen, ist ein konvexer Verlauf typisch (Disipal, Avacan). Bei den übrigen Präparaten, deren R_F-Werte zwischen diesen extremen Werten liegen, wurde eine annähernd lineare Abhängigkeit des R_F-Wertes von der Zusammensetzung der durchfliessenden Phase gefunden (Fig. 1).

Optimale Trenneffekte kann man in der mobilen Phase 3 oder 4 erreichen, in der mobilen Phase 5 schieden sich die R_F-Werte bei den beiden Präparaten mit dem niedrigsten R_F-Wert (Rodipal, Artan). Im Fliessmittelgemisch 5 und 2 stehen die R_F-Werte nah beieinander. Im letzteren und hauptsächlich in der Phase 1 (mit Ausnahme des Disipals und Avacans) sind die R_F-Werte der Verbindungen dieser Untergruppe sehr niedrig, so dass die Verwendung einer durchfliessenden Phase mit

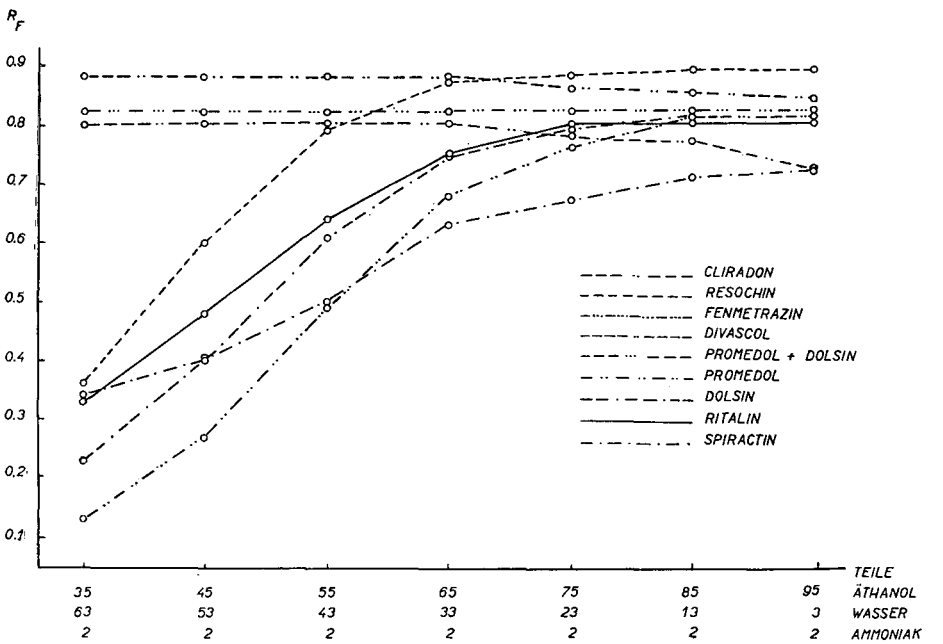


Fig. 2. Einfluss der Zusammensetzung der mobilen Phasen auf die R_F-Wert-Verschiebung von Arzneimitteln der zweiten Untergruppe.

noch niedrigerem Äthanolgehalt für die graphische Darstellung zwecklos ist.

Die zweite Untergruppe der Verbindungen mit tertiärem Stickstoff weist hohe R_F-Werte auf (in der mobilen Phase 3 höhere als 0.60): Ritalin, Spiractin, Promedol, Dolsin, Cliradon, Fenmetrazin und Divascol. Unterschiedliche R_F-Werte und dadurch optimale Trennmöglichkeiten wurden in der mobilen Phase 1 erzielt, während in den mobilen Phasen mit höherem Äthanolgehalt (3, 4, 5) alle R_F-Werte zu hoch gefunden wurden. Aus Kurvenextrapolationen konnte man auf eine weitere R_F-Wert-Abnahme und neue Trennmöglichkeiten von fünf angeführten Präparaten schliessen (Resochin, Spiractin, Ritalin, Dolsin und Promedol). Auf diese Weise wurden auch die Kurven der Verbindungen mit hohen R_F-Werten durch passende Vorbereitung der mobilen Phasen dargestellt. Dieselben besassen einen konvexen, die Stoffe der zweiten Untergruppe charakterisierenden Verlauf (Fig. 2).

Die R_F-Werte von drei Präparaten, Cliradon, Fenmetrazin und Divascol blieben durch weitere Abnahme des Äthanolgehaltes in der mobilen Phase unbeeinflusst. Diese drei Stoffe stellen eine Ausnahme unter den getesteten Verbindungen dar. Während die R_F-Werte des Fenmetrazins in allen durchfliessenden Phasen die gleichen

blieben, wurde bei der Chromatographie des Cliradons und Divascols eine ähnliche Abnahme wie bei quartären Basen festgestellt. Im Falle des Cliradons war der Unterschied von beiden Grenzwerten so gering ($\Delta R_F = 0.04$), dass ein absteigender Kurvenverlauf nicht mit Sicherheit erkannt werden konnte. Bei der Chromatographie des Divascols wurde eine ziemlich auffallende R_F-Wert-Abnahme bemerkt ($\Delta R_F = 0.08$), die also wirklich bestättigt werden kann.

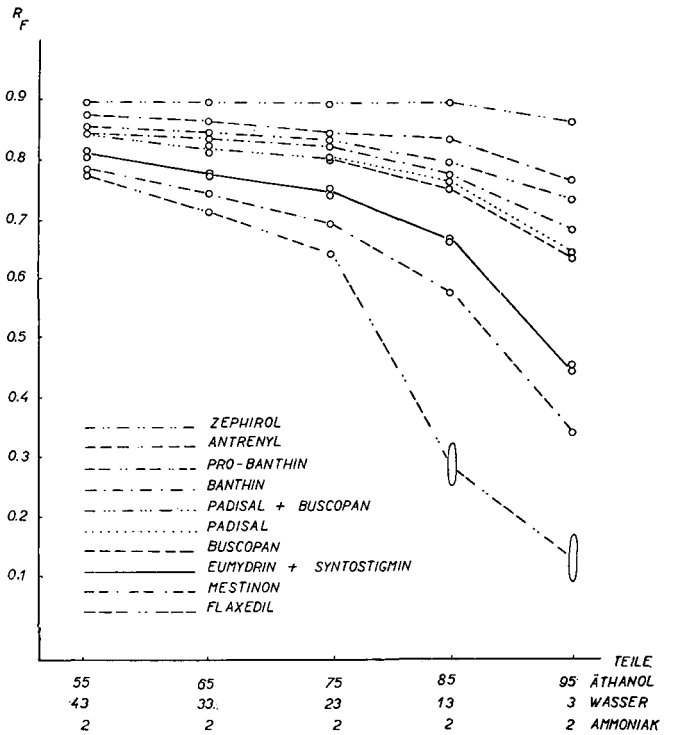


Fig. 3. Einfluss der Zusammensetzung der mobilen Phasen auf die R_F-Wert-Verschiebung von Arzneimitteln mit quartärem Stickstoff.

Die zweite Hauptgruppe umfasst 10 Präparate mit quartärem Stickstoff: Mestinon, Antrenyl, Buscopan, Syntostigmin, Zephirol, Banthin, Pro-Banthin, Eumydrin, Flaxedil und Padisal. Alle sind durch ein gemeinsames Merkmal, einen R_F-Wert-Abstieg mit zunehmendem Äthanolgehalt in der mobilen Phase gekennzeichnet (Fig. 3). Während man den R_F-Wert-Abstieg des Zephirols schwer nachweisen kann, ist er bei zahlreichen Präparaten ziemlich auffällig (Flaxedil, Mestinon, Syntostigmin, Eumydrin). Alle Stoffe dieser Gruppe zeigen in den durchfliessenden Phasen mit niedrigerem Äthanolgehalt hohe und annähernd gleiche R_F-Werte, die mit zunehmendem Äthanolgehalt unterschiedlich absinken. Daraus ergibt sich, dass die Chromatographie in der mobilen Phase 5, in der sehr verschiedene R_F-Werte erhalten wurden, die vorteilhafte Trennmöglichkeit für diesen Stofftypus darstellt.

Die Feststellung der Beziehung zwischen der Konstitution und dem chromatographischen Verhalten basischer Stoffe von verschiedenem Typus steht in Überein-

stimmung mit derjenigen, die bei der Chromatographie der Antihistaminika und Ataraktika beschrieben wurde. Auf Grund der Resultate beider Arbeiten ergibt sich, dass der R_F-Wert-Anstieg der tertiären Basen und der R_F-Wert-Abstieg der quartären Basen im angegebenen System mit zunehmendem Äthanolgehalt in der durchfliessenden Phase eine allgemein gültige Gesetzmässigkeit darstellt, welche jedoch gewisse Ausnahmen aufweisen kann. So wurden unter den tertiären sowie auch den

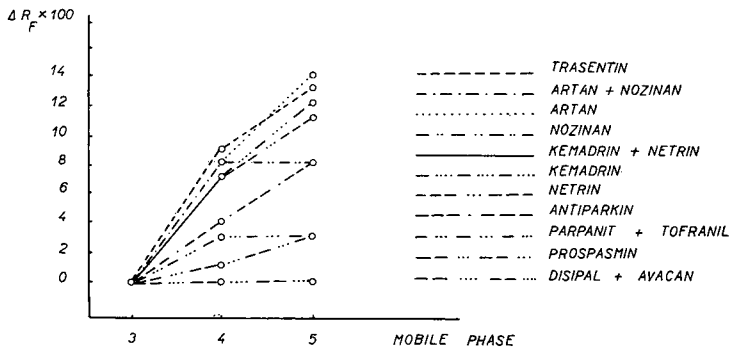


Fig. 4. Temperatureinfluss auf die R_F-Wert-Verschiebung der Arzneimittel der ersten Untergruppe.

quartären Basen Verbindungen gefunden, die keine auffallend steigenden oder fallenden Kurven besassen und deren R_F-Werte durch eine Veränderung in der mobilen Phase unbeeinflusst oder nur in geringem Masse beeinflusst wurden (Zephirol, Cliradon, Fenmetrazin). Schliesslich wurden tertiäre Basen gefunden, die einen R_F-Wert-Abstieg bei zunehmendem Äthanolgehalt aufweisen (Divascol). Es scheint, dass diese Ausnahmen selten sind, weil sich die festgestellte Gesetzmässigkeit bei der überwiegenden Zahl der getesteten Verbindungen als absolut gültig erwies (in 90–91 % der Fälle).

(*b*) *Temperatureinfluss.* Auf die absoluten R_F-Werte einiger Verbindungen übt die Temperatur einen gewissen Einfluss aus. Eine von der Temperaturveränderungen abhängige R_F-Wert-Verschiebung ist vor allem für die Präparate mit niedrigem R_F-Wert charakteristisch. Aus diesem Grunde wurde eine Versuchserie mit den Präparaten der ersten Untergruppe bei einer Temperatur von 12–13° durchgeführt. Die gewonnenen R_F-Werte waren in den meisten Fällen wesentlich niedriger als die bei einer Temperatur von 17°. Bei dem Vergleich beider Ergebnisse wurde für diese Verbindungen eine gewisse Abhängigkeit festgestellt. Die R_F-Werte aller Präparate blieben in den Fliessmittelgemischen 1, 2 und 3 ohne Veränderung. Ein erster R_F-Wert-Anstieg erschien in der mobilen Phase 4, ein zweiter im Fliessmittelgemisch 5. Die relativ grösste R_F-Wert-Zunahme wurde bei den Präparaten mit dem niedrigstem R_F-Wert gefunden (Artan, Kemadrin, Trasentin). Die Stoffe mit dem höchsten R_F-Wert wiesen im Gegensatz dazu keinen R_F-Wert-Anstieg auf (Disipal, Avacan), so dass die R_F-Wert-Zunahme in einem umgekehrten Verhältnis zu den absoluten R_F-Werten steht: je niedriger der R_F-Wert ist, desto grösser ist die R_F-Wert-Zunahme (Fig. 4). Die R_F-Wert-Verschiebungen von Präparaten anderer Gruppen konnten nicht nachgewiesen werden.

Bei der Chromatographie in diesem System sind Wärmestrahlungen oder Luftströmungen zu vermeiden; an der Stelle mit einer höheren Temperatur der Kammer zeigt sich eine Fliessmittelverdampfung aus dem Chromatogramm, das Fortschreiten der mobilen Phase wird aufgehalten und man erhält höhere R_F-Werte. Ähnliche Erscheinungen wurden auch bei unvollkommen verschlossenen Gefässen bemerkt. Im Gegensatz dazu, kondensieren sich an der Stelle mit einer niedrigeren Temperatur

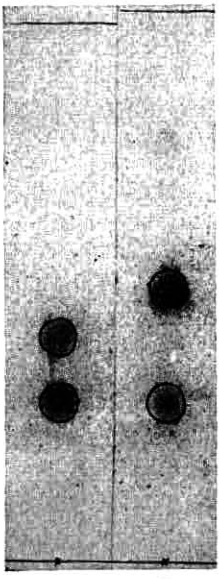


Fig. 5. Aufteilung des Gemisches Artan–Kemadrin und Rodipal–Nozinan in der mobilen Phase 4.

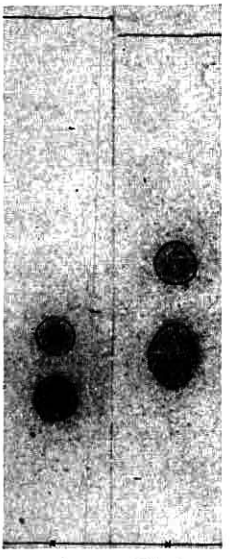


Fig. 6. Aufteilung des Gemisches Netrin–Parpanit und Disipal–Antiparkin in der mobilen Phase 3.

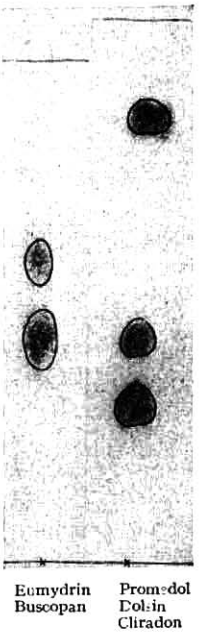


Fig. 7. Aufteilung des Gemisches Eumydrin–Buscopan in der mobilen Phase 5 und Cliradon–Promedol–Dolsin in der mobilen Phase A.

die Fliessmitteldämpfe nicht nur an den Gefässwänden, sondern auch auf dem Chromatogramm; das Fliessmittel läuft schneller und die R_F-Werte sind niedriger. Bei der Arbeit unter konstanten Bedingungen in gut verschlossenen Gefässen wurde eine gute Reproduzierbarkeit der R_F-Werte ($\pm$ 0.02) erreicht.

2. Fleckenform

Die tertiären sowie auch quartären Basen wiesen im allgemeinen eine vollkommen runde Fleckenform auf. Die Flecke von Verbindungen der ersten Untergruppe erschienen in der mobilen Phase 1 und 2 länglich oval, ähnlich wie die Flecke der quartären Basen im Fliessmittel 5. Bei den Verbindungen mit einem höheren R_F-Wert (zweite Untergruppe) wurden in den mobilen Phasen mit höherem Äthanolgehalt horizontal ovale, im Fliesmittelgemisch 1, 2, A und B runde Flecke gefunden. Auch die Fleckenform der Stoffe mit quartärem Stickstoff war befriedigend, nur die

Flecke des Flaxedils erschienen in der mobilen Phasen 4 und 5 so lang, dass es unmöglich war, genaue R_F-Werte zu bestimmen. Diese Erscheinung ist eine Folge der Trennstörung bei der Chromatographie von Verbindungen, deren Löslichkeit im Wasser wesentlich höher ist als im Äthanol. Bei der Chromatographie des Banthins und Pro-Banthins wurde noch ein Fleck mit niedrigerem R_F-Wert gefunden.

3. Trennmöglichkeiten

In diesem System kann ein Gemisch von Verbindungen getrennt werden, deren R_F-Werte sich mindestens um 0.1 unterscheiden, wozu man aus den graphischen Darstellungen eine vorteilhafte durchfliessende Phase bestimmt, in der die gegebenen Verbindungen am meisten unterschiedliche R_F-Werte aufweisen.

Weil die angeführten strukturell nahe stehenden Präparate diese Forderung erfüllen, kann ein Gemisch derselben in einer günstig gewählten Phase getrennt werden. Auf diese Weise wurden Artan–Kemadrin oder Rodipal–Nozinan in der mobilen Phase 4 (Fig. 5), Netrin–Parpanit und Disipal–Antiparkin im Fliessmittelgemisch 3 (Fig. 6) aufgeteilt. Für Eumydrin–Buscopan eignete sich die mobile Phase 5 am besten (Fig. 7). Für eine Trennung der Analgetikapräparate Cliradon–Promedol–Dolsin konnten mit gleichem Erfolg die Fliessmittel 1, A oder B (Fig. 7) angewendet werden.

ZUSAMMENFASSUNG

Es wurde eine Gesetzmässigkeit zwischen der Konstitution von 30 basischen Arzneimitteln und ihrem papierchromatographischen Verhalten im Umkehrphasensystem Petroleum/Äthanol–Wasser–Ammoniak untersucht. In sieben durchfliessenden Phasen mit unterschiedlichem Äthanolgehalt wurde der Einfluss der Zusammensetzung der mobilen Phase auf die R_F-Wert-Verschiebung verfolgt. Es wurde gefunden, dass tertiäre Basen durch einen R_F-Wert-Anstieg mit zunehmendem Äthanolgehalt in der durchfliessenden Phase charakterisiert sind, während die R_F-Werte quartärer Basen unter den gleichen Bedingungen absinken. Von den 30 getesteten Stoffen (20 Verbindungen mit tertiärem und 10 Präparate mit quartärem Stickstoff) wurden nur drei Ausnahmen gefunden.

Im gegebenen System wurde auch der Temperatureinfluss auf die R_F-Wert-Verschiebung beschrieben. Es wurden die Bedingungen für eine optimale Trennung von Verbindungen, die durch eine ähnliche Konstitution oder einen annähernd gleichen R_F-Wert gekennzeichnet sind, gefunden.

SUMMARY

For 30 basic drugs an investigation was made of the relationship between structure and behaviour on paper chromatography in the reverse phase system petroleum/ethanol–water–ammonia. Using seven mobile phases that differed in ethanol content, the influence of the composition of the mobile phase on the alteration of the R_F value was studied. It was found that for tertiary bases the R_F increased with increasing ethanol content of the mobile phase, whereas for quaternary bases the R_F decreased. Only three exceptions to this rule were found among the 30 compounds investigated (20 compounds with tertiary and 10 with quaternary nitrogen).

The influence of temperature on the change in R_F value was also investigated for this system. Conditions were established for obtaining the best separation of compounds that have similar structures or almost identical R_F values.

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THE PRESENCE OF A POSSIBLE CAFFEINE–STEROID COMPLEX IN HUMAN URINE*

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INTRODUCTION

Observations have been reported in the past[1–5] of an ultraviolet absorbing compound from various sources which has solubility characteristics similar to that of steroids. We have observed that such material can be extracted from human urine and that on occasion it can contain radioactivity when a subject is infused with cortisol-4-^{14}C. Despite the fact that radioactivity can be incorporated into the ultraviolet absorbing region, there were indications that the main part of this material was not a steroid. It therefore was desirable to determine the nature of this compound since it would be of interest to those concerned with corticosteroid work.

METHODS AND MATERIALS

Chemicals, unless otherwise specified, were reagent grade and all solvents were freshly distilled. The caffeine used for comparison purposes was resublimed.

The extraction of small volumes of urine (1 l) was carried out according to the method of EIK-NES[6] for the extraction of Porter-Silber chromogens from plasma. For the extraction of a larger batch of urine (21 l), a liquid–liquid continuous extractor was used. Each batch of 3 l of urine was extracted with methylene chloride for 24 h. The pooled methylene chloride was washed with 0.1 *N* sodium hydroxide, followed by water, dried and evaporated. The residue was partitioned between benzene and water (1:5). The water layer was re-extracted with methylene chloride, dried and the solvent evaporated. This residue from the small volumes of urine of individuals who had received cortisol-4-^{14}C was chromatographed directly on paper, whereas that from 21 l of urine was recrystallized from methanol, ether and finally from ether–hexane (1:2). The yield was 18.6 mg. The material from either source showed the same characteristics and is hereafter referred to as U-272.

Paper chromatography of U-272 was carried out in BUSH B-5[7], ZAFFARONI[8] and in a SCHNEIDER–LEWBART system (No. 6)[9].

Acetylation was done in a mixture of acetic anhydride and pyridine (1:5)[8] allowing the mixture to stand for 18 h. The reaction was then stopped by the addition of 1 ml of methanol and after one hour, the solvents were evaporated at 40° under nitrogen.

* This work was in part supported by research grants from the National Institutes of Health, Bethesda, Md.

