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PARTIAL SUBTRACTION CHROMATOGRAPHY

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The qualitative and quantitative analysis of certain mixtures can be carried out advantageously by combining gas chromatography with chemical treatment. One of the variants of this mode of operation is subtraction chromatography, in which one or more components are eliminated selectively, resulting in the disappearance of the corresponding peaks. By comparison with a conventional chromatogram, identification and quantitative analysis of the reacting compounds can be carried out readily. The reagents** employed in this method act rapidly; they are coated on the solid support and filled into a short piece of tubing attached to the main partition column. The emergence time of the non-reacting components is practically unaffected under these circumstances. Examples of suitable reagents are concentrated sulphuric acid, which is useful for the subtraction of olefins in mixtures with other hydrocarbons, or silver nitrate, which permits the analysis of secondary and tertiary alkyl bromides in the presence of primary isomers[1].

The procedure is of particular value when separation cannot be achieved by the usual gas chromatographic techniques. The analysis of argon and oxygen, for instance, has been carried out successfully only when the oxygen was burned in a precolumn loaded with palladium catalyst, using hydrogen as the carrier[2].

On the other hand, JANÁK AND NOVÁK[4], in developing an accurate procedure for the analysis of impurities accompanying butadiene, used the relatively slow acting maleic anhydride for subtracting the diene. A column containing about 10 g of the dienophile on 22 g of support was necessary to complete the reaction in 23 sec at 100°. In such a case the retention volume of the non-reacting components is considerably changed by the precolumn.

In the present paper we wish to discuss a procedure involving only partial subtraction of the reacting components. In this method chromatographic peaks are identified by observing either the occurrence or the rate of reaction with the stationary phase. The rate is determined from the peak areas corresponding to the residual amounts of components emerging from the column at different flows of the carrier gas.

The method is illustrated by experiments with conjugated dienes as the subtracted components, and the dienophilic chloromaleic anhydride (ClMA) as the stationary phase. The resulting adducts are relatively non-volatile and remain on the column.

* Part of a thesis presented by Y. HERZBERG-MINZLY to the Hebrew University, Jerusalem, in partial fulfilment of the requirements for the degree of Ph.D.

** It should be noted that molecular sieves[3] can be used effectively for the selective and irreversible absorption of *n*-aliphatic compounds.

The commercial ClMA was distilled before use, but, in general, not further purified by crystallization, since a small amount of residual impurities conveniently depresses the melting point below room temperature. When relative values only are required, the effect of the impurities on the rate of reaction can be disregarded, as long as care is taken to work with the same batch of reagent.

It has been shown[5,6] that for a pseudo-first-order reaction the rate of disappearance of the reacting component is given by $dx/dt = -kxH$, where x is the total amount of the reacting component at time t in the plug passing through the column, and H is a factor by which x has to be multiplied in order to give the amount of component dissolved in the stationary phase.

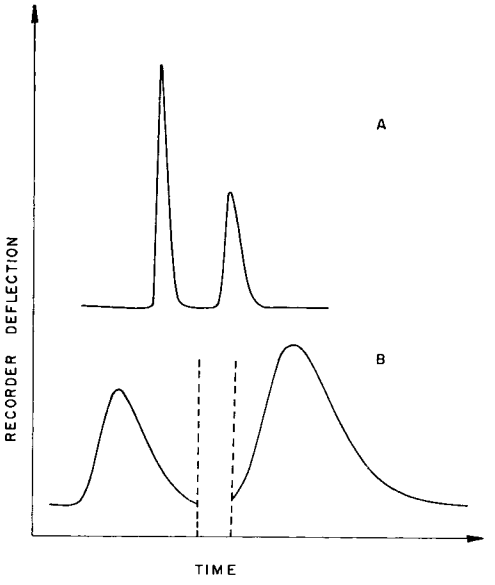

Fig. 1. Chromatography of commercial 1,3-pentadiene on a dual column containing silver nitrate and chloromaleic anhydride, respectively, as the stationary phases (see Table I). Temperature 40°. Curve A—flow rate of helium 77 ml/min, area of 1st peak (*trans* isomer) 60%; curve B—flow rate of helium 11.4 ml/min, area of 1st peak (*trans* isomer) 31%.

The conditions necessary for subtracting can be calculated by combining the equation $\log x^\circ/x = 0.4343\ k\ t\ H$ with the expression for the contact time $t = V_R/F$ where V_R is the uncorrected retention volume of the solute on the subtracting column, and F is the rate of flow of the mobile phase; hence

$$k = 2.3 \log x^\circ/x\ F/V_R H \qquad (1)$$

For complete subtraction $\log x^\circ/x$ can be taken as equal to 5, whereas for partial subtraction $\log x^\circ/x$ should be about 0.04 to 1.0. The rates of the reactions, which can be conveniently used in partial subtraction chromatography, cover a wide range under the chromatographic conditions employed (see Tables I and II for flow rates and description of columns, Fig. 2 for retention volumes, and EXPERIMENTAL for H

TABLE I

PARTIAL SUBTRACTION CHROMATOGRAPHY OF CONJUGATED DIENES[a]

Column	*1st Peak (% of total area)*	*2nd Peak (% of total area)*	*3rd Peak (% of total area)*	*Helium flow rate (ml/min)*
		1,3-Pentadiene		
$AgNO_3$–glycol[b]	65	35		
$AgNO_3$–glycol[b] and ClMA[c]	60	40		77
	31	69		11.4
		Crude 1,3-hexadien-5-yne		
Tri-*o*-tolyl phosphate[d]	55	45	[e]	
ClMA[f]	60	40	[e]	69
,,	65	35	[e]	31.5
,,	83	27	[e]	13
		Crude 2,4-hexadiene		
$AgNO_3$–glycol[g]	40	18	42	
		(*area/area n-hexane*[h])		
ClMA[c]	0.23	0.32	1.10	116
,,	0.09	0.26	1.13	58
,,	0.04	0.18	1.11	31

[a] Temperature 40°.
[b] Saturated silver nitrate–glycol solution; column length 1 m; weight ratio solid support to stationary phase 2:1.
[c] Non-crystalline chloromaleic anhydride; column length 1 m; i.d. 4 mm; weight ratio solid support to liquid phase 2:1.
[d] Column length 2 m; weight ratio solid support to liquid phase 2:1.
[e] The third peak, corresponding to the starting material, 1,5-hexadiyne (about 10%) was not taken into account in the above area calculations.
[f] As c, but column length 2 m.
[g] As b, but column length 2 m.
[h] Added inert reference substance.

TABLE II

SUBTRACTION CHROMATOGRAPHY[a] OF A MIXTURE OF ISOPRENE (I), *trans* (II)- AND *cis* (III)-1,3-PENTADIENE AND CYCLOPENTADIENE (IV)

Compound	*1st Peak*	*2nd Peak*	*3rd Peak*	*4th Peak*	*5th Peak*	*Helium flow rate (ml/min)*
	n-hexane	*I*	*II*	*III*	*IV*	
		Stationary phase—β,β′-oxydipropionitrile[b]				
% of total area	31.2	30	14	7.6	16.8	
		Stationary phase—chloromaleic anhydride[c]				
% of total area	48	32.2	8.8	11	nil	42.2
Area/area *n*-hexane		0.67	0.18	0.23		
% of total area	53.5	30.7	3.1	12.7	nil	14.8
Area/area *n*-hexane		0.58	0.058	0.24		

[a] Temperature 40°.
[b] Column length 1 m; weight ratio solid support to liquid phase 3:1.
[c] Crystalline chloromaleic anhydride; column length 1 m; weight ratio solid support to liquid phase 2:1.

values). In the present examples, for instance, the kinetic constants varied from $2.0 \cdot 10^{-2}$ to $1.4 \cdot 10^{-4}$ sec^{-1} (half life time 30 sec to 100 min respectively)[6].