Hydrolysis in alkali was performed by heating for 10 min in a boiling water bath with 0.5 *N* sodium hydroxide and acid hydrolysis was done by treatment with 0.5 *N* sulfuric acid or with Amberlite IR-120 and heating at 45° for 16 h[10]. The aqueous medium was then extracted with ethyl acetate.

The paper chromatograms were scanned for radioactivity in a thin window Geiger strip counter and ultraviolet absorbing spots on the paper were localized by a Haines type scanner[11]. Ultraviolet spectra were determined by a Beckmann DK_2 automatic recording spectrophotometer and the infrared spectra read in a Perkin-Elmer Model 21 recording infrared spectrophotometer.

RESULTS

The running rates of U-272 in various paper chromatographic systems are listed in Table I. In the urine of four out of eleven normal subjects infused* with cortisol-4-^{14}C, radioactivity was found moving with an ultraviolet absorbing compound and this radioactivity could not be separated from the ultraviolet absorbing material in any of the chromatographic systems listed in Table I. The material had an ultraviolet absorption maximum at 272 mμ in methanol. It did not give a positive test with any of the following reagents: Sulfuric acid–ethanol[12], sulfuric acid[13], 2,3,5-triphenyltetrazolium chloride[8], dinitrophenylhydrazine[14] and BARTON's reagent[15].

When acetylation of U-272 was attempted the mobility on paper did not change and the radioactivity remained still with the ultraviolet absorbing area (Table I).

TABLE I

R_F OF U-272 AND ACETYLATED U-272 IN VARIOUS CHROMATOGRAPHIC SYSTEMS

Paper chromatographic systems used	*U-272*	*U-272 after acetylation*	*Caffeine*	*Cortisone*	*Corti-costerone*
Bush B-5[7]	0.58	0.60	0.58	0.46	0.78
$CHCl_3$–formamide[8]	0.65	0.66	0.64	0.38	0.72
Benzene–formamide[8]	0.22				
Schneider-Lewbart[9]	0.84				

In a further effort to separate the radioactive material from the ultraviolet absorbing area, U-272 was hydrolyzed with alkali and acid. In alkali, the treatment was obviously too vigorous and the radioactive area now resided at the origin of the chromatogram in the Bush B-5 system and no ultraviolet spot was observed between the R_F's of cortisone and corticosterone. Upon hydrolysis of U-272 with sulfuric acid or following treatment[10] with IR-120, a radioactive area (U-272a) was detected with an R_F of cortisone and an ultraviolet spot (U-272b) was found which moved with an R_F similar to that of unhydrolyzed U-272 in Bush B-5 system of paper chromatography (Fig. 1).

The radioactive U-272a chromatographed like cortisone in both chloroform–formamide and in the Bush B-5 system. One third of U-272a was acetylated and chromatographed in benzene–formamide. The R_F was 0.27 which was identical with that of cortisone acetate run on a parallel strip. The remainder of the radioactive

* The authors would like to express their deepest gratitude to Dr. C. NUGENT, Department of Medicine, University of Utah, College of Medicine for his help in the infusion of cortisol-4-^{14}C.

material was crystallized to constant specific activity after adding carrier cortisone. The results are given in Table II. The ultraviolet absorbing compound U-272b had, however, the same running rates on paper and the same ultraviolet maximum as before the acid hydrolysis.

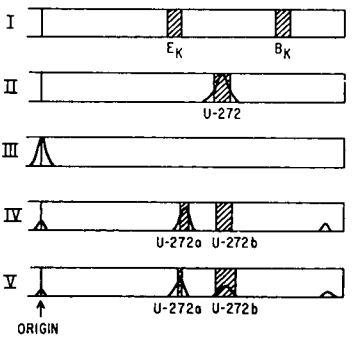

Fig. 1. Paper chromatographic pattern in Bush B-5 system of U-272 before and after hydrolysis. Shaded area represents ultraviolet absorption and the curve indicates radioactivity. (I) The relative mobilities of cortisone (E_K) and corticosterone (B_K); (II) mobility of U-272; (III) after hydrolysis with 0.5 *N* sodium hydroxide; (IV) after acid hydrolysis with 0.5 *N* sulfuric acid; (V) after treatment with Amberlite IR-120.

An elemental analysis of U-272b obtained after acid hydrolysis gave an empirical formula of $C_8H_{10}N_4$* which agrees with caffeine. The R_F's of caffeine in several systems of paper chromatography were similar (Table I). The melting point of U-272b and caffeine were identical (234.5–235.5°) and a mixed melting point determination showed

TABLE II

CRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY OF THE RADIOACTIVITY FROM ACID-HYDROLYZED U-272

No. of crystallization	*c.p.m./mg*	*Solvent*
0	112	
	117	
1	108	Methanol
	111	
2	124	Ether–hexane (1:2)
	122	
3	126	Ethanol
	124	
4	123	Methanol
	117	

the two compounds to be identical. The melting point of the picrate of caffeine and of U-272b were also the same (144–145°) and admixture of the two did not lower the melting point. The ultraviolet and infrared spectra of U-272b were identical in all respects with those of caffeine.

Finally, urine from an individual maintained on a caffeine-free diet yielded no U-272 when extracted and processed as outlined.

* Done by Elek Micro Analytical Laboratories, Los Angeles, Calif.

DISCUSSION

The finding of caffeine in urine or in plasma is not new[16,17], but it appeared interesting since in some cases steroid was observed bound to caffeine. The complexing of steroids with other substances which absorb ultraviolet light and/or which migrate on paper chromatograms like the corticosteroids have been observed by other workers. BERLINER *et al.*[2] have found that placental extracts contain a substance similar to caffeine. STAPLE *et al.*[3] reported a complexing of progesterone, sometimes as much as 75%, with a substance in beef adrenal. This complex migrated on paper between cortisone and corticosterone. The progesterone could be readily liberated by treatment with dilute acid.

The complexing of a number of steroids with purines has been studied by MUNCK, SCOTT AND ENGEL[18] and the postulate of ENGEL[19] on the physiological significance of such a complex is interesting. The physiological importance of the steroid–caffeine complex found in our investigation is questionable since complexing was not found to be general. In fact only about 36% of the normal individuals studied showed this phenomenon. However, that such a complex can be found in human urine and also that caffeine migrates similar to corticosteroids on paper chromatograms suggests that care must be exercised in the interpretation of quantitative and qualitative estimation of these steroids extracted from biological fluids and subjected to ultraviolet measurements only.

SUMMARY

A compound which behaves like a steroid in terms of solubilities and chromatographic behaviour in paper systems commonly used for the separation of C-21 steroids has been isolated and characterized. This compound was shown to be caffeine. Further, in four out of eleven cases where cortisol-4-^{14}C had been infused, cortisone-^{14}C was found bound to caffeine.

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CENTRIFUGAL CHROMATOGRAPHY

V. APPARATUS FOR PREPARATIVE-SCALE PAPER CHROMATOGRAPHY IN THE CENTRIFUGAL FIELD*

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The three main problems in preparative-scale paper chromatography are (1) lengthy separation, (2) the small amounts of substance that can be separated and (3) the discontinuous character of the separation. Conventional apparatus for centrifugal chromatography which are suitable for rapid chromatographic separation (CARONNA[1], MCDONALD *et al.*[2], TATA AND HEMMINGS[3], PAVLÍČEK *et al.*[4,5], DAUPHIN *et al.*[6], HERNDON *et al.*[7], MATTHEWS AND CERVANTES[8]) are useful only for analytical purposes; the possibility of semimicro preparation is limited by the capacity of the chromatographic paper. Another disadvantage of these apparatus is the impossibility of carrying out a continuous chromatographic separation.

The technique of continuous paper chromatography and the device used have already been described by SOLMS[9]; this method is very efficient in obtaining preparative separation, but the time required for separation is extremely long.

For achieving quick and continuous paper chromatography the apparatus for centrifugal chromatography as described in one of the previous communications[5] was adapted here.

PRINCIPLE OF CONSTRUCTION AND USE OF THE APPARATUS

The sample is continuously deposited by means of a capillary tube onto the rotating disc of chromatographic paper, close to the mobile phase inlet. The capillary tube undergoes only a slow relative rotary motion (1 rev./20 min–6 h). Assuming that the capillary tube dispenses the sample at time t at a point x (see Fig. 1), then the chromatographic trace at this point is directly on the start. If the continuous chromatograph has been in motion for some time prior to time t, at this moment the mobile phase flow will have caused a partial (or complete) chromatographic separation at a point y, located counter to the relative rotary motion. The trace of each of the separated components is a spiral, the curvature of which depends on the R_F value or rather on the nature and form of the separating function. The next factor bearing on the

* For Part IV, see M. PAVLÍČEK, J. ROSMUS AND Z. DEYL, *J. Chromatog.*, 9 (1962) 92.

course of the spiral is the rate of motion of the capillary tube with respect to the disc of chromatographic paper. If the period of one relative revolution of the capillary tube with respect to the paper disc just corresponds to the period within which the spot reaches the front of the paper, the spot (spiral) revolves once about an angle of

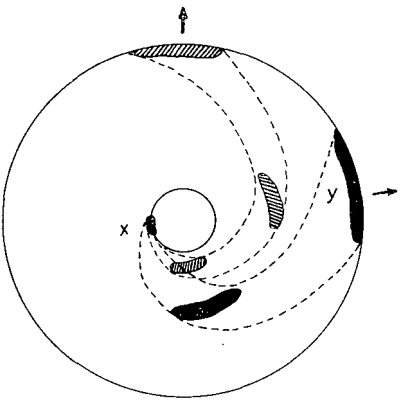


Fig. 1. Schematic representation of the continuous separation of two compounds with R_F values of 1.00 and 0.40.

360°; if this trace simultaneously corresponds to the substance with the highest R_F value the other substances (with lower R_F) revolve about an angle less than 360°. Practically speaking, it is not essential (because of unnecessary prolongation of the separation process and the possibility of the influence of diffusion or of the irregularity of the paper) that the substance with the highest R_F should revolve about 360°, but it may revolve about any central angle α, less than 360°, depending on the nature of the mixture to be separated. The scheme in Fig. 1 shows the separation of two substances of $R_F = 1.00$ and $R_F = 0.40$, the revolution time of the capillary tube being here just half the time necessary for the spot ($R_F = 1.00$) to reach the front of the paper under steady-state conditions.

The spiral of the substance to be separated follows a rotary course along the paper disc and the speed of the latter equals that of the capillary tube.

The fraction of the pure substance is collected from the front of the paper by various types of fraction collectors (see next section) which rotate at the same speed as the capillary tube. The capillary tube and a type of fraction collector form one construction unit. If the position of the capillary tube in this unit is denoted as radius vector r_0, then, according to a selected angular velocity of the capillary tube with respect to the paper disc, the fraction with the highest R_F in the fraction collector will be found lagging by the angle α behind the relative motion of the capillary tube, *i.e.* by the angle travelled by the capillary tube during the time required by the spot of the substance to cover the distance from the start to the front of the paper.

DESCRIPTION OF THE APPARATUS

The apparatus for the continuous paper chromatography is in principal the same as that described by PAVLÍČEK *et al.*[4,5]; the capillary type of apparatus has been used as distributor of the mobile phase[5].

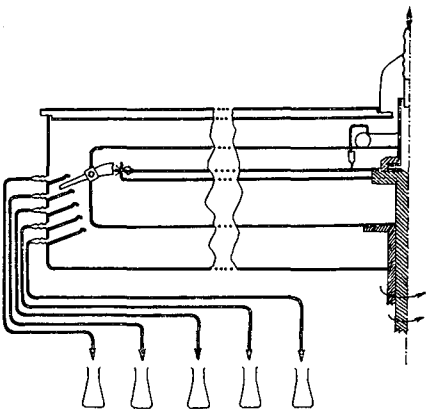


Fig. 2. Fraction collector; the separated fractions are collected along the wall of the chromatographic chamber.

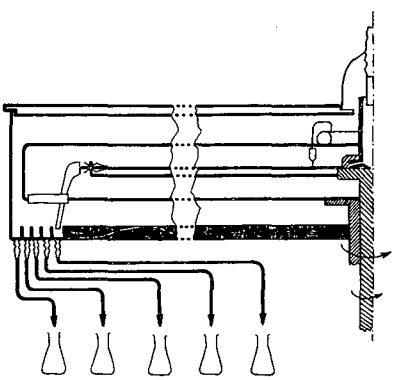


Fig. 3. Fraction collector; the separated fractions are collected on the bottom of the chromatographic chamber.

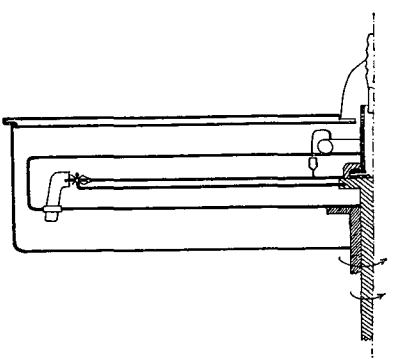
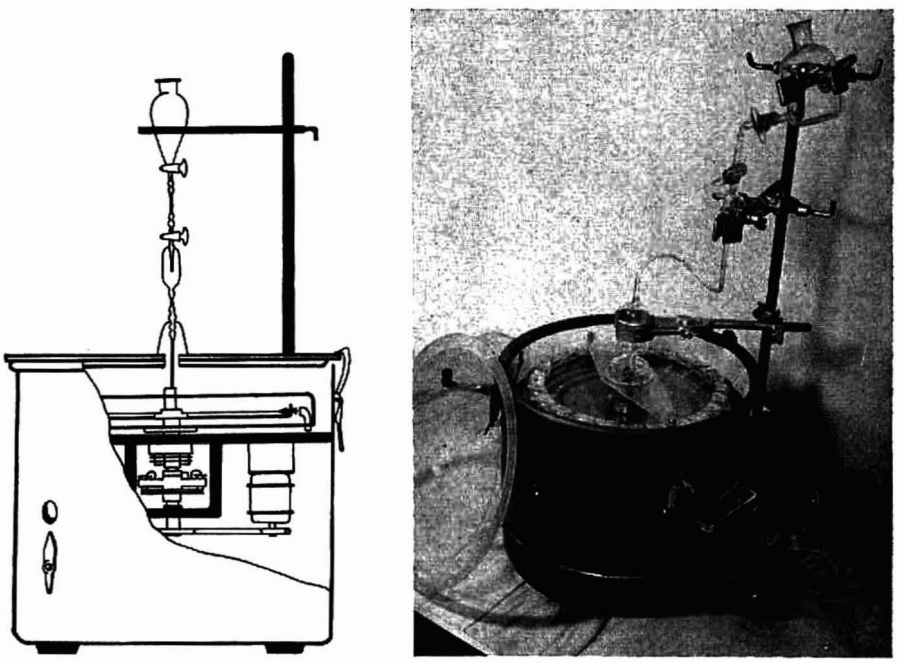


Fig. 4. Fraction collector; the separated fractions are collected in glass tubes along the disc of the chromatographic paper.

The apparatus is completed by one of three arrangements of fraction collectors; these are schematically shown in Figs. 2–4. The over-all scheme of the apparatus is given in Fig. 5. According to the arrangement of the fraction collector the separated fractions are collected either along the wall of the apparatus (Fig. 2), or on the bottom of the apparatus (Fig. 3). In the third arrangement the separated fractions remain in the glass tubes of the fraction collector. The last type is suitable only for separations where the volume of each fraction does not exceed approximately 1 ml.



a b

Fig. 5. (a) Overall scheme of the apparatus for continuous centrifugal chromatography. (b) Photograph. In this case the fraction collector shown in Fig. 4 is used.

The continuous centrifugal chromatography described reduces the time for separating 200–700 mg of substance to one-sixth in comparison with the descending technique.

SUMMARY

A simple technique and the necessary apparatus for quick preparative-scale paper chromatography is described. In order to reduce the time of separation the principle of centrifugal paper chromatography was used. The device permits a precise and controlled separation of the spotted sample into individual fractions within one-third to one-tenth of the time required for conventional chromatography.

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RARE EARTH CHROMATOGRAPHY USING BIS-(2-ETHYLHEXYL) ORTHOPHOSPHORIC ACID

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Bis-(2-ethylhexyl) orthophosphoric acid*, DEP, has been found by PEPPARD *et al.*[1], to form very selective complexes with the trivalent lanthanide and actinide ions. DEP, $(CH_3CH_2CH_2CH_2CH(CH_2CH_3)CH_2O)_2PO(OH)$, a monobasic acid insoluble in dilute acidic aqueous solutions, has been used in solvent extraction of the rare earths. In its pure form the separation factors for adjacent rare earths are uniformly close to 2.5, and the higher atomic number lanthanides are the more tightly complexed.

SIERKIERSKI AND FIDELIS[2,3] have carried out chromatographic separation of the rare earths using tributyl phosphate held on a column of diatomaceous silica, first made hydrophobic with dichlorodimethylsilane vapors. In their system elution was carried out with nitric acid.

In this work the rare earths are separated chromatographically using columns of DEP held on dichlorodimethylsilane-treated diatomaceous silica columns. Separation factors observed are similar to those in solvent extraction using DEP, and the kinetics of the elution are rapid. Columns are easily prepared and are stable and long-lived. Similar results were obtained independently by CERRAI *et al.*[4] using columns of cellulose.

EXPERIMENTAL

Column substrate material is Johns-Manville Celite Analytical Filter Aid, used directly as supplied by the manufacturer. The surface is made hydrophobic by placing several grams of Celite in a desiccator containing an open bottle of dichlorodimethylsilane, allowing about two days of contact time, and heating briefly and washing with methanol to remove hydrogen chloride released during the reaction. The dried product is slurried with an acetone solution of DEP such that the weight ratio of DEP to Celite is about 1:5. After a few minutes the acetone is evaporated with gentle heating until the odor of acetone is gone. The dry-looking product is then slurried with 0.01 *N* HCl containing a wetting agent (0.1 % of the polyester Tergitol Nonionic NPX, manufactured by the Union Carbide Co., Inc., is satisfactory) and held at 90° for two days until all particles settle out easily and no air bubbles remain. Removal of air bubbles is critical for subsequent column operation.

A column is packed by allowing the slurry to settle out in a suitable glass tube. Elution using columns of the dimensions reported in this paper requires a few pounds

* Also known as di-(2-ethylhexyl) orthophosphoric acid and di-(2-ethylhexyl) hydrogen phosphate and abbreviated HDEHP and D2EPA.

per square inch (several tenths of an atmosphere) of external pressure. If the column is properly packed and free of air bubbles, the bed should not be visibly compressed upon application of the pressure.

PEPPARD *et al.*[1] have stressed the necessity for purity of the DEP reagent in achieving the maximum separation factors, particularly for La and Ce. Small amounts of the dibasic acid, mono-(2-ethylhexyl) orthophosphoric acid, form strong and rather unselective complexes with the trivalent lanthanides. PEPPARD *et al.*[1] describe a solvent extraction procedure for purification of impure material. For this work, however, specially prepared DEP of high purity obtained from the Oak Ridge National Laboratory was used.

Loading the column is most easily carried out by treating the column with solution of the same acidity as that of the sample and allowing the sample in a small volume ($\sim$ 2 ml for columns reported here) to soak into the top of the bed. Pressure may be used in this operation, but care must be exercised not to allow any air to be forced into the column. The acidity of the solution during the loading operation must be such that the sample is strongly adsorbed. For rare earths in this work, 0.05 *N* HCl or HNO_3 is satisfactory. For efficient uptake of the rare earths by the column, the solution must be quite free of all other cations of charge 2+ and greater.

The heating jacket for columns used at elevated temperature consists of a 3 cm glass tubing surrounding the column and secured by rubber stoppers at the ends such that warm water can be passed into the bottom and out the top. The warm water line is heated by a constant temperature bath to about 70°. All eluent solutions are outgassed by heating on a steam bath prior to use. If air bubbles are inadvertently formed in the column, they can be resorbed by eluting with outgassed solution.

RESULTS

Rare earths in the presence of a large excess of DEP react to form complexes which may be described by equilibria of the type:

$$M^{3+} + 3HL = ML_3 + 3H^+$$

where HL represents the free acid and L^- the anion. Actually molecular weight determinations by BAES *et al.*[5] indicate that the complex contains 6 moles of DEP per mole of rare earth, but in dilute solutions the dependence of the equilibrium on acidity and reagent concentration is nevertheless accurately given by the above equation. Therefore, we may write an expression for an equilibrium constant

$$K = \frac{(ML_3)\,(H^+)^3}{(M^{3+})\,(HL)^3}$$

In a solvent extraction system the free reagent and the complex are present primarily in the organic phase, and the rare earth and hydrogen ions are concentrated in the aqueous phase. This expression therefore describes the ratio of rare earth concentrations in the two phases in terms of the acidity of the aqueous phase and the reagent concentration in the organic. Rewriting, the extraction coefficient,

$$E_a^{\,o} = \frac{(ML_3)}{(M^{3+})} = K\frac{(HL)^3}{(H^+)^3}$$

indicates that extraction is repressed by high acidity in the aqueous phase and enhanced by high reagent concentration in the organic. The equilibrium constant K is a characterisitic of each rare earth and increases rather regularly by a factor of 2.5 for each unit of atomic number, according to the extraction studies of PEPPARD *et al.*[1]. This discussion seems to apply to the organic phase composed of either the pure reagent or the reagent diluted with an inert solvent such as xylene and to either column operation or batch solvent extraction.