Many reagents should be suitable for use in subtraction chromatography, provided conditions in the subtracting column are adjusted in accordance with the requirements of eqn. (1). H can be taken as approximately equal to unity, except where the retention volume is relatively small (see EXPERIMENTAL). V_R can be varied

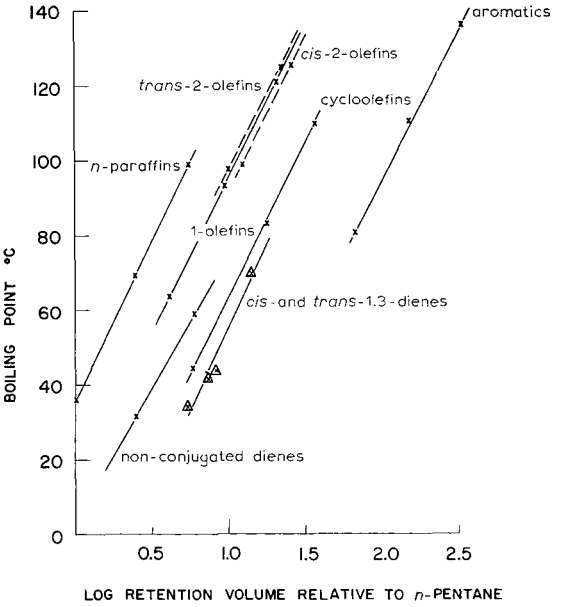

Fig. 2. Plot of log retention volume (relative to *n*-pentane) *versus* boiling point for different classes of hydrocarbons on non-crystalline chloromaleic anhydride at 40°. Net retention volume of *n*-pentane 11.3 ml, on a 1 m × 4 mm column containing 7.4 g of a mixture of solid support and ClMA in the ratio 2:1.

by the amount of stationary phase used, and the effective value of the kinetic constant can be modified by choice of temperature, by the addition of catalysts or by dilution. For example, when experimenting with mercury acetate as a reagent for the partial subtraction of mono-olefins[7], it was found necessary to dilute the acetate considerably with ethylene glycol in order to bring the half life time into a proper range (25 sec and 100 sec for 1-hexene and cyclohexene, respectively).

DISCUSSION OF RESULTS

Differentiation between cis- and trans-1,3-dienes

Chromatographic peaks due to *cis*- and *trans*-1,3-dienes can be distinguished readily from each other by partial subtraction with dienophiles. The *trans*-isomers, indeed, easily undergo Diels-Alder addition, whereas the *cis*-isomers[8,9] react very much more slowly or not at all.

Piperylene is the simplest example of a 1,3-diene showing geometric isomerism.

The commercial product gives two peaks on a column with silver nitrate–glycol as the stationary phase. When the mixture was run on a dual column containing silver nitrate–glycol and ClMA, respectively, the proportion of the first peak decreased relatively to the other, and with a higher contact time (helium flow rate, 11.4 ml/min) dropped below that of the second peak (see Table I and Fig. 1). It is easily seen from these data that the pentadienes emerge in the same order on both chromatograms, and that the first peak corresponds to the *trans*-isomer and the second peak to the *cis*-compound. In the same way it was possible to distinguish between the peaks due to *cis*- and *trans*-1,3-hexadiene. In both cases, as the flow rate altered, areas corresponding to the *cis*-isomer did not change with respect to an added inert substance.

The method has been used to prove the formation of *trans*-1,3-pentadiene in the elimination reaction of the tosylate of *trans*-2-methylcyclobutanol[10]. The nature of the by-products, accompanying the expected 3-methylcyclobutene, could thus be ascertained unambiguously.

Another interesting application is the identification of the geometric isomers of 1,3-hexadien-5-yne, prepared from 1,5-hexadiyne by action of potassium *tert.*-butoxide in *tert.*-butanol[11]. As shown by chromatography with a stationary phase such as tri-*o*-tolyl phosphate or Octoil, the product was found to consist of a small amount of unchanged starting material and two major components with peak areas in the ratio 5.5 : 4.5. On a ClMA column the peaks emerged in the same order, as could be seen by their respective areas. When the flow rate of the mobile phase was reduced (see Table I), the second of the large peaks gradually decreased relative to the other and, thus, was ascribed to *trans*-1,3-hexadien-5-yne*. The assignment was confirmed by preparative G.L.P.C. and infrared spectroscopy of the isolated fractions[11]. It is to be pointed out that the *trans*-form of the 1,3-hexadien-5-yne has the larger retention volume, contrary to the behaviour of the 1,3-penta- and hexadienes, as well as of some monoolefins on polar stationary phases[12].

Differentiation of structural types of conjugated dienes

Structural factors may markedly influence the rate of reaction of conjugated dienes with dienophilic reagents. In particular, alkyl substitution and inclusion of the diene system into a five-membered ring increase reactivity considerably. Available information in the literature[6,9,13] on the direction and magnitude of such effects may be used with advantage for the identification of chromatographic peaks, as shown below.

(a) Crude 2,4-hexadiene, prepared from crotonaldehyde and ethyl magnesium bromide[14], gave on a silver nitrate–glycol column a chromatogram consisting of three peaks. According to ALDER AND VOGT[14] there is formed, in fact, in addition to the expected *trans, trans*- and *trans, cis*-2,4-hexadienes, also *trans*-1,3-hexadiene. When the product was chromatographed at different flow rates on a column containing ClMA, it was found that the first peak decreased rapidly, the second peak more slowly, while the third peak remained unchanged (Table I). The last peak clearly corresponded to *cis, trans*-2,4-hexadiene, which does not react under mild conditions. The other peaks were due to *trans, trans*-2,4-hexadiene and *trans*-1,3-hexadiene; their assignment could be made on the ground that substitution at both ends of a diene

* It has been reported[11] that with maleic anhydride the dien-yne forms 1,2,3,6-tetrahydro-3-ethynyl phthalic anhydride.

system (*trans, trans*-2,4-hexadiene, first peak) more effectively promotes Diels-Alder addition than a similar substitution at one end only.

(b) To illustrate the distinctive behaviour of cyclopentadiene, a mixture consisting of isoprene, *cis*- and *trans*-1,3-pentadiene, cyclopentadiene, and *n*-hexane (reference substance) was passed over a ClMA column at 40° (Table II). Only four peaks were found on the chromatogram even at the higher rate of flow of the carrier gas, as the cyclopentadiene had reacted completely on passage through the column. The non-appearance of a fifth peak was not due to low efficiency of ClMA as a stationary phase, since, as shown on Figs. 2 and 3, the polar ClMA has about the same

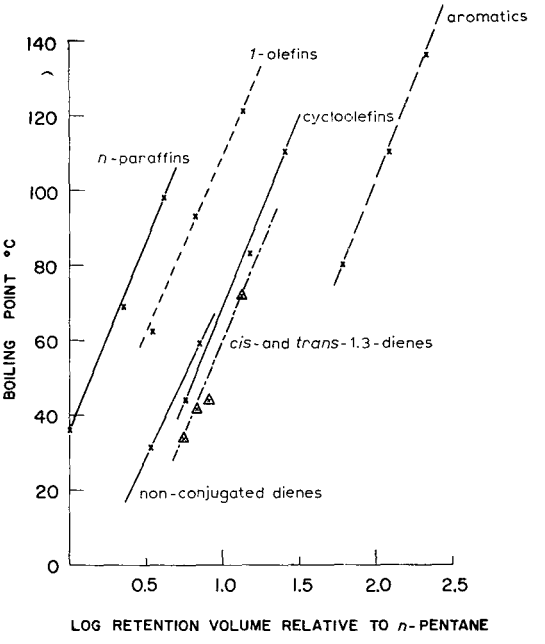

Fig. 3. Plot of log retention volume (relative to *n*-pentane) *versus* boiling point for different classes of hydrocarbons on β,β'-oxydipropionitrile at 40°.

selectivity towards various groups of hydrocarbons as β,β'-oxydipropionitrile, and all five compounds present in the sample were readily separated on the latter phase.