In two experiments designed to test column characteristics at room temperature, mixtures of carrier-free ^{141}Ce + ^{144}Ce, ^{143}Pr, ^{147}Pm and ^{91}Y radiotracers (obtained from the Isotopes Division, Oak Ridge National Laboratory) were loaded onto columns essentially as described under EXPERIMENTAL. In one of these experiments

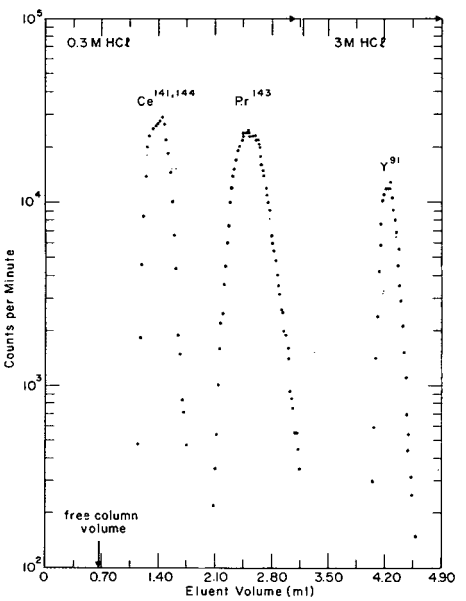

Fig. 1. Elution curve of carrier-free rare earth tracers. Column specifications: Celite + DEP (10:1). 3 cm × 0.24 cm² column, ~ 70 % void space. Flow rate, 0.037 ml/min or 0.154 cm/min.

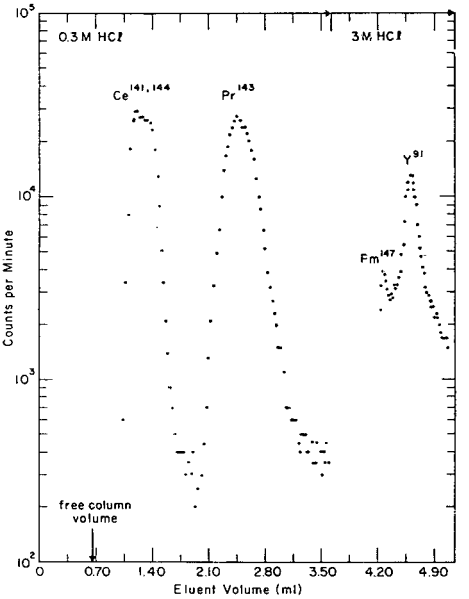

Fig. 2. Elution curve of rare earth tracers + 0.05 μmoles each of Ce, Pr, and Y, equivalent to about 1 % of the column capacity. Elution conditions same as Fig. 1.

the tracers were used in the carrier-free form and in the other they were diluted with 0.05 micromoles each of Ce, Pr and Y, an amount of total rare earth which represented theoretical saturation of about 1 % of the total column capacity. Elution was carried out with 0.3 N HCl until the Ce and Pr were completely removed from the column, and the acid concentration was changed to 3 N. (In the elution of the carrier-free tracer mixture, an intermediate elution with 0.5 N HCl was also included to remove the ^{147}Pm separately.) Elution was carried out slowly, the whole process requiring two hours, and the resulting elution curves, obtained by beta counting single drops collected and dried on a moving paper strip, are shown in Figs. 1 and 2. The separation of Ce from Pr is better for the carrier-free tracers, having a peak to valley ratio of the order of 10^3, but the separation of the tracers with added carriers gives a peak to

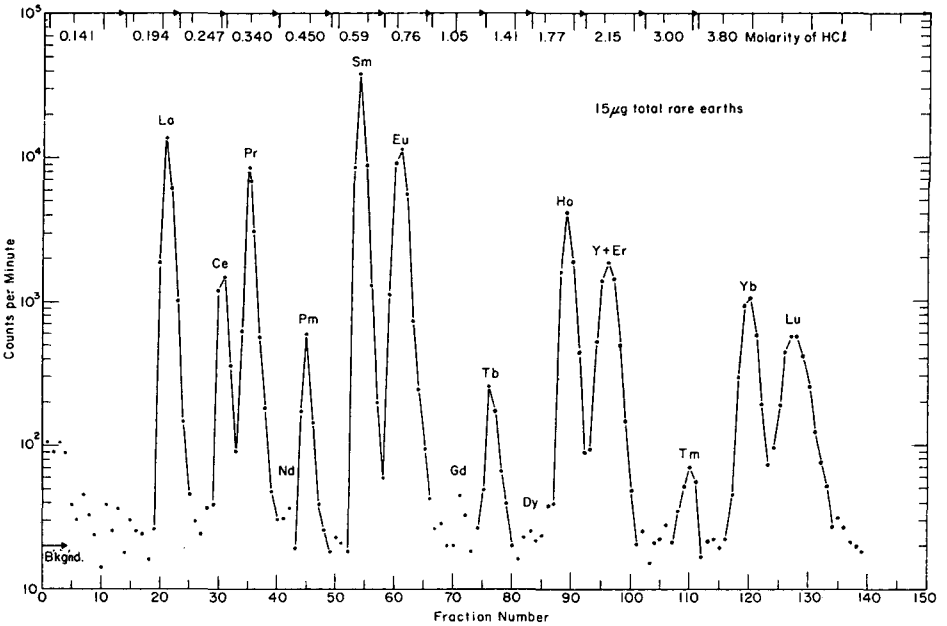

Fig. 3. Typical elution curve for rare earth separation. RE totalling 15 μg, in relative amounts as they occur in average shale (80 % Ce, Y, Nd, and La), were dissolved in 1 ml 0.05 *N* HNO_3, irradiated 1 h at 10^{13} n/cm^2 sec, and eluted 19–23 h later. Ten-drop fractions were evaporated and β-counted for 1 min each 38–41 h after irradiation. The column was 6 cm long and contained 1.9 ml total volume. Elution was carried out at 60–70°, 10 drops per fraction, and 1 fraction per minute. (Elution by 6 ml of 0.05 *N* HNO_3 preceded the HCl to remove traces of ^{24}Na and other radioactivities.)

valley ratio of about 10^2. It is interesting to note that the position of the peaks depends somewhat on the amount of rare earth present under high loading conditions.

In applying the procedure to separating the entire series of rare earths, a stepwise gradient of hydrochloric acid concentration was selected such that each concentration, if used alone, would bring one of the rare earths off the column in about two free column volumes, where the free column volume is about 70 % of the geometrical volume of the column bed. An elution curve at 70° for a mixture of rare earths, corresponding roughly to their abundance in 0.1 g of sedimentary rock and irradiated with pile neutrons for an hour, is shown in Fig. 3. The acid concentrations used in the gradient steps are indicated at the top of the figure. It appears that separations are regularly sharp along the series sufficient for graphical resolution and peak integration to an accuracy of several per cent in most cases.

Application of the DEP rare earth chromatographic procedure to neutron activation analysis in geochemistry is now in progress.

ACKNOWLEDGEMENTS

REGINA VOLFOVSKY AND DAVID G. TOWELL assisted in gathering data for Fig. 3. Support for this work by the Oak Ridge Institute of Nuclear Studies and the Oak Ridge National Laboratory and at M.I.T. by the National Science Foundation and the Office of Naval Research is gratefully acknowledged.

SUMMARY

Sharp separations of microgram amounts of the lanthanide elements as trivalent ions in aqueous hydrochloric acid solution are made chromatographically using a column of diatomaceous silica made hydrophobic with dichlorodimethylsilane vapors and coated with high purity bis-(2-ethylhexyl) orthophosphoric acid (DEP). Separation factors averaging 2.5 for adjacent rare earths are observed, as in solvent extraction using DEP, such that the higher atomic number elements are more tightly held by the column. The kinetics of the sorption–desorption process appear to be very fast, but elutions at 70° appear to be somewhat sharper than at 25°, especially at high flow rates and high loading. A heated column 5 cm long and 2 cm^3 total volume (70 % void space) has been used to separate 15 micrograms of mixed lanthanides from each other in 3 h using a stepwise gradient from 0.14 to 3.8 *N* HCl. La is eluted first, and Y is eluted together with Er.

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Short Communications

The preparation of ECTEOLA-celluloses of various capacities

In our work on the chromatographic purification of the antihaemophilic factor (AHF)[1] we used ECTEOLA-cellulose prepared from Whatman Standard Ashless Cellulose Powder by the method of PETERSON AND SOBER[2]. Several batches prepared in this way had capacities ranging from 0.36 to 0.42 mequiv./g. Although columns of these anion-exchangers worked satisfactorily only very limited quantities of AHF were actually bound. Eight grams of ECTEOLA-cellulose could bind no more AHF than the amount contained in 100 ml blood plasma which is a few mg at most. We therefore tried to increase the capacity of the ECTEOLA-cellulose. Using more NaOH or longer incubation at 0° for better mercerization (*cf.* PORATH[3]) was unsuccessful. Only processing ECTEOLA-cellulose (OH^-) of 0.38 mequiv./g a second time in exactly the same way resulted in an increase of the exchange capacity to 0.78 mequiv./g.

The AHF-binding capacity was not increased, however, or, at least, the recoveries were rather low, with the result that from this point of view working with ECTEOLA-cellulose of high capacity was not promising. At the same time the AHF-preparations obtained with this type of ECTEOLA-cellulose showed a greater density at 280 mμ, and thus contained more impurities. Because the aim of our work was to obtain AHF-preparations of the greatest possible purity, we then tried to prepare ECTEOLA-cellulose of low capacity. Halving the NaOH concentration proved ineffective. We then varied the ratio of epichlorohydrin:triethanolamine. In this way a series of ECTEOLA-celluloses of different capacities were prepared, ranging from 0.05 to 0.60 mequiv./g. Table I gives a survey of the results obtained.

TABLE I

Starting materials			*Capacity*
Cellulose material 30 g	*Epichlorohydrin ml*	*Triethanolamine ml*	*mequiv./g*
Cellulose	10	85	0.05
	15	40	0.14
	20	35	0.19
	22.5	32.5	0.27
	25	30	0.33
	30*	17.5*	0.36–0.42
	30	25	0.46
	35	20	0.60
	40	15	0.52
ECTEOLA-cellulose 0.38 mequiv./g	30*	17.5*	0.78

* Original method of PETERSON AND SOBER.

BOSCH *et al.*[4] only varied the amount of triethanolamine and thus obtained capacities ranging from 0.01 to 0.22 mequiv./g.

We started from 30 g of Whatman Standard Ashless Cellulose Powder, to which a cold solution of 30 g NaOH in 75 ml water was added in portions. The mixture was left standing overnight in the cold room, and the next morning the mixture of epichlorohydrin and triethanolamine was added. The ingredients were thoroughly mixed, and left at room temperature until a spontaneous reaction took place. After this had subsided the resulting product was washed and dried as described by PETERSON AND SOBER. In the first experiment of Table I, 85 ml triethanolamine was used erroneously instead of 45 ml. In the final experiment of that series it can be seen that

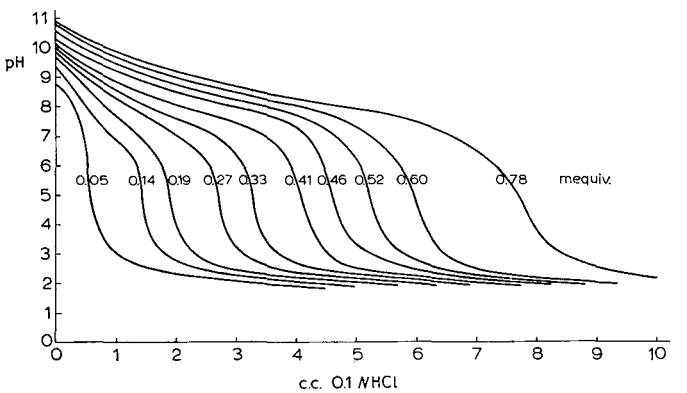

Fig. 1. Titration curves of several types of ECTEOLA-cellulose.

further increase of the ratio epichlorohydrin:triethanolamine only resulted in a lower capacity. This was to be expected.

The titration curves of the different types of ECTEOLA-cellulose are shown in Fig. 1. During these titrations we were struck by the very low exchange velocity in the pH-range 5.5 to 8.

The effect of different capacities on the chromatography of AHF will be described in another paper.

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* Head Prof. Dr. S. VAN CREVELD.

Polyphosphat-Cellulose, ein neuer Kationenaustauscher

Herstellung und Anwendung in der Dünnschicht-Chromatographie

Bei den handelsüblichen Cellulose-Kationenaustauschern handelt es sich ausnahmslos um sauer substituierte Cellulosen. Wir fanden, dass man Cellulose-Kationenaustauscher mit guter Trennfähigkeit erhält, wenn man basisch substituierte oder basisch imprägnierte[1,2] Cellulosen mit Polyphosphat behandelt. Das Polyphosphat wird salzartig gebunden und haftet unter den üblichen Elutionsbedingungen sehr fest.

In der Dünnschicht-Chromatographie sind Kationenaustauscher bisher noch nicht verwendet worden. Die Herstellung von Polyphosphat-Cellulose (PP-Cellulose) für Dünnschicht-Chromatographie ist besonders einfach, wenn man das Polyphosphat an Polyäthylenimin-Cellulose[1,2] bindet, wie das folgende Beispiel zeigt.

Herstellungsvorschrift

20 g Cellulosepulver MN 300 für Dünnschicht-Chromatographie* werden im Becherglas mit 120 ml einer 3 % Polyäthylenimin-Lösung** 5 min lang verrührt. Nach Zugabe von 200 ml dest. Wasser wird durch eine Glasfritte (G3) abgesaugt und zweimal mit je 120 ml dest. Wasser auf der Fritte gewaschen. Man kann auch auf der Zentrifuge waschen. Das noch feuchte Produkt wird im Becherglas mit 120 ml einer 20 % wässrigen Lösung von Grahamschem Salz*** 5 min lang verrührt. Man saugt wieder auf einer G3-Fritte ab, behandelt auf der Fritte mit 120 ml 0.25 % Salzsäure (ca. 2 min) und wäscht dann dreimal mit je 120 ml dest. Wasser. Man saugt die Waschflüssigkeit möglichst vollständig ab und schüttelt das noch feuchte Produkt in einem Erlenmeyerkolben mit 80 ml dest. Wasser 30 bis 45 sec lang kräftig durch. Die so erhaltene Suspension wird in üblicher Weise, entweder mit dem Stahlschen Streichgerät[3] oder mit dem Serva-Streichstab†, auf entfettete Glasplatten aufgetragen. Bei einer Schichtdicke von 500 μ erhält man etwa 12 Platten (10 × 20 cm). Man lässt die Schichten über Nacht bei Raumtemperatur trocknen. Um eine gerade Laufmittelfront zu erhalten, empfiehlt es sich, die Schicht an beiden Längsseiten mit einem Spatel etwa 5 mm breit abzustreifen. Ausserdem ritzt man am unteren Plattenrand parallel zu den Längsseiten etwa 2 cm lange und 0.5 mm breite Linien im Abstand von jeweils 5 mm in die Schicht ein.

Die so hergestellten PP-Cellulose-Schichten sind vollständig homogen und mechanisch so stabil, dass man auf ihnen wie auf Papier schreiben kann.

Trennbeispiele

Vor Ausführung der Chromatographie entwickelt man die Platten mit dest. Wasser vor und lässt sie anschliessend 15–20 Stunden lang an der Luft trocknen.

Die Kationenaustauscher-Schichten sind beispielsweise zur Trennung von Nucleobasen und Nucleosiden geeignet. Die Verbindungen werden 3 cm vom unteren Plattenrand entfernt aufgetragen.

---

* Fa. Macherey und Nagel, Düren, Rheinland, Deutschland.

** Eine 50 % Polyäthylenimin-Lösung ("Polymin P") wird von der Badischen Anilin- und Sodafabrik, Ludwigshafen a. Rh., Deutschland, hergestellt.

*** Natriummetaphosphat, Fa. Benckiser, Ludwigshafen a. Rh., Deutschland.

† Serva-Entwicklungslabor, Heidelberg, Deutschland.

Trennung von Nucleobasen. Laufmittel: 100 ml Citrat–HCl-Puffer nach Sörensen pH 3.7 [49,9 ml 0.1 *N* Salzsäure + 50.1 ml 0.1 *M* Natriumcitrat-Lösung (21.008 g Citronensäure-Monohydrat + 200 ml *N*-Natronlauge, aufgefüllt auf 1 l] werden im Messkolben nach Zugabe von 0.01 Mol $MgSO_4$ (1.2039 g) mit dest. Wasser auf 500 ml aufgefüllt. Bei einer Laufstrecke von 7 cm (Laufzeit 25 bis 30 min im geschlossenen Gefäss) werden die Nucleobasen Uracil, Cytosin, Adenin und Guanin getrennt. Die R_F-Werte liegen zwischen 0.4 und 0.8, sie nehmen in der Reihenfolge Uracil > Cytosin > Adenin > Guanin ab. Nur Guanin zeigt eine gewisse Neigung zur Schwanzbildung; diese stört jedoch nicht, da Guanin die am langsamsten laufende Nucleobase ist.

Trennung von Nucleosiden. Laufmittel: 100 ml Citrat–HCl-Puffer nach Sörensen pH 3.7 (s. oben) werden nach Zugabe von 0.01 Mol $MgSO_4$ (1.2039 g) mit dest. Wasser auf 300 ml aufgefüllt. Bei einer Laufstrecke von 7 cm werden die Nucleoside Uridin, Cytidin, Adenosin und Guanosin getrennt. Die R_F-Werte liegen zwischen 0.5 und 0.9, sie nehmen in der Reihenfolge Uridin > Cytidin > Adenosin > Guanosin ab.

Zum Nachweis der Verbindungen betrachtet man das Chromatogramm im kurzwelligen U.V.-Licht*. Noch etwa 3.10^{-4} μmol (0.04 μg) Adenin lassen sich unter den angegebenen Bedingungen in einem vollständig dunklen Raum mit blossem Auge erkennen. Die untere Nachweisgrenze von Adenin liegt bei der Papier-Chromatographie dagegen bei etwa 1 μg (Photoprintverfahren); bei Betrachtung mit blossem Auge ist die Empfindlichkeit noch geringer[4]. Bei Dünnschicht-Chromatographie auf nichtmodifizierter Cellulose lassen sich etwa 0.1 μg Adenin (mit blossem Auge) eben erkennen[5].

Besonders vorteilhaft ist, dass sich auf PP-Cellulose-Schichten wesentlich grössere Mengen ohne Schwanzbildung chromatographieren lassen als auf Schichten aus nichtmodifizierter Cellulose. Die Chromatographie an den Kationenaustauscher-Schichten ist deshalb besonders zum Spurennachweis geeignet.

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Eingegangen den 8. Januar 1963

* Mineralight-Lampe der Fa. Ultraviolet Products, Inc., San Gabriel, Calif., U.S.A.

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Notes

A new method for the detection of some aromatic acids

The classical indicator methods for the detection of aromatic acids on paper chromatograms are relatively insensitive and yield transient colours. A recent method[1] involving the detection of the sodium salts of acids with chloranilic acid is sensitive to 1 μg but its use is limited to chromatograms developed with unbuffered and non-acidic solvent systems. This paper describes a new sensitive method based on the formation of fluorescent hydroxy derivatives by means of hydroxyl radicals photochemically generated in dilute aqueous hydrogen peroxide.

Experimental

All compounds, except terephthalic acid (for which pyridine was the solvent) were dissolved in ethanol and 5 μl aliquots (5 μg) were applied to Whatman No. 1 paper for descending chromatography. The chromatograms after development and drying were lightly sprayed with an aqueous 0.3% solution of hydrogen peroxide. The wet chromatograms were placed on a glass plate and were illuminated with a Mineralight U.V. lamp (Model V-41) equipped with a filter to absorb most of the emitted visible radiation above 400 mμ. Within a few minutes blue fluorescent spots became visible.