Peak identification by accurate kinetic data

Accurate measurement of the rate of subtraction of a peak, and comparison with that of the component assumed to be present, can be used as a method of identification. Such a procedure is of interest in cases in which preliminary peak assignment has been made, *e.g.* on the basis of the retention volume, but further confirmatory evidence is required. The reproducibility and accuracy of the method is seen in Fig. 4, in which comparison is made of the isoprene and *trans*-1,3-pentadiene peaks appearing in the chromatogram of the mixture described in Table II. The values of log $x°/x$ for the second and third peak of the sample closely fit the curves of the respective pure components. Special care must of course be taken to operate under the same con-

ditions; thus in all cases ClMA purified by crystallization was used, and the temperature was kept at 40°.

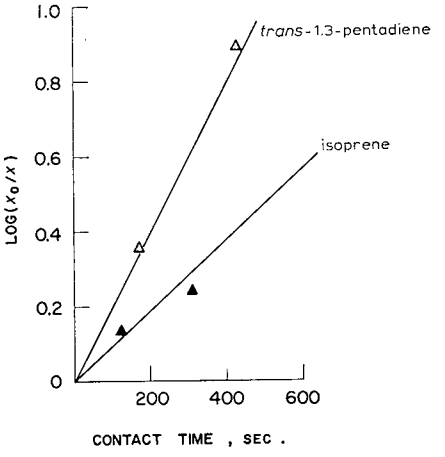

Fig. 4. Plot of log $x°/x$ *versus* contact time for isoprene and *trans*-1,3-pentadiene on a chloromaleic anhydride column. (——) Pure isoprene and pure *trans*-1,3-pentadiene; points correspond to 2nd peak (▲) and 3rd peak (△) of the mixture described in Table II. Stationary phase crystalline chloromaleic anhydride; column length 1 m; weight ratio of solid support to liquid phase 2:1; temperature 40°.

Quantitative analysis

For quantitative analysis a parallel run has to be made on a non-reacting stationary phase, and the order of emergence of the peaks, as compared with that on the subtracting column, has to be checked. Such a comparison of peaks corresponding to non-reacting substances can be made on the basis of their respective areas. As to reacting components, no difficulty arises if only one of the components is partially subtracted. If, however, more than one peak varies, assignment can be made after estimating, to a first approximation, the initial amounts of subtracted components by extrapolating to zero time the corresponding values of log x (with respect to an inert reference substance). An example of such a case is given in the experimental section.

EXPERIMENTAL

Materials

1,3- and 2,4-Hexadiene were prepared according to ALDER AND VOGT[14]. Samples of *1,3-hexadien-5-yne* were kindly supplied by Dr. F. SONDHEIMER and Dr. D. BEN-EFRAIM. All other dienes used were commercial products; *cyclopentadiene* was obtained from dicyclopentadiene by distillation.

Chloromaleic anhydride was distilled at 96°/25 mm, and, where necessary, purified by crystallization from ether[15] at —20°, m.p. 31–32°.

Apparatus and procedure

A Perkin-Elmer Model 154 A Fractometer was used in the experiments. The solid support was powdered Johns-Manville C-22 firebrick of 50–80 mesh, prepared as described previously[16]. The firebrick powder was coated with ClMA, using a small

amount of ether as solvent, and immediately poured into the chromatographic column. The ether was evaporated by passing carrier gas through the column after installation in the apparatus, care being taken to avoid moisture.

The quantities of material injected into the column were adjusted so as to obtain symmetrical peaks, and the areas were calculated by multiplying heights by peak width at half height.

In experiments in which only a ClMA column was employed, the contact time (t) was obtained directly from the chromatogram by dividing the distance from the injection point to the peak maximum by the speed of the recorder paper. For dual columns t was calculated from the equation $V^{\circ}_R = j\,Ft.V^{\circ}_R$, the corrected retention volume was determined by a separate measurement using the ClMA column only; j, the pressure gradient factor, was obtained by estimating the pressure (p_i) at the head of the ClMA section of the dual column on the assumption that resistance to flow was proportional to length[17]; F, the flow of carrier gas at the exit from the ClMA column at atmospheric pressure (p_o), was measured with a soap bubble meter (the ClMA was always placed at the exit side of the dual column system).

Determination of the peak order of the components of crude 2,4-hexadiene

This was carried out on a 2 m column with a saturated silver nitrate–glycol solution as the stationary phase (chromatogram A) and on a 1 m ClMA column (chromatogram B). The ratio of solid support to liquid phase was 2:1 on both columns. The ClMA was not purified by crystallization and was liquid at room temperature. On A the areas of the peaks with respect to *n*-hexane were in their order of emergence, (1) 0.95, (2) 0.42, (3) 1.02. The first peak on B had the same relative area with respect to the inert reference substance as the third peak on A, and was therefore ascribed to the non-reacting *cis*, *trans*-2,4-hexadiene. As to the first and second peaks on B, extrapolation of the values of log area x/area *n*-hexane (Table I) showed that at zero time the first component had a larger area than the second component (log relative area $\sim$ 0 and -0.4, respectively). Hence it followed that on both chromatograms the order of emergence of the peaks was the same.

TABLE III

H VALUES OF DIENES ON A CHLOROMALEIC ANHYDRIDE COLUMN AT 40°*

Compound	*H*
Butadiene	0.68
Isoprene	0.84
trans-1,3-Pentadiene	0.89
trans-1,3-Hexadiene	0.89
trans,trans-2,4-Hexadiene	0.94
trans-1,3-Hexadien-5-yne	0.98

* For details on column see Table I, footnote f.

H values of dienes on a chloromaleic anhydride column at 40°

H is calculated from the retention volume according to the equation:

$$H = \frac{V'_R}{V_R} \qquad (2)$$

where V'_R = adjusted retention volume and V_R = uncorrected retention volume. The values are given in Table III.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. C. GRÜNER and Mrs. A. JACOB for valuable laboratory assistance.

SUMMARY

Partial subtraction chromatography is defined as a procedure in which stationary phases are employed capable, under the chromatographic conditions, of reacting at a measurable rate with certain classes of compounds. The reaction occurring can be followed as a function of time by determining the areas of the corresponding peaks emerging from the column at different flow rates of carrier gas.

Partial subtraction is proposed as a method of peak identification. Its application is illustrated by a series of examples in which conjugated dienes are the reacting components and chloromaleic anhydride is the stationary phase.

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EIN GERÄT ZUR MARKIERUNG VON CHROMATOGRAMMEN

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ALLGEMEINES

Eine Kombination von Papierchromatographie mit radiometrischen Methoden hat zur Entstehung einer neuen Technik geführt, die als Radiochromatographie bekannt ist. Es gibt prinzipiell zwei Wege bei dem radiochromatographischen Verfahren. In dem ersten Falle wird eine Mischung von radioaktiven Bestandteilen auf dem Filterpapier getrennt. In dem zweiten kann man eine nichtradioaktive Mischung chromatographieren und die in den Flecken enthaltenden getrennten Substanzen unmittelbar auf dem Filterpapier markieren.

Um die Markierung der Chromatogramme durchzuführen, ist es notwendig, sie in eine radioaktive Lösung einzutauchen. Ein Überschuss des markierenden Stoffes wird nachher durch mehrmalige Spülung entfernt. Zu diesem Zwecke wurde eine spezielle Vorrichtung entwickelt, in der die obengenannten Tätigkeiten, gleichzeitig mit einer grossen Zahl von Chromatogrammen, vorteilhaft durchzuführen sind.

EINZELHEITEN DES GERÄTES

Das Prinzip der Vorrichtung geht von der Voraussetzung aus, dass bei der Markierung der Chromatogramme Eintauchen von grossen Oberflächen des Filterpapiers in kleine Volumen der radioaktiven Lösung erwünscht ist.

Diese Bedingung erfüllt ein hohes und schmales Gefäss, vom Querschnitt eines flachen Sechseckes. Das Chromatogramm wird in dieses Gefäss entlang der grösseren Diagonale hineingeschoben und kann in ihm mit radioaktiven Lösungen behandelt sowie mit Spülflüssigkeiten gewaschen werden.