TABLE I

R_F VALUES AND SENSITIVITIES OF AROMATIC ACIDS TO THE U.V.–HYDROGEN PEROXIDE DETECTION METHOD

Compound	$R_F \times 100$			*Sensitivity* (μg)
	Solvent A	*Solvent B*	*Solvent C*	
Terephthalic acid	0	24*	40*	0.2
Isophthalic acid	0	31*	52	1
o-Phthalic acid	0	23*	31	2
Benzoic acid	47	not detected	not detected	2
Trimesic acid	0	1	10	0.5
Phenylacetic acid	not detected	not detected	not detected	(>>20)**
o-Toluic acid	not detected	not detected	not detected	(>>20)**
m-Toluic acid	59	not detected	90	2
p-Toluic acid	57	95	90	2
o-Chlorobenzoic acid	not detected	not detected	not detected	(>>20)**
m-Chlorobenzoic acid	69	91	89	3
p-Chlorobenzoic acid	68	91	89	3
2,4-Dichlorobenzoic acid	74	not detected	88	4
3,4-Dichlorobenzoic acid	75	90	88	3
3,4,5-Trimethoxybenzoic acid	44		84	1

Solvent A: *n*-butanol saturated with 5 *N* NH_4OH[2]; Solvent B: solvent "B" of REIO[3]; Solvent C: benzene–acetic acid–water (2:2:1)[4].

* Streaky spot.

** 20 μg gives no detectable fluorescence.

Results and discussion

R_F values and sensitivities for the aromatic acids investigated are shown in Table I. The sensitivity is the minimum weight of compound found to yield an easily seen fluorescence in a spot approximately 1 cm^2 in area when viewed in a darkened room. No solvent development was done in those experiments in which sensitivity was measured.

The maximum fluorescence from terephthalic acid, isophthalic acid, trimesic acid and 3,4,5-trimethoxybenzoic acid was usually attained after illumination for about 1/2 min. For the other compounds yielding a positive reaction illumination for at least 3 min was required for maximum fluorescence. Prolonged illumination of terephthalic acid spots changed the colour of their fluorescence from blue to white.

The failure to detect benzoic acid, *m*-toluic acid, and 2,4-dichlorobenzoic acid in some solvent systems may be due to interference from the fluorescent solvent front.

Since the 6-methylsalicylic acid at least is known to be fluorescent[3], the apparent absence of reaction with *o*-toluic acid must be attributed to preferential radical attack on the methyl group to produce non-fluorescent products. It is noteworthy that BATES AND URI[5] found no evidence for hydroxylation of *o*-toluic acid by radicals photochemically generated from the ferric ion-pair complex $Fe^{3+}OH^-$. Also in the case of phenylacetic acid, it is probable that radicals attack the side-chain in preference to the ring.

Attempts were made to produce fluorescent reaction products by photochemical decomposition of the compounds in the absence of hydrogen peroxide. For this purpose unfiltered radiation from the Mineralight lamp was used to illuminate wet (sprayed lightly with water) samples of the acids. In only one compound 3,4-dichlorobenzoic acid was an intense fluorescence (white) detected. Very faint fluorescence was detected for *p*-chlorobenzoic acid, 2,4-dichlorobenzoic acid, trimesic acid, and terephthalic acid.

Although the method is by no means general for aromatic acids, a number of acids can be detected because many hydroxy acids fluoresce. Even if specific isomers are non-fluorescent, the mixture of isomeric hydroxy acids produced may well fluoresce. Prolonged illumination and respraying with peroxide to produce dihydroxy acids might prove useful in cases where the monohydroxy acids do not fluoresce. The hydroxylation technique under carefully controlled conditions followed by subsequent rechromatography might yield unique reproducible product patterns useful for the identification of complex aromatic molecules.

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Detection of compounds on paper by fluorescence and phosphorescence at liquid nitrogen temperatures

We would like to call attention to a little used but valuable technique suggested by SZENT-GYÖRGYI[1]. This technique consists of the detection of substances on paper chromatograms at liquid nitrogen temperature by means of fluorescence or phosphorescence in ultraviolet light. The paper sheet is simply placed in a pyrex baking dish, cooled with a liberal quantity of liquid nitrogen, and examined with an ultraviolet light.

A Mineralight, Model V-41, Ultra-Violet Products, Inc., San Gabriel, Calif., maximum output at 2537Å, is used as the source of exciting radiation. Some compounds fluoresce strongly when directly illuminated, while other compounds can be more readily seen by means of the slowly decaying phosphorescence which persists after the lamp is turned off. The phosphorescence is accentuated by the dark background of the paper. Examples are seen in Fig. 1, 2, and 3. Since the fluorescence

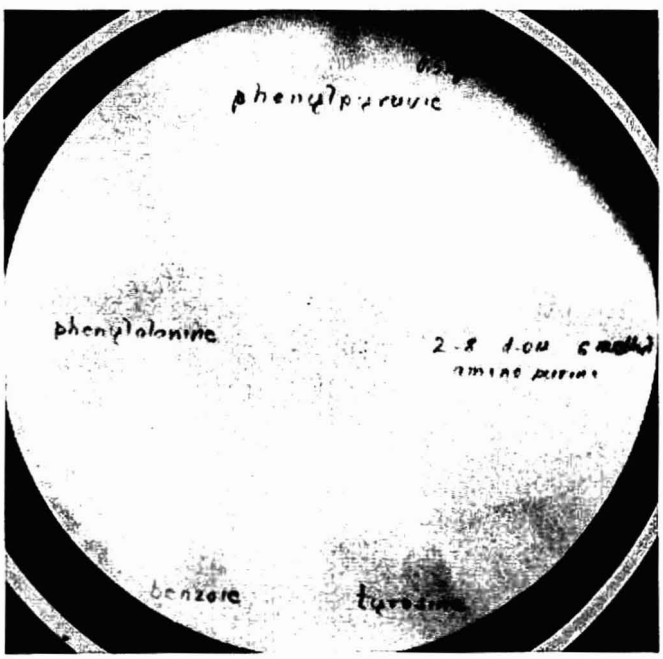

Fig. 1. Compounds photographed at room temperature with the exciting source on. The compounds, 5 μg per spot, are given in clockwise order starting at 12:00: (1) Phenylpyruvic acid; (2) 2,8-dihydroxy-6-methylaminopurine; (3) L-tyrosine; (4) benzoic acid; (5) L-phenylalanine. The film was Ansco, "Super Hypan"; a 155 mm lens at F/4, and a Wratten G filter were used. Exposure time: 15 sec. Photography was used to obtain a permanent record.

of various ionic species of a given compound can vary, exposure of the paper to ammonia or hydrochloric acid fumes may intensify the fluorescence. It is important to note that this technique is usually not applicable when the paper has been exposed to ultraviolet absorbing solvents such as phenol or pyridine.

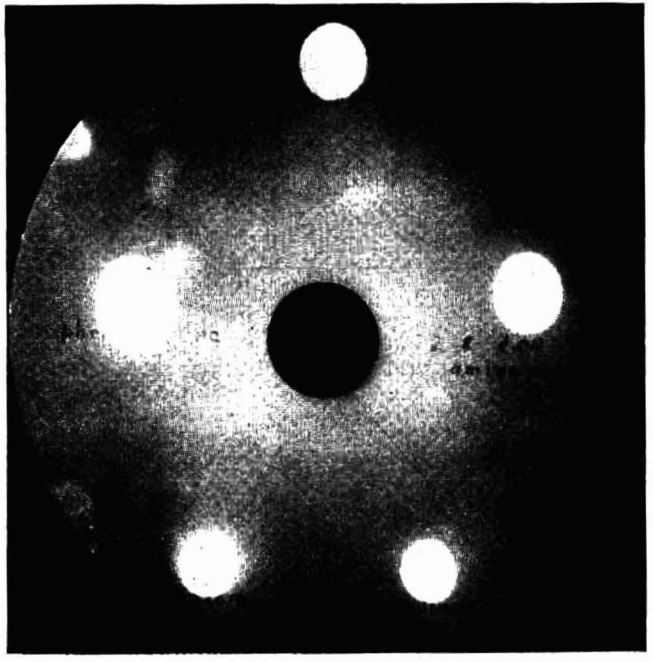

Fig. 3. Compounds photographed at liquid nitrogen temperature with the exciting source removed. No filter was used. Exposure time 40 sec. For other conditions see legend to Fig. 1. Note the intense phosphorescence of all the compounds.

Fig. 2. Compounds photographed at liquid nitrogen temperature with the exciting source on. A Wratten G filter was used. Exposure time 30 sec. For other conditions see legend to Fig. 1. Note the intense fluorescence of the substituted purine. (The dark spot is a coin to hold the paper steady during photography.)

In our experience, any compound, including proteins, containing an aromatic ring system may be readily detected in trace amounts by these procedures. The great advantage of this procedure is that the compounds are not destroyed or altered in any way. The fluorescence and phosphorescence are easily visible with the naked eye.

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Methyl yellow as a spray reagent in the paper chromatography of chlorinated hydrocarbon pesticides

N,N-Dimethyl-*p*-phenylazoaniline (methyl yellow) has been reported to undergo a color change when a chloroform solution of the dye is irradiated with X-rays[1]. This reaction suggested a possible application in the detection of chlorinated hydrocarbon pesticides on paper chromatograms. When the chromatograms were sprayed with a solution of methyl yellow and exposed to ultra-violet radiation, the pesticides appeared as red spots against a yellow background. Fourteen pesticides containing chlorine were tested and all were easily detected by the reagent.

The spray reagent was prepared by dissolving 100 mg of methyl yellow in 60 ml of ethanol in a 100 ml volumetric flask. Twenty-five ml deionized water was added and the solution brought to volume with ethanol. Paper chromatograms were prepared by spotting known amounts of the pesticide solution (1 μg/μl) on Whatman No. 1 chromatographic paper (8 × 8 in.). The papers were then impregnated with a 5 % solution of cottonseed oil in ethyl ether[2], and developed with pyridine–water (6:4, v/v).

Upon removal from the chromatographic tank, the chromatograms were dried in air and sprayed with the methyl yellow solution until the paper appeared uniformly wet. After being again dried in air, the sheets were finally exposed for five minutes to 30 W of ultra-violet radiation. The ultra-violet source, consisting of two 15 W 20-in. germicidal lamps in a reflector housing, was placed 2 to 4 in. above the paper chromatograms.

Minimum amounts of chlorinated pesticides which could be detected ranged from 2 to 8 μg per spot (Table I). All pesticides tested could be detected above a level of 8 μg in the chromatographic system described, the spots ranging from 5 to 10 mm in diameter. The red color formed by the methyl yellow and pesticide was quite

intense at first but gradually faded over a period of one hour. Additional exposure to ultra-violet radiation did not restore the color. However, the sprayed chromatograms could be held as long as two days before being exposed to ultraviolet radiation with no loss of the original intensity of the spots.

TABLE I

SENSITIVITY OF SPRAY REAGENT

*Pesticide**	*Minimum amount detectable μg***
Toxaphene	4
DDT	2
Aldrin	4
Chlordane	4
Endrin	4
Heptachlor	4
Methoxychlor	4
Perthane	4
Rothane	4
2,4-D	8
Dieldrin	4
Chlorobenzilate	8
Kelthane	2
Lindane	4

* Standard solutions of pure chlorinated hydrocarbon pesticides.
** Spots ranged 5–10 mm in diameter.

Although hexane, alcohol and acetone were good solvents for methyl yellow, aqueous ethanol (25 % water) was the most suitable and gave the most sensitive spray reagent. Addition of 2-phenoxyethanol, which was reported to increase the sensitivity of the $AgNO_3$ spray reagent[3], gave no improvement in either sensitivity or the time required for the appearance of the spots. The methyl yellow spray reagent, although not as sensitive as the $AgNO_3$ reagent, has the advantage that it is not affected by impurities in the paper as is the $AgNO_3$ reagent. Furthermore, the "curtain" effect which appears with the $AgNO_3$ reagent and which masks spots found near the solvent front, is eliminated so that the spots having high R_F values are clearly visible.

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Dünnschicht-Chromatographie *cis-trans* isomerer Carbonsäuren

Während unserer Versuche zur Dünnschicht-Chromatographie von Dicarbonsäuren[1] beobachteten wir das unterschiedliche chromatographische Verhalten von Malein- und Fumarsäure. Dies veranlasste uns zur Untersuchung anderer *cis-trans* isomerer Carbonsäuren.

Bereits 1943 beschrieben KAUFMANN UND WOLF[2] eine chromatographische Trennung stereoisomerer Säuren. Später wurden derartige Versuche papierchromatographisch bearbeitet. Auch die Dünnschicht-Chromatographie wurde schon zur Unterscheidung von Stereoisomeren herangezogen, so von PETROWITZ[3] und MORRIS *et al.*[4]; auch MANGOLD[5] weist auf diese Möglichkeit hin.

Wir führten unsere Versuche auf 0.3 mm starken Kieselgelschichten durch. Als Fliessmittel wurden die bewährten Gemische von Benzol–Methanol–Eisessig (45:8:4; v/v) (BME) und Benzol–Dioxan–Eisessig (90:25:4) (BDE) benutzt. Die Steighöhe betrug 10 cm, die Laufzeit der Chromatogramme etwa 30 min. Die Fehlergrenze beträgt $\pm$ 0.02. Die Flecke wurden durch Besprühen mit sodaalkalischer Permanganatlösung sichtbar gemacht. In Tabelle I sind die R_F-Werte zusammengestellt.

TABELLE I

Substanz	*R_F-Werte in:*	
	BME	*BDE*
Isocrotonsäure	0.70	0.71
Crotonsäure	0.73	0.73
Tiglinsäure	0.71	0.79
Maleinsäure	0.13	0.06
Fumarsäure	0.43	0.22
Citraconsäure	0.18	0.07
Mesaconsäure	0.55	0.53
Itaconsäure	0.46	0.34
cis-Aconitsäure	0.03	0.04
trans-Aconitsäure	0.12	0.04

Vergleicht man die Konfiguration der Säuren mit ihrem chromatographischen Verhalten, so erkennt man leicht, dass in allen Fällen die *trans*-Verbindungen schneller wandern als die *cis*-Verbindungen. Aber auch weitere Unterschiede in den Wanderungsgeschwindigkeiten können durch Betrachtung der Konstitutionen erklärt werden. Ausser der allen beschriebenen Verbindungen gemeinsamen Doppelbindung haben die Crotonsäuren nur eine stark adsorptionsfähige Carboxylgruppe. Die Adsorption ist relativ gering und daher liegen die R_F-Werte hoch. Tritt eine Methylgruppe in die Molekel ein, so wird die Adsorption etwas herabgesetzt. Die Tiglinsäure (Methylcrotonsäure) besitzt daher einen höheren R_F-Wert. Die stärkere Adsorption von zwei Carboxylgruppen in der Malein- und Fumarsäure wird in der kürzeren Wanderungsstrecke deutlich. Tritt eine Methylgruppe in die Molekel ein, so steigen die R_F-Werte auch hier an, wie es an der Citraconsäure (Methylmaleinsäure) und an der Mesaconsäure (Methylfumarsäure) zu erkennen ist. Interessant ist der im Verhältnis zur Citraconsäure höher liegende R_F-Wert der Itaconsäure (Methylenbernsteinsäure). Als Grund dafür kann die stärkere Konjugation der Doppelbin-

dungen in der Citraconsäure angesehen werden; durch sie wird eine stärkere Adsorption und damit ein kleinerer R_F-Wert bewirkt. Die geringere Konjugation der Itaconsäure wird im grösseren R_F-Wert deutlich. Die Aconitsäuren haben drei Carboxylgruppen in der Molekel; die R_F-Werte sind nur noch sehr klein.

Die stark adsorbierende Wirkung von Hydroxygruppen ist auch bei Carbonsäuren zu erkennen. So hat die Citronensäure in beiden Fliessmitteln die R_F-Werte 0.02 und die Dihydroxyfumarsäure bleibt völlig am Startpunkt zurück.

Auch zwei Säureanhydride konnten in die Versuche einbezogen werden, Maleinsäureanhydrid und Citraconsäureanhydrid.

Maleinsäureanhydrid zeigt im Chromatogramm neben Maleinsäure einen Fleck mit den R_F-Werten 0.67 (BME) und 0.56 (BDE). Ein käufliches Citraconsäureanhydrid liess vier Flecke erkennen. Neben Citraconsäure zeichnet sich vor allem ein deutlicher Fleck bei 0.77 (BME) und 0.86 (BDE) ab. Daneben konnten noch drei weitere Flecke erkannt werden, von denen einer eventuell der Mesaconsäure zugeordnet werden kann. Es ist unklar, ob die anderen Flecken durch Verunreinigungen bedingt wurden.

Erkennbar wird jedoch die geringere Adsorption der Säureanhydride am Kieselgel im Vergleich zu den Carbonsäuren.

Anmerkung

Während der Drucklegung veröffentlichten E. KNAPPE UND D. PETERI (*Z. Anal. Chem.*, 190 (1962) 380) eine Arbeit über die dünnschicht-chromatographsche Trennung ungesättigter Dicarbonsäuren.

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Eingegangen den 20. August 1962

A simple saturation chamber for thin layer chromatography

It has often been reported that the R_F of a substance spotted near the edge of a thin layer chromatogram[1] is different from that when spotted at the centre. This *edge effect* was at first attributed to a difference in the layer thickness between the edges and the centre of the plate[1,2]. The effect, however, persisted even when adsorbent layers of constant thickness were prepared, so the phenomenon was reinvestigated. It has been found that the critical factor is the degree of saturation of the air in the

chromatography chamber with solvent vapour; the less saturated the atmosphere, the more pronounced is the effect. The effect can be reduced by running the ascending chromatogram in a chamber fitted with layers of thick filter paper which soak up the solvent which then quickly saturates the gaseous phase. A straight solvent front is obtained when the adsorbent is scraped from the edges of the plate prior to chromatography[3]. The *edge effect* can also be significantly reduced, if not eliminated, by reducing the size of the chamber[4].

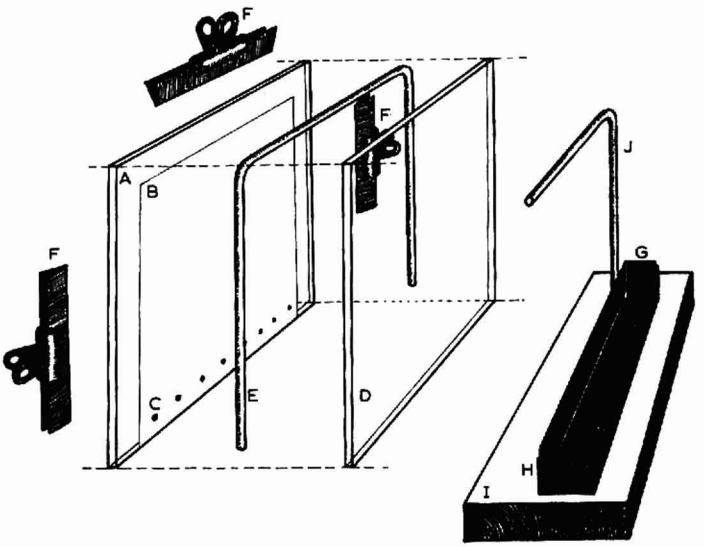

Fig. 1. Components of the chamber.

This communication describes the construction of a simple and inexpensive, yet highly efficient chamber for developing thin layer chromatograms under conditions of high chamber saturation with the solvent, brought about by a reduction of the size of the chamber. It is based on the principle of STAHL's *S-Chamber*[5].

Description of apparatus

The chamber is formed from two plates of 32 oz. glass (20 × 20 cm), one of which (A) is the plate supporting the solid phase, the thin layer of adsorbent (B) (see Fig. 1). The adsorbent is removed from three edges of the adsorbent layer to leave a clear margin 1.5 cm wide at the top and two sides. The samples (C) are now applied to the adsorbent layer along a line 1.5 cm from the bottom edge. The cover plate (D) is placed over the adsorbent layer, but is held just under 3 mm away from it by a glass spacer (E). This is made from a 55 cm length of 3 mm diameter glass rod which is bent at right angles 18 cm from each end so that it forms three sides of a rectangle. It is held between the two sheets of glass with its 19 cm middle section at the top. The two glass plates are held firmly by three large (5.5 in.) "Bulldog" clips (F). It is important that the bends in the glass rod should be flush with the glass plates; any projection of the glass can be removed with a file. The chamber is now open at only the lower end, and this end is placed in the solvent trough (see Fig. 2).

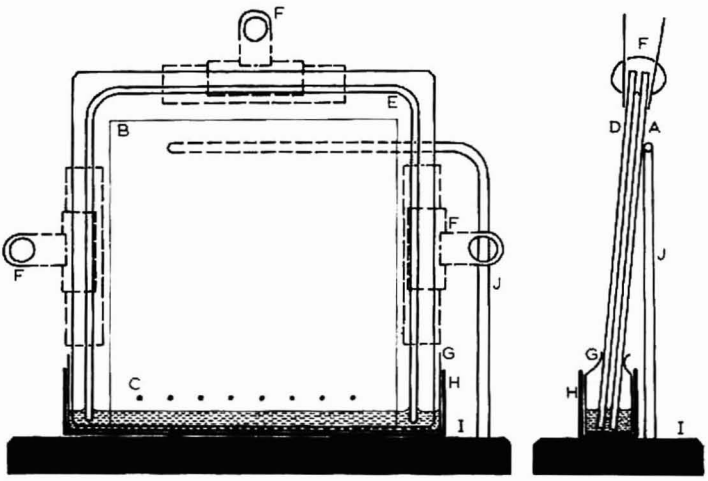

Fig. 2. Construction of the apparatus.

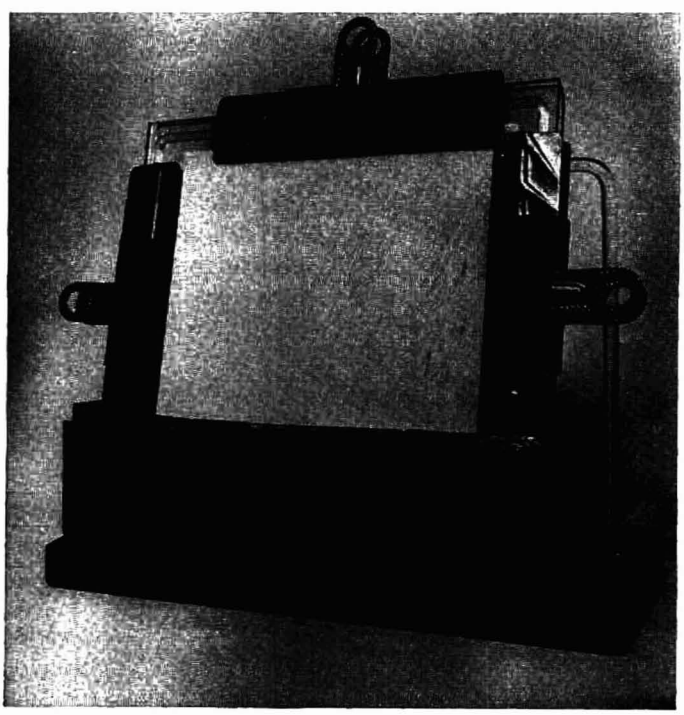

Fig. 3. Front view of the assembled apparatus.

The trough which has been used for organic solvents is made from several layers of aluminium foil (G). This is placed inside a strong cardboard trough (H) which is held to a wooden baseboard (I) with drawing pins. A 5 mm diameter glass rod with a single right angled bend (J), which is fitted into a hole drilled in the baseboard, is sufficient to hold the chamber vertically. The solvent is placed in the trough to a depth of 0.5–1.0 cm. The advantage of using an aluminium foil trough for organic solvents is that its edges can be bent over on to the chamber to reduce evaporation of the solvent from the trough (see Fig. 2). The foil trough can be replaced by a glass one for use with aqueous solvents.

The size of the chamber is, of course, not limited to 20 × 20 cm. Glass plates as long as 50 cm have been used to carry out the simultaneous chromatographic separation of up to 50 samples. In this case, a 60 cm glass trough was used to hold the solvent.

Discussion

The results obtained with this technique have been compared with those from experiments using a standard thin layer chromatography chamber (22 × 22 × 7 cm) fitted with filter paper. In the case of lipid samples chromatographed with non-aqueous solvents, the 3 mm chamber described above gave a much better separation. The spots are much smaller, and the complete separation of mixtures of carotenes[6] or of other isoprenoid hydrocarbons[7,8] can be accomplished in 15 min. The high chamber saturation with solvent vapour results in the absence of the *edge effect*, so that R_F values are constant over the whole length of the chromatograms.

This technique has also been used to separate substances requiring the use of aqueous solvents. In this case, there is little difference between these results and those obtained using the larger standard chamber, since the running time of chromatograms with aqueous solvents is necessarily longer than with organic ones.

The author is grateful to his colleagues who have thoroughly tested this technique for their various requirements, to Professor T. W. GOODWIN for his interest and encouragement, and to Professor E. STAHL for demonstrating the principle of his "S-chamber" on a recent visit to this laboratory.

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CHROMATOGRAPHIC DATA

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TABLE 1

R_F VALUES (RELATIVE) OF SOME AMINO SUGARS

(R. W. JEANLOZ, Z. T. GLAZER AND D. A. JEANLOZ, *J. Org. Chem.*, 26 (1961) 532)

Solvent: Pyridine–ethyl acetate–water–acetic acid (5:5:3:1) (F. G. FISCHER AND H. J. NEBEL, *Z. Physiol. Chem.*, 302 (1955) 10).
Paper: Whatman Nos. 1 and 54 (not specified which was used); descending.
Detection: Not specified.

Hydrochloride of	$R_{Glucosamine}$*
D-Glucosamine	1.00
D-Talosamine	1.06, 1.07
D-Gulosamine	1.04
D-Galactosamine	0.89

* $R_{Glucosamine} = R_F$ compound/R_F glucosamine.

TABLE 2

R_F AND RELATIVE R_F VALUES OF DERIVATIVES OF AMINO-DEOXY-SUGARS AND RELATED COMPOUNDS

(R. W. JEANLOZ AND D. A. JEANLOZ, *J. Org. Chem.*, 26 (1961) 537)

Solvents: S_1 = Pyridine–ethyl acetate–acetic acid–water (5:5:1:3); pyridine–ethyl acetate–water (11:40:6) at bottom of tank (F. G. FISCHER AND H. J. NEBEL, *Z. Physiol. Chem.*, 302 (1955) 10).
S_2 = Propan-1-ol–water–ammonia (70:29:1).
Paper: P_1 = Whatman No. 54 (descending).
P_2 = Whatman No. 1 (descending).
Detection: D_1 = Alkaline $AgNO_3$ reagent.
D_2 = Ninhydrin reagent.
D_3 = Aniline hydrogen phthalate reagent.

Compound	R_F			$R_{Glucosamine}$*	
	S_1P_1	S_1P_2	S_2P_2	S_1P_1	S_2P_1
3-Acetamido-3-deoxy-D-idose				1.60	1.13
N-Acetyl-D-glucosamine				1.52	1.10
3-Amino-1,6-anhydro-3-deoxy-β-D-idopyranose·HCl		0.46	0.67		
D-Glucosamine·HCl		0.29	0.52		
D-Galactosamine·HCl		0.25	0.47		
2-Amino-1,6-anhydro-2-deoxy-β-D-galactopyranose·HCl		0.38	0.58		
2-Amino-1,6-anhydro-2-deoxy-β-D-gulopyranose·HCl		0.41	0.65		
Glucosamine	0.85				
Galactosamine				0.85	
2-Amino-1,6-anhydro-2-deoxy-β-D-gulopyranose	1.46				
3-Amino-1,6-anhydro-3-deoxy-β-D-gulopyranose	1.59				
3-Amino-3-deoxy-D-gulose	1.05–1.06				

* $R_{Glucosamine} = R_F$ compound/R_F glucosamine.

TABLE 3

R_F VALUES OF ARABINOSE AND BENZYL DERIVATIVES

(F. WOLD, *J. Org. Chem.*, 26 (1961) 197)

Solvent: Butan-1-ol–water (3:2) with enough acetic acid to make a one-phase system.
Paper: Not specified (ascending).
Detection: D_1 = Benzidine spray (R. H. HORROCKS, *Nature*, 164 (1949) 444).
D_2 = Benzidine–periodate spray (J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132).

Compound	R_F	*Colour**	
		D_1	D_2
Arabinose	0.35	+	
Benzylarabinoside	0.85	+	
Benzyl-2-O-benzylarabinopyranoside	0.95	—	
Hydrochloric acid	0.50		YB

* + = positive; — = negative; Y = yellow; B = brown.

TABLE 4

R_F AND RELATIVE R_F VALUES OF NEOSAMINE C (2,6-DIAMINO-2,6-DIDEOXY-D-GLUCOSE) DERIVATIVES AND RELATED COMPOUNDS

(K. L. RINEHART, M. HICHENS, K. STRIEGLER, K. R. ROVER, T. P. CULBERTSON, S. TATSUOKA, S. HORII, T. YAMAGUCHI, H. HITOMI AND A. MIYAKE, *J. Am. Chem. Soc.*, 83 (1961) 2964)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5).
S_2 = Pyridine–ethyl acetate–water (1:2:2).
S_3 = Butan-1-ol–acetic acid–water (2:2:1) (K. L. RINEHART AND P. W. K. WOO, *J. Am. Chem. Soc.*, 83 (1961) 643).
Paper: *cf.* K. L. RINEHART AND P. W. K. WOO, *loc. cit.*
Detection: U.V. light (presumed).

Compound	R_{NAG}* S_1	R_F	
		S_2	S_3
N,N′-Diacetylneosamine C	1.33		
N-Acetyl-6-O-tosyl-α-D-glucosaminide		0.90	
2,6-Diacetamido-2,6-dideoxy-α-D-glucosamide			0.77
2,6-Diamino-2,6-dideoxy-D-glucose·diHCl			0.17
N,N′-Diacetyldiaminoglucose	1.32		
Neosamine C·diHCl			0.17

* R_{NAG} = R_F compound/R_F N-acetyl-glucosamine.

TABLE 5

R_F VALUES OF PHENYLAZO DERIVATIVES OF SIMPLE PHENOLS

(A. I. YUDINTSKAYA, *Zhur. Priklad. Khim.*, 34 (1961) 395)

Solvent: Methyl ethyl ketone–water (1:1).

Paper: Leningrad Paper Factory No. 2, type B (exposed to atmosphere in tank saturated with water vapour for 1.5 h) (descending).

Detection: Visible light (phenols converted to phenylazo derivatives prior to run, *cf.* D. SHLEEDE, *Referat. Zhur. Khim. i Khim. Tekhnol.*, 1 (1956) 82 and R. L. HOSFIELD, *J. Am. Chem. Soc.*, 73 (1951) 852).

Original phenol	R_F	*Colour**
Pyrogallol	0	B
Hydroquinone	0	B
Catechol	0	RB
Hydroquinone dimethyl ether	0	Oc
Resorcinol	0.05 (0.04–0.06)**	SY
Dimethylguiacol	0.06	Ra
Propylguiacol	0.09	Ra
Methylpyrogallol dimethyl ether	0.10	Ra
Pyrogallol dimethyl ether	0.10	Ra
Guiacol	0.15 (0.13–0.17)	ORe
Phenol	0.25 (0.19–0.30)	LY
m-Cresol	0.41 (0.32–0.44)	Y
o-Cresol	0.43 (0.30–0.51)	Oc
Methylguiacol	0.45 (0.38–0.56)	WR
α-Naphthol	0.47 (0.41–0.53)	Ra
p-Cresol	0.82 (0.79–0.89)	ReY
β-Naphthol	0.88 (0.80–0.91)	Br
Thymol	0.93 (0.89–0.97)	O

* B = brown; R = red; Oc = ochre; S = scarlet; Y = yellow; Ra = raspberry; O = orange; Re = rose; L = lemon; W = wine; Br = brick.

** Range given in parentheses.

TABLE 6

R_F VALUES OF PRODUCTS FROM ALTERNATE SYNTHESES OF N-ACETYL-3,5-DIIODO-4-CYCLOHEXYLOXY-DL-PHENYLALANINE

(E. C. JORGENSEN AND P. A. LEHMAN, *J. Org. Chem.*, 26 (1961) 894)

Solvent: Butan-1-ol–dioxan–2 *N* ammonia (4:1:5).

Paper: Whatman No. 3 MM (ascending).

Detection: D_1 = U.V. light (quartz envelope).

D_2 = Ceric sulphate–sodium arsenite reagent (C. H. BOWDEN, N. F. MACLAGAN AND J. H. WILKINSON, *Biochem. J.*, 59 (1955) 93).

Compound	R_F
N-Acetyl-3,5-diiodo-DL-tyrosine	0.22, 0.29
N-Acetyl-DL-thyroxine*	0.92
N-Acetyl-3,5-diiodo-4-cyclohexyloxy-DL-phenylalanine	0.75
N-Acetyl-3,5-diiodo-4-(3-cyclohexenyloxy)-DL-phenylalanine	0.71

* Tentative identification.

TABLE 7

R_F VALUES OF IODIDE, IODOTYROSINES, IODOTHYRONINES AND THEIR DERIVATIVES

(F. BJÖRKSTÉN, R. GRÄSBECK AND B.-A. LAMBERG, *Acta Chem. Scand.*, 15 (1961) 1165)

Solvents: S_1 = Butan-1-ol–0.5 *M* NH_4OH–ethanol-water (20 vol. butan-1-ol added to 20 vol. 0.5 *M* NH_4OH; shaken and phases separated; 2 vol. 94% (w/w) ethanol and 1 vol. H_2O added to organic phase). All aqueous phase and 40% organic phase used for saturation of atmosphere. Aqueous phase must be 0.3–0.4 *M* in NH_4OH (R. GRÄSBECK, B.-A. LAMBERG AND F. BJÖRKSTÉN, *Acta Endocrinol.*, 34 (1960) 113).
S_2 = Butan-1-ol–ethanol–2 *M* NH_4OH (10:3:10).
S_3 = Toluene–formic acid–ethanol (10:3:3).
S_4 = Water–formic acid (5:1).

Paper: Whatman No. 1.

Detection: D_1 = Pauly reagent (A. TAUROG AND I. L. CHAIKOFF, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 4, Academic Press Inc., New York, 1957, p. 856).
D_2 = Ceric sulphate–arsenious acid reagent (*cf.* R. M. MANDL AND R. J. BLOCK, *Arch. Biochem. Biophys.*, 81 (1959) 25).
D_3 = Ferric chloride–potassium ferricyanide–sodium arsenite reagent (R. GMELIN AND A. I. VIRTANEN, *Acta Chem. Scand.*, 13 (1959) 1469); used with S_4; paper pre-washed with S_4 by elution; 1–2 h.
D_4 = Ninhydrin reagent (J. ROCHE, S. LISSITZKY AND R. MICHEL, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 1, Interscience, New York, 1954, p. 243).
D_5 = Palladous chloride reagent (J. GROSS AND C. P. LEBLOND, *Endocrinology*, 48 (1951) 714).

*Compound**	R_F			
	S_1	S_2	S_3	S_4
I^-	0.27	0.44	0.03	0.79
3-MIT	0.17	0.34	0.14	0.77
3,5-DIT	0.10	0.40	0.17	0.67
3,5-T_2	0.71	0.72	0.18	0.68
3,5,3′-T_3	0.70	0.65	0.22	0.54
3,5,3′,5′-T_4	0.54	0.53	0.29	0.35
3,5-T_2A	0.80	0.78	0.94	0.57
3,5,3′-T_3A	0.79	0.75	0.98	0.36
3,5,3′,5′-T_4A	0.64	0.63	0.98	0.14
3,5,3′-T_3F	0.81	0.74	0.98	0.20
3,5,3′,5′-T_4F	0.70	0.67	0.99	0.12
3,5,3′-T_3P	0.79	0.75	0.97	0.28
3,5,3′,5′-T_4P	0.66	0.72	0.98	0.08

* MIT and DIT = mono- and di-iodotyrosine respectively; T = thyronine; TA = thyroacetic acid; TF = thyroformic acid; TP = thyropropionic acid; numerical subscript = number of iodo-substituents (see D. H. SOLOMON AND J. T. DOWLING, *Ann. Rev. Physiol.*, 22 (1960) 615).

TABLE 8

ELECTROPHORETIC MOBILITIES OF IODIDE, IODOTYROSINES, IODOTHYRONINES AND THEIR DERIVATIVES

(F. BJÖRKSTÉN, R. GRÄSBECK AND B.-A. LAMBERG, *Acta Chem. Scand.*, 15 (1961) 1165)

Electrolyte: Diethyl barbiturate buffer (pH. 8.0; μ = 0.06).
Paper: Whatman No. 1 (presumed).
Potential applied: 7 V/cm.
Mobility: M_{SA} (mobility relative to serum albumin).
Detection: See Table 7 for reagents.

*Compound**	M_{SA}
I^-	3.4
3-MIT	0.59
3,5-DIT	1.09
3,5-T_2	0.23
3,5,3′-T_3	0.10
3,5,3′,5′-T_4	0.20
3,5-T_2A	0.96
3,5,3′-T_3A	0.88
3,5,3′,5′-T_4A	1.34
3,5,3′-T_3F	0.74
3,5,3′,5′-T_4F	1.16
3,5,3′-T_3P	0.80
3,5,3′,5′-T_4P	1.15

* Abbreviations: see Table 7.

TABLE 9

R_F VALUES (RELATIVE) OF SEVERAL TYROSINE DERIVATIVES

(J. W. DAVIES AND G. HARRIS, *J. Chem. Soc.*, (1961) 3193)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:1, v/v).
S_2 = Ethanol–2 *N* ammonia (9:1, v/v).
Paper: See J. W. DAVIES, G. HARRIS AND R. PARSONS, *J. Inst. Brewing*, 62 (1956) 38; (ascending).
Detection: Ninhydrin (presumed).

Compound	R_{Tyr}*	
	S_1	S_2
3-Chlorotyrosine	1.26	0.78
Di-*p*-(2-amino-2-carboxyethyl)-phenyl peroxide	1.26	0.78
Di-[4-(2-amino-2-carboxyethyl)-3-chlorophenyl] peroxide	1.26	0.78
3,5-Dichlorotyrosine	1.58	0.46
Di-[4-(2-amino-2-carboxyethyl)-3,5-dichlorophenyl] peroxide	1.58	0.46

* R_{Tyr} = R_F compound/R_F tyrosine.

TABLE 10

R_F VALUES OF VARIOUS PURINES, THEIR 3-METHYL DERIVATIVES AND RELATED COMPOUNDS

(F. BERGMANN, G. LEVIN, A. KALMUS AND H. KWIETNY-GOVRIN, *J. Org. Chem.*, 26 (1961) 1504)

Solvents: S_1 = 95% ethanol–glacial acetic acid–water (85:5:10).
S_2 = 95% ethanol–pyridine–water (70:20:10).
S_3 = Propan-2-ol–dimethylformamide–25% NH_4OH (65:25:10).
(All by vol.)
Paper: Whatman No. 1 (descending).
Detection: U.V. light (Mineralite U.V. lamp; emission at about 255 mμ).

Compound	R_F			*Colour* (fluorescence)*
	S_1	S_2	S_3	
2-Hydroxypurine	0.25	0.22		sB
3-Methyl deriv.	0.48	0.39		sB
6-Hydroxypurine	0.47	0.40		dB
3-Methyl deriv.	0.35	0.33		dB
8-Hydroxypurine	0.55	0.63		dB
3-Methyl deriv.	0.42	0.41		dB
Xanthine	0.27	0.31		dB
3-Methyl deriv.	0.42	0.49		dB
2,8-Dihydroxypurine	0.22	0.20		B
3-Methyl deriv.	0.39	0.42		B
6,8-Dihydroxypurine	0.30	0.34		dB
3-Methyl deriv.	0.20	0.06		dB
6-Thiopurine	0.52	0.60		B-Y
3-Methyl deriv.	0.40	0.43		Y
6-Methyl thiopurine			0.80	G-Y
3-Methyl deriv.			0.71	G-Y
8-Hydroxy-3-methyl-6-purinone	0.30			dB
3-Methyluric acid	0.40			V

* B = blue; Y = yellow; G = green; V = violet; s = sky; d = dark.

TABLE 11

R_F VALUES OF SOME HYDROXYPURINES AND CERTAIN MERCAPTO DERIVATIVES

(F. BERGMANN AND A. KALMUS, *J. Org. Chem.*, 26 (1961) 1660)

Solvent: 95% ethanol–glacial acetic acid–water (85:5:10).
Paper: Whatman No. 1 (descending).
Detection: U.V. light (Mineralite U.V. lamp; 255 mμ).

Compound	R_F	*Colour* (fluorescence)*
6,8-Dihydroxypurine	0.28	V
8-Hydroxypurine-6-thione	0.42	B
8-Hydroxy-6-methylmercaptopurine	0.73	W
8-Hydroxy-6-benzylmercaptopurine	0.81	B
8-Hydroxypurine	0.55	dB

* V = violet; B = blue; W = white; d = dark.

TABLE 12

R_F AND RELATIVE R_F VALUES OF ADENOSINE POLYNUCLEOTIDES

(R. K. RALPH AND H. G. KHORANA, *J. Am. Chem. Soc.*, 83 (1961) 2926)

Solvents: S_1 = Isobutyric acid–1 *M* NH_4OH–0.1 *M* Na_2EDTA (100:60:1.6, v/v).
S_2 = Propan-2-ol–conc. NH_4OH–water (7:1:2, v/v).
S_3 = Ethanol–0.1 *M* $AcONH_4$ (pH 7.5) (5:2, v/v).
S_4 = Propan-1-ol–conc. NH_4OH–water (55:10:35, v/v).
Paper: Whatman No. 1 or 40 (double acid washed) not more closely specified (descending).
Detection: U.V. light.