Das Gerät wird aus zwei verschiedenartigen Grundelementen zusammengesetzt:

(1) aus zwei äusseren Grundelementen, von denen eine Oberfläche gefräst wird; die zweite Oberfläche bleibt glatt und ist nach aussen gerichtet,

(2) aus einer beliebigen Zahl von inneren Grundelementen, deren beide Oberflächen gefräst werden.

Nach der Zusammenstellung der Grundelemente entstehen lange, flache, sechseckige Gefässe; ihr Volumen beträgt etwa 5.5 ml pro 10 cm Länge. Die Dicke der Flüssigkeitschicht macht beiderseits des Chromatogramms kaum etwa 2 mm aus. Die Fig. 1 ist eine Skizze der beiden Grundelemente sowie eines runden Sockels, an dem die zusammengefügten Grundelemente befestigt werden.

Die beiden Grundelemente werden aus Platten von Polymethacrylat (Plexiglas, 1 cm dick) hergestellt. Dieses Material zeichnet sich nicht nur durch gute Durchsichtigkeit aus, sondern besitzt auch hohe chemische Widerstandsfähigkeit und schlechte Netzbarkeit. Parallele Aushöhlungen sollen mit einer Genauigkeit von ± 0.1 mm gefräst und nachher leicht poliert werden. Es besteht natürlich eine Möglichkeit

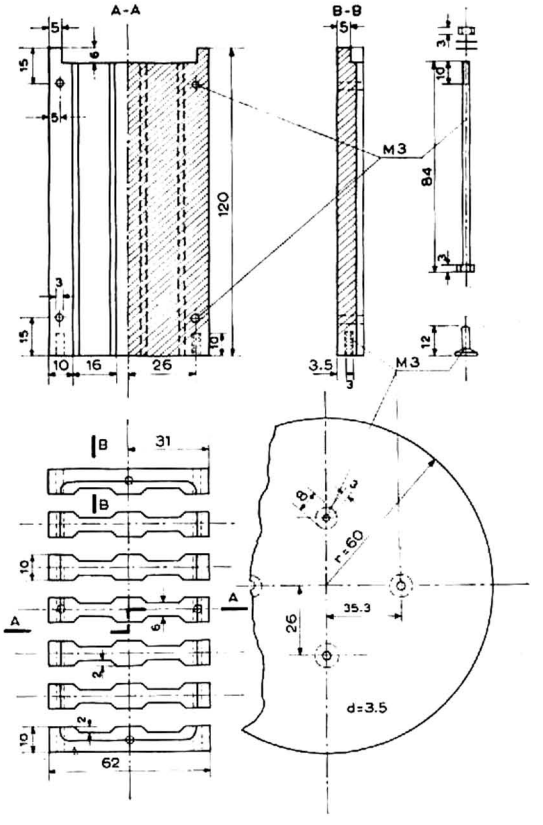

Fig. 1. Skizze der äusseren und inneren Grundelemente von 12 cm Höhe sowie des Sockels.

alle die aufzustellenden Grundelemente mit Hilfe von einem Bindemittel zusammenzukleben, was sich allerdings nicht bewährt hat. Es ist vorteilhafter alle die aufzustellenden Grundelemente mit einem Silikonfett derart zu schmieren, dass die in Berührung kommenden Flächen abgedichtet werden. Sie werden danach mit Hilfe von 4 langen Schrauben mit sechseckigem Kopfe (M3 × 84) zusammengesetzt. Dadurch wird eine gute Reinigung gesichert und überdies noch eine Möglichkeit geschaffen eine Zusammenstellung einer beliebigen Zahl von Grundelementen zu erhalten. Der runde Sockel, der aus einer Polymethacrylatplatte von etwa 3.5 mm Dicke ausgedrechselt wird, wird an den zusammengesetzten Grundelementen mit Hilfe von 4 Schrauben mit flachem Kopfe (M3 × 12) befestigt; der Sockel soll vorher mit einem Silikonfett geschmiert werden. Die obere Flansche des Gerätes wird erst nach der Zusammenstellung