*Compound**	*R values*							
	S_1		S_2		S_3		S_4	
	R_F	R_{pA}**	R_F	R_{pA}	R_F	R_{pA}	R_F	R_{pA}
(d)pA	0.61	1.0	0.145	1.0	0.22	1.0	0.42	1.0
(d)pApA	0.583	0.956	0.073	0.500	—	0.13	—	0.76
(d)pApApA	0.558	0.915	0.029	0.2	—	0.037	—	0.585
(d)pA(pA)$_2$pA	0.544	0.892	—	0.1	—	—	—	0.43
(d)pA(pA)$_3$pA	0.520	0.850	—	—	—	—	—	0.31
(d)pA(pA)$_4$pA	0.475	0.780	—	—	—	—	—	0.207
(d)pA(pA)$_5$pA	0.415	0.680	—	—	—	—	—	0.136
(d)pA(pA)$_6$pA	0.378	0.620	—	—	—	—	—	0.10
(d)ApA	0.795	1.29	0.394	2.72	—	1.51	—	1.4
(d)ApApA	0.693	1.125	—	—	—	0.65	—	1.125
(d)A(pA)$_2$pA	0.628	1.02	—	—	—	0.21	—	0.85
(d)A(pA)$_3$pA	0.565	0.92	—	—	—	0.046	—	0.65
(d)A(pA)$_4$pA	0.545	0.885	—	—	—	0.01	—	0.475
(d)A(pA)$_5$pA	0.488	0.79	—	—	—	—	—	0.275
(d)A(pA)$_6$pA	—	—	—	—	—	—	—	0.092
Deoxyadenosine-3′,5′-cyclic phosphate	0.702	1.14	0.38	2.6	—	1.85	0.6	1.43
Cyclic trinucleotide***	0.685	1.11	0.202	1.39	—	0.96	—	1.24

* Abbreviations are those currently used in *J. Biol. Chem.*, and as further developed by H. G. KHORANA AND J. P. VIZSOLYI, *J. Am. Chem. Soc.*, 83 (1961) 675.

** R_{pA} = R_F of compound/R_F of pA.

*** Cyclic trinucleotide:

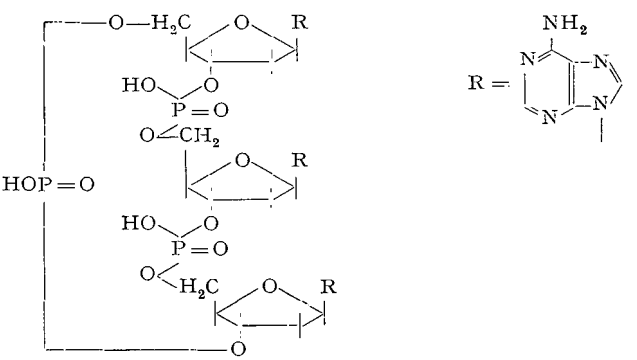


TABLE 13

R_F VALUES OF FOOD DYES

(V. SADINI, *Rass. Chim.*, (1960) 27)

A = Azorubin
B = Echtrot E
C = Amaranth
D = Ponceau 4 R
E = Ponceau 6 R
F = Scharlach GN
G = Erythrosine
H = Sunset Yellow (Gelborange S)
I = Tartrazine
L = C.I. Acid Yellow 3 (Chinolingelb)
M = Indigo Carmine
N = C.I. Acid Blue 3
O = C.I. Food Black 1

50 ml water added to:

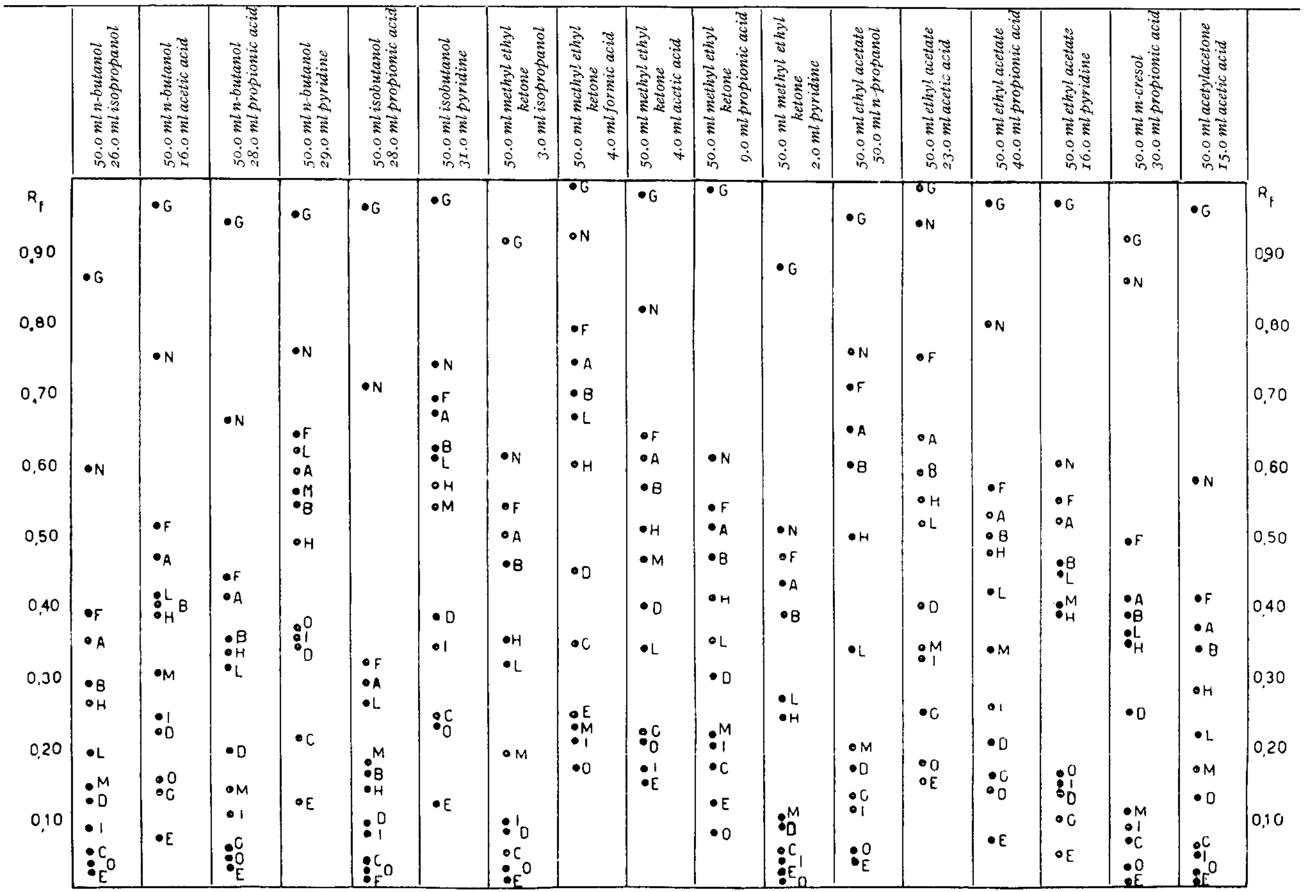


TABLE 14

R_F VALUES OF ALIPHATIC AMINES. COMPARISON OF METHODS

(Cited by J. GASPARIČ, *Chem. Listy*, 55 (1961) 1439)

Solvents: S_1 = *n*-Butanol–acetic acid–water (4:1:5).
S_2 = *n*-Butanol–saturated KCl solution.
S_3 = Formamide–cyclohexane.
Paper: See individual references.
Impregnation: I = KCl.
Detection: See individual references.

Amine	R_F		
	S_1[1]	S_2I[2]	S_3*,[3]
Methylamine	0.34	0.14	0.11
Dimethylamine	0.28	0.18	0.59
Ethylamine	0.38	0.32	0.27
Diethylamine	0.57	0.54	0.88

* As 3,5-dinitrobenzamides.

[1] W. DIHLMAN, *Biochem. Z.*, 325 (1954) 295.
[2] M. VEČEŘA AND J. GASPARIČ, *Chem. Listy*, 52 (1958) 611; *Collection Czech. Chem. Commun.*, 24 (1959) 465).
[3] M. VEČEŘA AND J. GASPARIČ, *Chem. & Ind. (London)*, (1957) 263.

TABLE 15

R_F VALUES OF β-ALANINE AND α-FLUORO-β-ALANINE

(E. D. BERGMANN AND S. COHEN, *J. Chem. Soc.*, (1961) 4669)

Solvents: S_1 = Butan-1-ol–acetic acid–water (75:15:10).
S_2 = Phenol saturated with water.
Paper: Whatman No. 1.
Detection: Not specified.

Compound	R_F	
	S_1	S_2
β-Alanine	0.23	0.57
α-Fluoro-β-alanine	0.08	0.37

TABLE 16

R_F VALUES OF AMINO ACIDS AND THEIR 2,4-DINITROPHENYL AND 2,4-DINITRO-5-AMINOPHENYL DERIVATIVES

(E. D. BERGMANN AND M. BENTOV, *J. Org. Chem.*, 26 (1961) 1480)

Solvents: S_1 = Phenol–water (R. J. BLOCK, R. LESTRANGE AND G. ZWEIG, *Paper Chromatography*, Academic Press Inc., New York, 1952, p. 67).
S_2 = Collidine–lutidine (R. J. BLOCK *et al.*, *loc. cit.*).
S_3 = Butan-1-ol–acetic acid (R. J. BLOCK *et al.*, *loc. cit.*).
S_4 = Butan-1-ol–3% NH_3 (R. J. BLOCK *et al.*, *loc. cit.*).
S_5 = Propan-1-ol–cyclohexane (3:7) (R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224).
S_6 = *tert.*-Amyl alcohol (S. BLACKBURN AND A. G. LOWTHER, *Biochem. J.*, 48 (1951) 126).
S_7 = Ethanol–benzyl alcohol (1:9) (S. BLACKBURN AND A. G. LOWTHER, *loc. cit.*).
S_8 = Butan-1-ol saturated with water (E. F. MELLON, A. H. KORN AND S. R. HOOVER, *J. Am. Chem. Soc.*, 75 (1953) 1675).

Paper: Whatman No. 1.

Detection: Diazotisation agent for dinitroaminophenyl derivatives; spray successively with 10% HCl, 1% aq.$NaNO_2$ and alkaline α-naphthol (1 g α-naphthol in 10 ml 10% NaOH). Chromatogram dried at 50-60°.

Compound	R_F							
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
Free acid								
Glutamic acid	0.31	0.20	0.37	0.01				
Phenylalanine	0.85	0.48	0.66	0.46				
Serine	0.36	0.28	0.31	0.05				
Isoleucine	0.84	0.45	0.68	0.40				
Methionine	0.81	0.42	0.57	0.05				
2,4-Dinitrophenyl derivative								
Glutamic acid					0.05	0.04	0.07	0.14
Phenylalanine					0.74	0.74	0.63	0.71
Serine					0.05	0.21	0.18	0.32
Isoleucine								0.73
Methionine								0.65
2,4-Dinitro-5-aminophenyl derivative								
Glutamic acid								0.26
Phenylalanine								0.56
Serine								0.15
Isoleucine								0.52
Methionine								0.76

TABLE 17

ELECTROPHORETIC MOBILITIES OF VARIOUS AMINO ACIDS AT HIGH POTENTIAL

(G. N. ATFIELD AND C. J. O. R. MORRIS, *Biochem. J.*, 81 (1961) 606)

Electrolytes: E_1 = 2.5% formic acid–7.8% acetic acid, pH 1.85.
E_2 = 2.0% formic acid–20% acetic acid–4 m*M* cadmium acetate, pH 8.1.
E_3 = Pyridine–acetic acid, pH 5.2.
Paper: Whatman No. 3 MM (120 × 13.5 cm).
Apparatus: Modified apparatus (from D. GROSS, *Nature*, 178 (1956) 29; *J. Chromatog.*, 5 (1961) 194).
Potential applied: P_1 = 100 V/cm.
P_2 = 70 V/cm.
Time of run: T_1 = 270 min.
T_2 = 420 min.
T_3 = 200 min.
Mobility: M_1 = Mobility relative to alanine.
M_2 = Mobility relative to arginine.
M_3 = Mobility relative to glutamic acid.
Temperature of run: 2° ± 1°.
Detection: D_1 = Cadmium–ninhydrin reagent (CdOAc (0.050 g) in H_2O (5.0 ml) and HOAc (1.0 ml); acetone (50 ml) added, then ninhydrin (0.500 g); fresh daily).
D_2 = Cadmium–isatin reagent (CdOAc (0.25 g) in H_2O (1.25 ml) and HOAc (0.25 ml) with propan-2-ol (23.75 ml); isatin (0.25 g) added; fresh daily).
Analytical reagent grade chemicals used.

Compound	*Mobility*			
	$E_1P_1M_1T_3$	$E_2P_1M_1T_2$	$E_3P_2M_2T_3$	$E_3P_2M_3T_3$
Alanine	1.00	1.00		
β-Alanine	1.45			
α-Aminobutyric acid	0.90			
Arginine	1.31		1	
Asparagine	0.71			
Aspartic acid	0.61	0.59		1.21
Cysteine	0.60			
Gystine	0.59			
Glutamic acid	0.67	0.66		1
Glutamine	0.69			
Glycine	1.14			
Histidine	1.31		1.06	
Hydroxyproline	0.54			
Leucine	0.77	0.78		
Isoleucine	0.77	0.80		
Alloisoleucine		0.76		
Lysine	1.47		1.11	
Methionine	0.71			
Methionine sulphone	0.57	0.55		
Methionine sulphoxide	0.60			
Ornithine	1.52			
Phenylalanine	0.61	0.61		
Proline	0.69	0.69		
Serine	0.83	0.81		
Taurine	0.03			
Threonine	0.75	0.73		
Tryptophan	0.46			
Tyrosine	0.53	0.53		
Valine	0.81	0.81		
Hydroxylysine			1.04	
Cysteic acid				1.30

TABLE 18

R_F VALUES OF γ-GUANIDINOBUTYRIC ACID

(R. L. BARNES, *Nature*, 193 (1962) 781)

Solvents: S_1 = Ethanol–ammonia–water (18:1:1).
S_2 = Butan-1-ol–propionic acid–water (*cf.* A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS AND W. STEPKA, *J. Am. Chem. Soc.*, 72 (1950) 1710).
S_3 = Ethyl acetate–acetic acid–water (3:1:1).
S_4 = Phenol–water (71:29, w/v).
Paper: Not specified.
Detection: Location of ^{14}C labelled compound.

Compound	R_F			
	S_1	S_2	S_3	S_4
γ-Guanidinobutyric acid	0.23	0.48	0.57	0.90

TABLE 19

R_F VALUES OF GUANIDINE DERIVATIVES DEVELOPED WITH DIFFERENT SOLVENT SYSTEMS

(R. PANT AND S. S. DUBEY, *Biochim. Biophys. Acta*, 41 (1960) 536)

Solvents: S_1 = *n*-Butanol–acetic acid–water (40:10:50).
S_2 = Pyridine–isoamyl alcohol–water (80:40:70).
S_3 = Pyridine–isoamyl alcohol–water–20% (v/v) aq.NH_3, sp. gr. 0.88 (80:40:40:10).
S_4 = Phenol (80%, w/v)–NH_3 (atmosphere).
S_5 = Methanol–water–acetic acid (80:20:10).
Paper: Whatman No. 1.
Temperature of run: 31°.
Time of run: 14 h (S_1–S_4); 6 h (S_5).
Length of run: About 25 cm.
Detection: D_1 = Sakaguchi reagent spray (R. PANT, *Biochem. J.*, 73 (1959) 30) for guanidine derivatives.
D_2 = Fresh 1% (w/v) α-naphthol (15 ml) in 6% (w/v) NaOH + diacetyl (0.1 ml) added just before spraying (J. ROCHE, N. VAN THOAI AND J. L. HATT, *Biochim. Biophys. Acta*, 14 (1954) 71).

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Taurocyamine	0.24	0.63	0.54	0.57	0.56
Lombricine	0.07	0.21	0.18	0.48	0.36
Glycocyamine	0.36	0.43	0.40	0.61	0.70
Guanidino-ethyl phosphate	0.17	0.33	0.29	0.49	0.66
Guanidino-ethanol	0.35	0.50	0.65	0.82	0.72
Creatine	0.33	0.51	0.44	0.82	0.72
Arginine	0.15	0.44	0.37	0.76	0.68

TABLE 20

R_F VALUES OF CERTAIN AMINO ACIDS OF THE CYCLOHEXANE SERIES

(L. MUNDAY, *J. Chem. Soc.*, (1961) 4372)

Solvents: S_1 = Butan-1-ol–acetic acid (4:1) saturated with water.
S_2 = *sym.*-Collidine.
Paper: Whatman No. 3 MM (ascending).
Detection: 0.1% ninhydrin in butan-1-ol (1-amino-2-methylcyclohexanecarboxylic acids only detected in S_2).

Compound	R_F	
	S_1	S_2
1-Amino-*cis*-4-methylcyclohexanecarboxylic acid	0.69	
1-Amino-*trans*-4-methylcyclohexanecarboxylic acid	0.57	
1-Amino-*trans*-3-methylcyclohexanecarboxylic acid	0.73	
1-Amino-*cis*-3-methylcyclohexanecarboxylic acid	0.65	
1-Amino-*cis*-2-methylcyclohexanecarboxylic acid		0.33
1-Amino-*trans*-2-methylcyclohexanecarboxylic acid		0.27

TABLE 21

R_F VALUES OF ISOMERIC TOLUIDINES. COMPARISON OF METHODS

(J. GASPARIČ, *Chem. Listy*, 55 (1961) 1439)

Solvents: S_1 = *n*-Butanol–25% acetic acid.
S_2 = Butyl acetate–pyridine–water (1:5:10).
S_3 = Ethanol–water–acetic acid (20:14:1).
S_4 = Cyclohexane.
S_5 = Ethanol–ammonia (4:1).
S_6 = Hexane.
Paper: P = Acetylated paper. See individual references for others.
Impregnation: I_1 = Liquid paraffin. I_2 = Formamide.
Detection: See individual references.

Compound	R_F						
	S_1[1]	S_2P	S_3I_1[a,2]	S_4I_2[b,3]	S_5I_1[c,4]	S_6I_2[d,4]	*Colour (in S_6)*
o-Toluidine	0.85	0.11	0.50	0.05	0.42	0.30	red
m-Toluidine	0.84	0.17	0.13	0.10	0.42	0.25	red
p-Toluidine	0.75	0.22	0.28	0.10	0.42	0.03	yellow
Aniline derivative							
Aniline				0.04			
N-Methylalanine				0.28			
N-Ethylaniline				0.53			

[a] As bromo derivatives.
[b] As 3,5-dinitrobenzamides.
[c] As toluyl-azo-2-naphthols.
[d] After coupling with 1-diazo-2-chloro-4-nitrobenzene.

[1] J. BERTETTI, *Ann. Chim. (Paris)*, 44 (1954) 54.
[2] J. LATINÁK, *Chem. Listy*, 51 (1957) 1493.
[3] J. GASPARIČ AND M. VEČEŘA, *Mikrochim. Acta*, (1958) 68.
[4] J. GASPARIČ, M. NOVOTNÁ AND M. JUREČEK, *Collection Czech. Chem. Commun.*, 25 (1960) 2757.

TABLE 22

R_F VALUES OF MONOHYDRIC PHENOLS. COMPARISON OF METHODS

(Cited by J. GASPARIČ, *Chem. Listy*, 55 (1961) 1439)

Solvents: S_1 = Cyclohexane.
S_2 = Isoamyl alcohol–ammonia–water (6:3:1).
S_3 = *sec.*-Butanol/2 % Na_2CO_3.
(See individual references for details.)
Paper: See individual references.
Impregnation: I = Formamide.
Detection: See individual references.

Phenol	R_F		
	$S_1 I$[1]	S_2*,[2]	S_3**,[3]
m-Cresol	0.19	0.31	0.43
p-Cresol	0.19	0.31	0.89
m-Ethylphenol	0.37	0.49	0.76
p-Ethylphenol	0.35	0.49	0.95

* As aryloxyacetic acids.
** As azo dyes (coupling with diazotised sulphanilic acid).

[1] J. FRANC, *Chem. Listy*, 52 (1958) 55.
[2] K. SCHLÖGL AND A. SIEGEL, *Mikrochem.*, 40 (1953) 202.
[3] *Cf.* W. H. CHANG, R. L. HOSSFIELD AND W. M. SANDSTROM, *J. Am. Chem. Soc.*, 74 (1952) 5766; R. L. HOSSFIELD, *J. Am. Chem. Soc.*, 73 (1951) 732.

TABLE 23

R_F VALUES OF RESORCINOL GLYCOSIDES

(J. B. PRIDHAM, *Chem. & Ind. (London)*, (1961) 1172)

Solvents: S_1 = Butan-1-ol–ethanol–water (40:11:19, v/v).
S_2 = Ethyl acetate–acetic acid–water (9:2:2, v/v).
Paper: Whatman No. 3.
Detection: Diazotised *p*-nitroaniline–NaOH reagent (T. SWAIN, *Biochem. J.*, 53 (1953) 200).

Compound	R_F	
	S_1	S_2
Resorcinol α-glucoside	0.55	0.64
Resorcinol β-glucoside	0.52	0.63
Resorcinol α-isomaltoside*	0.27	0.38

* Tentative identification.

TABLE 24

ELECTROPHORETIC MOBILITIES OF RESORCINOL GLYCOSIDES

(J. B. PRIDHAM, *Chem. & Ind. (London)*, (1961) 1172)

Electrolyte: 0.1 *M* sodium borate buffer (pH 10).
Paper: Whatman No. 3 (?).
Potential applied: Not given.
Apparatus: Not specified.
Mobility: M_{SA} = Rate of movement relative to salicylic acid (J. B. PRIDHAM, *J. Chromatog.*, 2 (1959) 605).
Detection: Diazotised *p*-nitroaniline–NaOH reagent (T. SWAIN, *Biochem. J.*, 53 (1953) 200).

Compound	M_{SA}
Resorcinol α-glucoside	0.45
Resorcinol β-glucoside	0.50
Resorcinol α-isomaltoside*	0.39

* Tentative identification.

TABLE 25

R_F VALUES OF GALLOTANNINS AND RELATED COMPOUNDS

(R. ARMITAGE, G. S. BAYLISS, J. W. GRAMSHAW, E. HASLAM, R. D. HAWORTH, K. JONES, H. J. RODGERS AND T. SEARLE, *J. Chem. Soc.*, (1961) 1842)

Solvents: S_1 = 6% Acetic acid.
S_2 = Butan-2-ol–acetic acid–water (14:1:5).
Paper: Whatman No. 2.
Temperature of run: 20° ± 3°.
Detection: D_1 = Ferric chloride–potassium ferricyanide reagent (K. S. KIRBY, E. KNOWLES AND T. WHITE, *J. Soc. Leather Trades' Chemists*, 37 (1953) 283).
D_2 = Citric acid–boric acid spray reagent (WILSON, *J. Am. Chem. Soc.*, 61 (1939) 2303).
D_3 = U.V. light.
D_4 = U.V. light in presence of NH_3 vapour.

Compound	R_F		*Colour**			
	S_1	S_2	D_1	D_2	D_3	D_4
Gallotannin	0.00–0.30 0.02–0.37	0.32–0.54 0.36–0.50	+	—	a	a
m-Digallic acid	0.29, 0.33, 0.36	0.63, 0.70, 0.72, 0.69	+	—	a	B
Gallic acid	0.45, 0.47, 0.49	0.64, 0.65, 0.70	+	—	—	B-V,B
Ellagic acid	0.02	0.27–0.41	—			B-G
Methyl gallate	0.52	0.82				B
Tara tannin	0.02–0.37	0.35–0.50	+			a
Trigallic acid**	0.17	0.70	+			B-V
Shikimic acid	0.83	0.48	—			—
Quinic acid	0.84	0.18	—			—
β-2,3,4,6-Tetra-O-galloyl-glucose**	0.20	0.64				a
β-Penta-O-galloylglucose	0.02–0.14	0.58				a

* a = absorbs; B = blue; G = green; V = violet; + = positive; — = negative.
** Tentative identification.

TABLE 26

R_F VALUES OF NATURALLY OCCURRING CAFFEIC ACID GLYCOSIDES AND RELATED COMPOUNDS

(J. B. HARBORNE AND J. J. CORNER, *Biochem. J.*, 81 (1961) 242).

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase).
S_2 = Water.
S_3 = Butan-1-ol–2 *N* NH_4OH (1:1, v/v; upper phase).
S_4 = Butan-1-ol–ethanol–water (4:1:2.2, by vol.).
Paper: Whatman Nos. 1 and 3, chromatography grade (number not specified more closely).
Detection: U.V. light: 253 mμ and > 300 mμ in presence and absence of NH_3 vapour.

Compound	R_F			
	S_1	S_2	S_3	S_4
Caffeic acid 4β-glucoside*	0.29	—	—	0.46
Echinacoside	0.44	—	—	0.31
CA_5 (caffeic acid glucose derivative)**	0.29	—	—	0.18
Orobanchin	0.56	—	—	0.63
p-Coumarylquinic acid	0.71	0.92	0.09	0.54
Caffeoylquinic acid (chlorogenic acid)	0.59	0.67, 0.84	0.01	0.50
1-*p*-Coumaroyl glucose	0.66	0.73, 0.81	0.30	0.75
1-*p*-Coumaroylrutinose	0.49	0.84	0.16	0.64
1-Caffeoylglucose	0.51	0.61, 0.72	0.08	0.63
Caffeic acid 3β-glucoside	0.46	0.46	0.02	0.55
1-Caffeoylgentiobiose	0.29	0.77, 0.83	0.03	0.46
1-Feruloylglucose	0.57	0.64, 0.73	0.19	0.68
1-Sinapoylglucose	0.47	0.47, 0.57	0.16	0.66

* Only occurs naturally in bound form as far as known.
** Not more closely characterised.

TABLE 27

R_F VALUES OF ANTHOCYANINS FROM *Bryum cryophilum* O. MÅRT. (*B. obtusifolium* LINDB.)

(G. BENDZ AND O. MÅRTENSSON, *Acta Chem. Scand.*, 15 (1961) 1185)

Solvent: 1% aq. HCl.
Paper: Not specified.
Detection: Not specified.

Compound	R_F
Luteolinidin-5-diglucoside*	0.44
Luteolinidin-5-monoglucoside*	0.13
Luteolinidin (3′,4′,5,7-tetrahydroxyflavylium chloride)	0.03

* Tentative identification.

TABLE 28

R_F VALUES (THIN LAYER) OF SOME α-GLYCOL COMPOUNDS AND RELATED SUBSTANCES

(L. D. BERGELSON, E. V. DYATLOVITSKAYA AND V. V. VORONKOVA, *Dokl. Akad. Nauk SSSR*, 141 (1961) 84)

Solvents: S_1 = Methanol–chloroform (1:9).
S_2 = Ethanol–water (95:5).
S_3 = Ethanol–acetic acid–water (60:0.25:10).
S_4 = Ethanol–25% ammonia–water (80:5:10).

Thin-layer carrier: Silica gel Type KSK (150–200 mesh).

Binder: Plaster of Paris (6 g silica gel + 0.35 g plaster of Paris + 15 ml water; dried in air, 6–12 h; then at 104–106° for 40 min; 13 × 18 cm plates).

Detection: D_1 = 5% $AgNO_3$–25% NH_4OH (I. M. HAIS AND K. MACEK (Editors), *Handbuch der Papierchromatographie*, Vol. I, Gustav Fischer Verlag, Jena, 1958, p. 736).
D_2 = Alkaline sodium periodate and potassium permanganate solution (R. U. LEMIEUX AND H. F. BAUER, *Anal. Chem.*, 26 (1954) 920).
D_3 = Lead tetraacetate after spraying with rosaniline (J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162; K. SAMPSON, F. SCHILD AND R. J. WICKER, *Chem. & Ind. (London)*, (1961) 82).
D_4 = Potassium periodate with benzidine (J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132).

Compound	R_F				*Detection**			
	S_1	S_2	S_3	S_4	D_1	D_2	D_3	D_4
Ethylene glycol	0.3				10 (S_1)	10 (S_1)	50 (S_1)	50 (S_1)
1,2-Propylene glycol	0.35				10 (S_1)	20 (S_1)	40 (S_1)	40 (S_1)
Glycerol	0.1	0.8	0.8	0.7	2 (S_1), 4 (S_2), 4 (S_3), 3 (S_4)	4 (S_1), 15 (S_2), 20 (S_3), 10 (S_4)	10 (S_1), 20 (S_2, S_4), 15 (S_3)	6 (S_1), 15 (S_2), 50 (S_3), 16 (S_4)
Glucosaccharin (glucosaccharic acid)	0.15	0.8	0.8	0.15	20 (S_1, S_2, S_3), 15 (S_4)	40 (S_1, S_2, S_3, S_4)	50 (S_1), 32 (S_2, S_4), 40 (S_3)	40 (S_1, S_4), 50 (S_2, S_3)
3-Methylhexyne-1-diol-3,4	0.5				20** (S_1)	10 (S_1)	40 (S_1)	50 (S_1)
2,3-Dihydroxy-2-methyl-pentanoic acid	0.05	0.5	***	0.8	12 (S_1), 20 (S_2, S_3), 15 (S_4)	40 (S_2, S_3, S_4)	32 (S_2, S_4) 40 (S_3)	50 (S_2, S_3) 40 (S_4)
Tartaric acid		0	0	0.05	1.5 (S_2), 2 (S_3, S_4)	5 (S_2, S_3, S_4)	10 (S_2, S_3, S_4)	10 (S_2, S_3), 8 (S_4)
Lactose		0.5	0.6	0.1	1.5 (S_2), 2 (S_3, S_4)	15 (S_2), 10 (S_3, S_4)	20 (S_2, S_3), 16 (S_4)	20 (S_2), 50 (S_3), 32 (S_4)
Glucose		0.7	0.7	0.4	1.5 (S_2), 2 (S_3, S_4)	15 (S_2, S_3), 12 (S_4)	20 (S_2, S_3, S_4)	15 (S_2), 50 (S_3), 25 (S_4)

* Minimum amount (μg) detected in system.
** Detected as a white spot (dependent on formation of ammoniacal complex with silver acetylide).
*** Does not give a discrete spot.

TABLE 29

R_F VALUES OF 1-PIPERIDINO-PROPANE-2,3-DIOL AND GLYCEROL

(J. GIERER AND I. KUNZE, *Acta Chem. Scand.*, 15 (1961) 803)

Solvent: Butan-1-ol–ethanol–water (4:1:5).
Paper: Whatman No. 1.
Detection: $AgNO_3$–NaOH reagent (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444).

Compound	R_F
1-Piperidino-propane-2,3-diol	0.28
Glycerol	0.36

TABLE 30

R_F VALUES OF SOME LONG-CHAIN FATTY ACIDS

(I. A. PEARL AND P. F. MCCOY, *J. Org. Chem.*, 26 (1961) 550)

Solvent: 85% acetic acid.
Paper: Not specified.
Impregnation: Mineral oil.
Detection: D_1 = U.V. light.
D_2 = U.V. light after iodination.
D_3 = Mercury stain (M. A. BUCHANAN, *Anal. Chem.*, 31 (1959) 1616).

Compound	R_F
Myristic acid	0.65
Palmitic acid	0.54
Stearic acid	0.15
Arachidic acid	0.09
Behenic acid	0.04
Lignoceric acid and/or higher acids	0.00
Arachidonic acid	0.39
Oleic acid	0.50
Linoleic acid	0.66
Linolenic acid	0.74

TABLE 31

R_F VALUES OF BROMO-DERIVATIVES OF SOME HIGHER UNSATURATED FATTY ACIDS

(M. H. HASHMI, E. ALI AND M. UMAR, *Pakistan J. Sci. Res.*, 14 (1962) 103)

Solvent: 90% acetic acid.
Paper: Whatman No. 4 impregnated with liquid paraffin (10% in ether).

Acid (bromo-derivative)	R_F
Erucic	0.05
Ricinoleic	0.16
Linolenic	0.22
Linoleic	0.42
Oleic	0.52

TABLE 32

R_F VALUES OF SOYA BEAN TRIGLYCERIDES AND RELATED COMPOUNDS

(G. A. SEREBRENNIKOVA, T. K. MITROFANOVA, A. A. KRAEVSKII, I. K. SARYCHEVA AND N. A. PREOBRAZHENSKII, *Dokl. Akad. Nauk SSSR*, 140 (1961) 1083)

Solvents: S_1 = Chloroform–methanol (1:3).
S_2 = Butan-1-ol–chloroform–25% aq. NH_4OH (10:5:2).
S_3 = Butan-1-ol–10% aq. NH_4OH (9:2).
Paper: Not specified.
Detection: Not specified.

Compound (triglyceride unless otherwise specified)	R_F		
	S_1	S_2	S_3
α-Linolenoyl-β,α'-distearin	0.23		
α-Linoleoyl-β,α'-distearin	0.14		
α-Linoleoyl-β,α'-dipalmitin	0.166		
α-Oleoyl-β-stearoyl-α'-linolein	0.228		
α,α'-Dioleoyl-β-stearin	0.15		
Triolein	0.24		
α-Oleoyl-β,α'-distearin	0.125		
α-Stearoyl-β-oleoyl-α'-linolein	0.18		
α-Stearoyl-β-linoleoyl-α'-olein	0.234		
α,α'-Distearoyl-β-linolenin	0.185		
α,α'-Distearoyl-β-linolein	0.218		
α,α'-Distearoyl-β-olein	0.13		
α-Stearoyl-β,α'-diolein	0.20		
Octadecadiyn-9,12-oic-1-acid		0.84	
cis-cis-Octadecadien-9,12-oic-1-acid			0.71

TABLE 33

R_F VALUES OF OXIDATION PRODUCTS OF DIFFERENT TYPES OF LIGNINS (VANILLIN DERIVATIVES)

(A. A. SOKOLOVA, E. V. NAZAR'EVA AND L. A. SEMAKOVA, *Zh. Prikl. Khim.*, 34 (1961) 2084)

Solvents: S_1 = Butan-1-ol–3% NH_4OH (3:1).
S_2 = Butan-1-ol–pyridine–water (10:3:3).
S_3 = Butan-1-ol–acetic acid–water (4:1:5).
Paper: Leningrad Paper Factory (grades: "fast", "slow" and No. 2; one used, not specified which).
Detection: 0.1% 2,4-dinitrophenylhydrazine in 2 *N* HCl.

Compound	R_F		
	S_1	S_2	S_3
Vanillin	0.44	0.87	
5-Carboxyvanillin	0.10, 0.12, 0.15	0.35, 0.30, 0.36, 0.37	
5-Carboxyvanillic acid	0.04	0.18, 0.25	
5-Formylvanillin	0.18, 0.20, 0 27		
Dehydrovanillin	0.30		
Phenol			0.96, 0.97
Guiacol			0.95, 0.97
Vanillic acid	0.10	0.42	0.92
Dehydrodivanillic acid		0.04, 0.05, 0.06	

TABLE 34

R_F VALUES OF SEVERAL ANALOGUES OF THE CHLORAMBUCIL ISOSTERE 3-{*p*-[BIS-(2-CHLOROETHYL)-AMINO]-PHENOXY}-PROPIONIC ACID

(W. A. SKINNER, A. P. MARTINEZ AND B. R. BAKER, *J. Org. Chem.*, 26 (1961) 152)

Solvent: Benzene–methanol–water (2:6:1).
Paper: P_1 = Schleicher & Schüll No. 2495 acetylated paper.
P_2 = Whatman No. 1.
(Both descending.)
Detection: U.V. light.

Compound	R_F	
	P_1	P_2
Ethyl 3-(*m*-aminophenoxy)-propionate·HCl	0.57	
Ethyl 3-(*m*-aminophenoxy)-acetate·HCl	0.45	
Ethyl 3-(*o*-aminophenoxy)-propionate·HCl	0.46	
Ethyl *m*-[bis-(2-hydroxyethyl)-amino]-phenoxy-acetate	0.61	
Ethyl 3-{*m*-[bis-(2-hydroxyethyl)-amino]-phenoxy}-propionate	0.65	
Ethyl 3-{*o*-[bis-(2-hydroxyethyl)-amino]-phenoxy}-propionate	0.74	
Ethyl *m*-[bis-(2-chloroethyl)-amino]-phenoxy-acetate	0.52	
Ethyl 3-{*m*-[bis-(2-chloroethyl)-amino]-phenoxy}-propionate	0.61	
Ethyl 3-{*o*-[bis-(2-chloroethyl)-amino]-phenoxy}-propionate	0.71	
{*m*-[Bis-(2-chloroethyl)-amino]-phenoxy}-acetic acid		0.87
3-{*m*-[Bis-(2-chloroethyl)-amino]-phenoxy}-propionic acid	0.70	
3-{*o*-[Bis-(2-chloroethyl)-amino]-phenoxy}-propionic acid	0.77	

TABLE 35

R_F VALUES OF ETHYL *o*-AMINO-α-BENZAMIDOCINNAMATE AND ITS TRANSFORMATION PRODUCTS

(A. P. MARTINEZ, W. A. SKINNER, W. W. LEE, L. GOODMAN AND B. R. BAKER, *J. Org. Chem.*, 26 (1961) 860)

Solvents: S_1 = Benzene–methanol–water (2:6:1).
S_2 = Butan-1-ol–water (saturation presumed).
S_3 = Butan-1-ol–methyl ethyl ketone–water (5:3:2).
Paper: P_1 = Schleicher & Schüll No. 2492, acetylated (descending).
P_2 = Whatman No. 1.
Detection: D_1 = U.V. light.
D_2 = Tollens reagent.

Compound	R_F			*Colour**	
	S_1P_1	S_2P_2	S_3P_2	D_1	D_2
Ethyl α-benzamido-*o*-nitrocinnamate	0.64				
Ethyl *o*-amino-α-benzamidocinnamate	0.76				
Ethyl α-benzamido-*o*-hydroxylaminocinnamate	0.76			Gf	+
3-Benzamidocarbostyril	0.56			Bf	
Ethyl 3-(*o*-benzamidophenyl)-pyruvate	0.74				
3-Benzamido-3,4-dihydrocarbostyril		0.91	0.91	Pa	
o-Amino-α-benzamidocinnamoylhydrazide**					

* G = green; B = blue; P = purple; f = fluorescence; a = absorption; + = positive.
** Unsatisfactory behaviour in several solvent systems.

TABLE 36

R_F VALUES OF SYMMETRICAL DIARYL ORGANOTIN COMPOUNDS

(O. A. REUTOV, O. A. PTITSYNA AND M. F. TURCHINSKII, *Dokl. Akad. Nauk SSSR*, 139 (1961) 146)

Solvents (% methanol in 1 *N* HCl):
S_1 = 20%.
S_2 = 30%.
S_3 = 40%.
S_4 = 50%.
S_5 = 60%.

Paper: Leningrad Factory Chromatographic Paper (rapid filtration type; substances applied in petrol ether, as 10% soln.).

Temperature of run: 20°.

Detection: Saturated soln. of diphenylcarbazone in 50% aqueous methanol.

Ar_2SnCl_2	R_F*				
	S_1	S_2	S_3	S_4	S_5
$(C_6H_5)_2SnCl_2$	0.33	0.40	0.50	0.60	—
$(p\text{-}CH_3C_6H_4)_2SnCl_2$	0.33	0.07	0.15	0.29	—
$(p\text{-}ClC_6H_4)_2SnCl_2$	0.04	0.10	0.19	0.34	—
$(p\text{-}BrC_6H_4)_2SnCl_2$	0.01	0.02	0.14	0.24	0.38
$(p\text{-}IC_6H_4)_2SnCl_2$	—	0.02	0.04	0.11	0.20
$(p\text{-}CH_3OC_6H_4)_2SnCl_2$	—	0.23	0.31	0.50	—
$(p\text{-}C_2H_5OCOC_6H_4)_2SnCl_2$	0.19	0.29	0.48	0.70	—
$(m\text{-}C_2H_5OCOC_6H_4)_2SnCl_2$	0.23	0.38	0.55	0.75	—

* $SnCl_2$ and $SnCl_4$ have R_F values of 0.80–0.90 in all solvents.

TABLE 37

R_F VALUES OF UNSYMMETRICAL DIARYL ORGANOTIN COMPOUNDS

(O. A. REUTOV, O. A. PTITSYNA AND M. F. TURCHINSKII, *Dokl. Akad. Nauk SSSR*, 139 (1961) 146)

Solvents (% methanol in 1 *N* HCl):
S_1 = 20%.
S_2 = 30%.
S_3 = 40%.
S_4 = 50%.

Paper: Leningrad Factory Chromatographic Paper (rapid filtration type; substances applied in petrol ether, as 10% soln.).

Temperature of run: 20°.

Detection: Saturated soln. of diphenylcarbazone in 50% aqueous methanol.

$ArAr'SnCl_2$	R_F			
	S_1	S_2	S_3	S_4
$(p\text{-}ClC_6H_4)(C_6H_5)SnCl_2$	—	0.20	0.33	0.45
$(p\text{-}BrC_6H_4)(C_6H_5)SnCl_2$	—	0.11	0.22	0.39
$(p\text{-}IC_6H_4)(C_6H_5)SnCl_2$	0.05	0.11	0.20	0.34
$(p\text{-}CH_3OC_6H_4)(C_6H_5)SnCl_2$	—	0.31	0.42	—
$(p\text{-}C_2H_5OCOC_6H_4)(p\text{-}CH_3OC_6H_4)SnCl_2$	—	—	0.39	0.62
$(p\text{-}C_2H_5OCOC_6H_4)(C_6H_5)SnCl_2$	0.25	—	—	—
$(p\text{-}C_2H_5OCOC_6H_4)(p\text{-}CH_3C_6H_4)SnCl_2$	—	0.15	0.29	—
$(m\text{-}C_2H_5OCOC_6H_4)(p\text{-}CH_3C_6H_4)SnCl_2$	—	0.18	0.32	—

TABLE 38

R_F VALUES OF THE 2,4-DINITROPHENYLHYDRAZONES OF METHYL AND ETHYL 3-PYRIDYL KETONES

(L. D. QUIN, B. S. MENEFEE AND N. A. PAPPAS, *J. Org. Chem.*, 26 (1961) 267)

Solvents: S_1 = Chloroform–*n*-heptane (40:15, v/v).
S_2 = Carbon tetrachloride.
Paper: Whatman No. 1 (ascending).
Detection: 10% NaOH spray (brown colour).

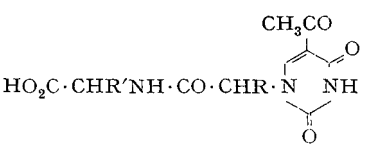
2,4-Dinitrophenylhydrazone of	R_F	
	S_1	S_2
Methyl 3-pyridyl ketone	0.50	0.40
Ethyl 3-pyridyl ketone	0.62	0.48

TABLE 39

R_F VALUES OF 5-ACETYLURACIL PEPTIDES

(J. H. DEWAR AND G. SHAW, *J. Chem. Soc.*, (1961) 3254)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5).
S_2 = Propan-2-ol–NH_4OH (sp. gr. 0.88)–water (7:1:3).
S_3 = Butan-1-ol saturated with water.
(All volume ratios.)
Paper: Whatman No. 1 (ascending).
Detection: D_1 = U.V. light (Hanovia, Chromatolite) (dark absorption).
D_2 = 2,4-Dinitrophenylhydrazine·HCl (yellow spots).



(I)

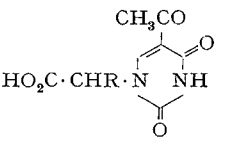
5-Acetyluracil peptide (I)			R_F		
R	*R'*	*Generating acid*	S_1	S_2	S_3
H	H	Glycylglycine	0.48	0.63	0.02
H	Me	Glycyl-DL-alanine	0.84	0.85	0.04
H	Bu^i	Glycyl-DL-leucine	0.82	0.78	0.15
H	$Ph\cdot CH_2$	Glycyl-DL-phenylalanine	0.79	0.77	0.12
H	$p\text{-}HO\cdot C_6H_4\cdot CH_2$	Glycyl-L-tyrosine	0.70	0.68	0.06
H	$C_8H_6N\cdot CH_2$	Glycyl-L-tryptophan	0.77	0.72	0.09
Bu^i	H	DL-Leucylglycine	0.83	0.83	0.16

TABLE 40

R_F VALUES OF 5-ACETYL-1-URACIL-ACETIC ACID DERIVATIVES AND THEIR 2,4-DINITROPHENYLHYDRAZONES

(J. H. DEWAR AND G. SHAW, *J. Chem. Soc.*, (1961) 3254)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5).
S_2 = Propan-2-ol–NH_4OH (sp. gr. 0.88)–water (7:1:3).
S_3 = Butan-1-ol saturated with water.
Paper: Whatman No. 1 (ascending).
Detection: D_1 = U.V. light (Hanovia, Chromatolite) (dark absorbing spots).
D_2 = 2,4-Dinitrophenylhydrazine·HCl (yellow spots).



(I)

5-Acetyl-1-uracil-acetic acid (I)		*R_F of ketone*			*R_F of 2,4-dinitrophenylhydrazone*		
R	*Generating acid (L-conf.)*	S_1	S_2	S_3	S_1	S_2	S_3
H	Glycine	0.48	0.59	0.04	0.73	0.70	0.24
Me	Alanine	0.66	0.66	0.07	0.82	0.78	0.28
Pr^i	Valine	0.84	0.77	0.21	0.89	0.86	0.42
Bu^i	Leucine	0.85	0.80	0.31	0.90	0.92	0.47
Bu^s	Isoleucine	0.87	0.82	0.27	0.90	0.91	0.45
$Ph\cdot CH_2$	Phenylalanine	0.83	0.79	0.25	0.89	0.88	0.41
$p\text{-}HO\cdot C_6H_4\cdot CH_2$	Tyrosine	0.78	0.71	0.17	0.86	0.83	0.34
$HO\cdot CH_2$	Serine	0.51	0.64	0.04	0.72	0.76	0.20
$HS\cdot CH_2$	Cysteine	0.65	0.55	0.08	0.74	0.68	0.30
$C_9H_9N_2O_5S\cdot S\cdot CH_2$	Cystine	0.52	0.56	0.01	0.89	0.70	0.04
$Me\cdot CH(OH)$	Threonine	0.63	0.70	0.08	0.81	0.80	0.30
$MeS\cdot CH_2\cdot CH_2$	Methionine	0.79	0.79	0.16	0.88	0.83	0.34
$C_8H_6N\cdot CH_2$	Tryptophan	0.82	0.74	0.20	0.89	0.79	0.37
$HO_2C\cdot CH_2$	Aspartic acid	0.58	0.57	0.01	0.75	0.68	0.09
$H_2N\cdot CO\cdot CH_2$	Asparagine	0.51	0.66	0.04	0.65	0.70	0.13
$HO_2C\cdot CH_2\cdot CH_2$	Glutamic acid	0.62	0.63	0.01	0.79	0.71	0.03
$H_2N\cdot CO\cdot CH_2\cdot CH_2$	Glutamine	0.39	0.65	0.03	0.68	0.74	0.14
$H_2N\cdot C(:NH)\cdot NH\cdot (CH_2)_3$	Arginine	0.51	0.74	0.06	0.74	0.76	0.25
$C_6H_5N_2O_3\cdot (CH_2)_4$	Lysine	0.62	0.70	0.06	0.75	0.73	0.20
$C_3H_3N_2\cdot CH_2$	Histidine	0.25	0.69	0.06	0.51	0.73	0.23

TABLE 41

R_F VALUES OF CERTAIN URIDINE DERIVATIVES

(J. SMRT AND F. ŠORM, *Collection Czech. Chem. Commun.*, 27 (1962) 73)

Solvents: S_1 = Propan-2-ol–ammonia–water (7:1:2).
S_2 = Butan-1-ol–water (satd.?).
Paper: Whatman No. 1 (descending), except where otherwise stated.
Detection: Not specified.

Compound	R_F	
	S_1	S_2
5′-O-Acetyluridine-3′-phosphate	0.1	
2′-O-Tetrahydropyranyl-5′-O-acetyluridine-3′-phosphate (Ca salt)	0.23	
2′,3′-O-Benzylideneazauridylyl-(5′→3′)-uridine	0.55*	
2′,3′-O-Benzylidene-6-azauridylyl-(5′→3′)-2′-O-tetrahydropyranyl-uridine	0.6*	
6-Azauridylyl-(5′→3′)-uridine	0.1*	
6-Azauridylyl-(5′→3′)-2′-O-tetrahydropyranyl-uridine	0.25*	
2′,3′-Di-O-acetyl-6-azauridine		0.69
Uridylic acid	0.09	
5′-O-Acetyluridylic acid	0.1	
Azauridine-5′-phosphate	0.02	

* Paper: Whatman No. 3.

TABLE 42

R_F VALUES OF AZAURIDINE, AZACYTIDINE AND AZACYTOSINE DERIVATIVES

(V. ČERNĚCKIJ, S. CHLÁDEK, F. ŠORM AND J. SMRT, *Collection Czech. Chem. Commun.*, 27 (1962) 87)

Solvents: S_1 = Butan-1-ol–water (satd.?).
S_2 = Propan-2-ol–ammonia–water (7:1:2).
S_3 = Butan-1-ol–water–acetic acid (5:3:2).
S_4 = Methylcellosolve–water (8:2).
Paper: Whatman No. 1, except where otherwise stated.
Detection: Not specified.

Compound	R_F			
	S_1	S_2	S_3	S_4
2′,3′,5′-Tri-O-acetyl-6-azauridine	0.71	0.73		
Tetraacetyl-6-azauridine	0.77	0.85		
2′,3′,5′-Tri-O-benzoyl-4-thio-6-aza-uridine		0.89		
Tetraacetyl-4-thio-6-azauridine	0.85	0.92		
4-Thio-6-azauridine	0.36*			
6-Azacytidine	0.11	0.49*, 0.55		
N^4-Hydroxy-6-azacytidine	0.14	0.43		
N^4-Amino-6-azacytidine		0.34	0.39	0.78
N^4-Amino-6-azacytosine		0.24	0.82	
N^4-*n*-Butyl-6-azacytidine	0.70	0.78		

* Paper: Whatman No. 3.

TABLE 43

R_F AND RELATIVE R_F VALUES OF SOME DEOXY SUGARS

(M. ČERNY AND J. PACAK, *Collection Czech. Chem. Commun.*, 27 (1962) 94)

Solvent: Butan-1-ol–water.
Paper: Whatman No. 1 (descending).
Temperature of run: 20–22°.
Length of run: 30–40 cm.
Detection: D_1 = Bonner reagent (T. G. BONNER, *Chem. & Ind. (London)*, (1960) 345).
D_2 = Ammoniacal $AgNO_3$ solution.

Compound	R_F	R_{DR}*
4-Deoxy-D-glucose	0.17	0.57
2-Deoxy-D-glucose	0.24	0.79
3-Deoxy-D-glucose	0.22	0.76
6-Deoxy-D-glucose	0.25	0.82
4-Deoxy-D-altrose	0.25	0.82
2-Deoxy-D-ribose	0.30	1.0
1,6-Anhydro-4-deoxy-β-D-xylohexopyranose	0.45	—
1,6-Anhydro-4-deoxy-β-D-arabinohexopyranose	0.45	—

* R_{DR} = R_F compound/R_F 2-deoxy-D-ribose.

TABLE 44

R_F VALUES OF SOME N-AMINO-ACYL DERIVATIVES OF AMINO SUGARS

(N. K. KOCHETKOV, V. A. DEREVITSKAYA AND N. A. MOLODTSEV, *Chem. & Ind. (London)*, (1961) 1159)

Solvents: S_1 = *n*-Butanol–water–acetic acid (4:5:1).
S_2 = Isobutanol saturated with water.
Paper: Not specified.
Detection: Aniline phthalate reagent (for free hydroxyl at C-1) and fluorescein reagent (for carbobenzoxy group); ninhydrin negative.

Compound	R_F	
	S_1	S_2
N-(N′-Carbobenzoxyglycyl)-glucosamine	0.69	0.63
N-(N′-Carbobenzoxyalanyl)-glucosamine	0.76	0.77
N-(N′-Carbobenzoxyglycyl)-galactosamine	0.75	0.63

TABLE 45

R_F AND RELATIVE R_F VALUES OF SOME *Anogeissus schimperi* GUM OLIGOSACCHARIDES AND RELATED COMPOUNDS

(G. O. ASPINALL AND T. B. CHRISTENSEN, *J. Chem. Soc.*, (1961) 3461)

Solvents: S_1 = Ethyl acetate–pyridine–water (10:4:3).
S_2 = Ethyl acetate–acetic acid–formic acid–water (18:3:1:4).
S_3 = Butan-1-ol–ethanol–water (4:1:5, upper layer).
S_4 = Butan-1-ol–ethanol–water (1:1:1).
S_5 = Ethyl acetate–acetic acid–formic acid–water (18:8:3:9).
Papers: Whatman Nos. 1, 3 MM and 31 (not specified).
Detection: Not specified.

Compound	R_{Gal}*			R_G**	R_F
	S_1	S_2	S_5	S_3	S_4
2,3,5-Tri-O-methyl-L-arabinose				0.95	
2,5-Di-O-methyl-L-arabinose				0.83	
2,3,4-Tri-O-methyl-L-arabinose				0.82	
2,4-Di-O-methyl-L-arabinose				0.60	
3,4-Di-O-methyl-L-arabinose				0.51	
3-O-β-L-Arabinopyranosyl-L-arabinose	0.8	0.7			
3-O-β-D-Galactopyranosyl-L-arabinose	0.6	0.4			
6-O-β-D-Galactopyranosyl-D-galactose	0.3				
3-O-β-D-Galactopyranosyl-D-galactose	0.4				
O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→6)-D-galactose					0.2
O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→3)-L-arabinose					0.25
O-β-D-Galactopyranosyl-[(1→6)-O-β-D-galactopyranosyl]$_2$-(1→3)-L-arabinose					0.16
O-β-D-Galactopyranosyl-[(1→6)-O-β-D-galactopyranosyl]$_3$-(1→3)-L-arabinose					0.10
2-O-(Glucosyluronic acid)-mannose		0.35	0.74		
2,3,4,6-Tetra-O-methyl-D-glucose			1.0		
3,4,6-Tri-O-methyl-D-mannose			0.85		
6-O-(β-D-Glucopyranosyluronic acid)-D-galactose		0.15			0.51
O-D-Glucopyranosyluronic acid-(1→2)-O-D-mannopyranosyl-(1→2)-D-mannose		0.07			0.32

* R_{Gal} = R_F compound/R_F galactose.
** R_G is not defined.

TABLE 46

R_F VALUES (RELATIVE) OF CERTAIN OLIGOSACCHARIDES FROM *Virgilia oroboides* GUM

(F. SMITH AND A. M. STEPHEN, *J. Chem. Soc.*, (1961) 4892)

Solvents: S_1 = Butan-1-ol–ethanol–water (4:1:5, upper layer).
S_2 = Ethyl acetate–acetic acid–formic acid–water (18:3:1:4).
S_3 = Butan-1-ol–pyridine–water (9:2:2).
S_4 = Ethyl acetate–pyridine–water (10:4:3).
S_5 = Butan-1-ol–ethanol–water (1:1:1).
S_6 = Butan-1-ol–acetic acid–water (2:1:1).
Papers: Whatman Nos. 1 and 6 (not specified).
Detection: D_1 = *p*-Anisidine·HCl in wet butan-1-ol; D_2 = Aniline oxalate in water; D_3 = Tollen's ammoniacal $AgNO_3$; D_4 = Periodate–benzidine. (All spray reagents.)

Compound	R_{Gal}*					
	S_1	S_2	S_3	S_4	S_5	S_6
5-O-α-L-Arabinopyranosyl-L-arabinose	0.9	0.85	0.8		1.0	
5-O-α-L-Arabinofuranosyl-L-arabinose	1.42	1.40	2.0			
3-O-α-L-Arabinofuranosyl-L-arabinose	1.23	1.28	1.47			
5-O-β-D-Xylopyranosyl-L-arabinose	1.07	1.0	1.15			
Arabinotriose	0.50	0.50	0.42			
6-O-β-D-Galactopyranosyl-D-galactose				0.62	0.65	
3-O-β-D-Galactopyranosyl-D-galactose				0.76	0.75	
O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→6)-D-galactose				0.30	0.40	
(2-D-Mannose-β-D-glucopyranosid)-uronic acid		0.50				0.55
(6-D-Galactose-β-D-glucopyranosid)-uronic acid		0.25				0.35

* R_{Gal} = R_F compound/R_F galactose.

TABLE 47

ELECTROPHORETIC MOBILITIES OF CERTAIN OLIGOSACCHARIDES FROM *Virgilia oroboides* GUM

(F. SMITH AND A. M. STEPHEN, *J. Chem. Soc.*, (1961) 4892)

Electrolyte: 0.1 *M* borate buffer.
Paper: Whatman No. 1.
Apparatus: See R. CONSDEN AND W. M. STANIER, *Nature*, 169 (1952) 783 (and for other conditions).
Mobility: M_G = Mobility relative to glucose.
Detection: D_1 = *p*-Anisidine·HCl in wet butan-1-ol; D_2 = Aniline oxalate in water; D_3 = Tollen's ammoniacal $AgNO_3$; D_4 = Periodate–benzidine. (All spray reagents.)

Compound	M_G
5-O-α-L-Arabinofuranosyl-L-arabinose	0.45
3-O-α-L-Arabinofuranosyl-L-arabinose	0.45
5-O-β-D-Xylopyranosyl-L-arabinose	0.50
Arabinotriose	0.60
6-O-β-D-Galactopyranosyl-D-galactose	0.85
3-O-β-D-Galactopyranosyl-D-galactose	0.75
O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→6)-D-galactose	0.85
(2-D-Mannose-β-D-glucopyranosid)-uronic acid	0.85
3,4,6-Tri-O-methyl-D-mannose	0.45
(6-D-Galactose-β-D-glucopyranosid)-uronic acid	1.0

TABLE 48

R_F VALUES (RELATIVE) OF THE CYCLITOL, LIRIODENDRITOL, AND RELATED COMPOUNDS
(S. J. ANGYAL AND V. BENDER, *J. Chem. Soc.*, (1961) 4718)

Solvent: Butan-1-ol–pyridine–water (6:4:3).
Paper: Not specified.
Detection: Not specified.

Compound	R_G*
Glucose	1.0
Liriodendritol [(1*S*)-1,4-di-O-methylmyoinositol]	0.52
4-O-Methylmyoinositol	0.31
1-O-Methylmyoinositol	0.19
Myoinositol	0.06

* R_G = R_F compound/R_F glucose.

TABLE 49

R_F VALUES OF SOME 2-BENZYL-2-HYDROXYCOUMARAN-3-ONES AND RELATED COMPOUNDS
(H. G. C. KING, T. WHITE AND R. B. HUGHES, *J. Chem. Soc.*, (1961) 3234)

Solvents: S_1 = 6% acetic acid.
S_2 = Butan-2-ol–acetic acid–water (14:1:5).
S_3 = 25% acetic acid.
S_4 = Acetic acid–water–HCl (30:15:1) (E. BATE-SMITH, *Biochem. J.*, 58 (1954) 122).
Paper: Not specified.
Detection: D_1 = U.V. light.
D_2 = U.V. light after NH_3 vapour.
D_3 = 5% aq. NaOH.
D_4 = Bisdiazotised benzidine.

Compound	R_F				*Colour**			
	S_1	S_2	S_3	S_4	D_1	D_2	D_3	D_4
2-Benzyl-2,6,3′-trihydroxy-4′-methoxycoumaran-3-one (A)	0.74	0.94		1.00	d	V		
Coumaran-3-one triacetate (corresponding to A)			0.97				Y	
Aurone diacetate (corresponding to A)			0.71				Y	
Coumaran-3-one tetraacetate**			0.91					
Sulphuretin triacetate**			0.79					
6,3′-Dihydroxy-4′-methoxy-aurone		0.92		0.76, 0.73	YGf	lGf		
Sulphuretin				0.55				
2-Benzyl-6,3′-dihydroxy-2,4′-dimethoxycoumaran-3-one	0.79				a	dV		Y

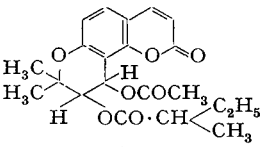
* V = violet; Y = yellow; G = green; d = dark; f = fluorescence; l = light; a = absorbs.
** Corresponding to 2-benzyl-2,6,3′,4′-tetrahydroxycoumaran-3-one.

TABLE 50

R_F VALUES OF PROVISMINE (VISNADIN) AND ACCOMPANYING CHROMONES OF *Ammi visnaga* L.

(A. MUSTAFA, N. A. STARKOVSKY AND T. I. SALAMA, *J. Org. Chem.*, 26 (1961) 890)

Solvent: Water.
Paper: Not specified.
Temperature of run: 20° ± 3°.
Detection: D_1 = U.V. light.
D_2 = D_1 after spraying with 4% NaOH and drying at 80°.



(I)

Compound	R_F	*Colour**	
		D_1	D_2
Provismine (I)**	0.18	—	B
Visnagin	0.32	iB	V
Khellin	0.42	Y-G	Y-Gr
Khellol glucoside	0.53	pGr	pB-Gr

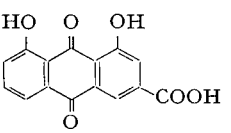
* B = blue; Y = yellow; G = green; Gr = gray; V = violet; i = intense; p = pale; — = negative.
** Visnadin.

TABLE 51

R_F VALUES OF RHEIN AND ITS METHYL ESTER

(H. NAWA, M. UCHIBAYASHI AND T. MATSUOKA, *J. Org. Chem.*, 26 (1961) 979)

Solvent: Butan-1-ol saturated with water.
Paper: Not specified (ascending).
Detection: U.V. light (presumed).



(I)

Compound	R_F
Rhein (I)	0.32
Rhein methyl ester	0.84

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