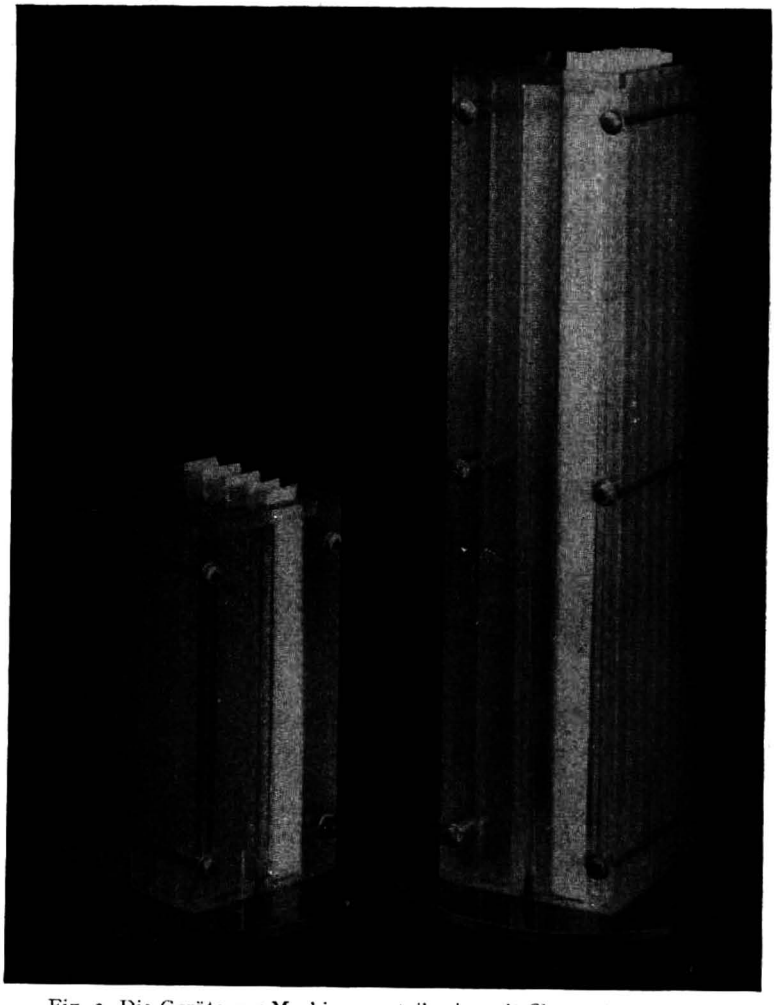

Fig. 2. Die Geräte zur Markierung, teilweise mit Chromatogrammen.

und Befestigung gefräst. In unserem Laboratorium werden Geräte von 12, 16, 20 und 26 cm Höhe verwendet. Das niedrigste und das höchste wurden in der Fig. 2 dargestellt.

MARKIERUNGSMETHODIK

In unserem Laboratorium wird das beschriebene Gerät folgenderweise verwendet. Die Chromatogramme werden auf 15 mm breiten Filterpapierstreifen hergestellt. Dann werden trockene Streifen in die lufttrockenen Spalten der entsprechend hohen Geräte hineingeschoben. Vorspringende Teile der Chromatogramme werden derart abgeschnitten, dass sie 4–5 mm hervorragen. Das Gerät mit den Chromatogrammen wird unter einem radiochemischen Abzug mit radioaktiver Lösung so gefüllt, dass die Spalten vollkommen überschwemmen; danach wird es mit einer Glasplatte bedeckt. Nach Beendigung der Reaktion wird das Gerät mit der Hand oder mit einem Manipulator

umgekippt. Dabei fliesst lediglich die Lösung heraus, die markierten Chromatogramme bleiben dagegen drinnen. In derselben Weise werden die Radiochromatogramme mit geeigneter Waschlösung gespült. Die letzte Waschlösung wird nicht entfernt, sondern die Radiochromatogramme werden aus der Lösung mit Hilfe einer Pinzette herausgezogen. Sie werden nachher auf einer Glasplatte unter dem Abzug getrocknet.

Es soll betont werden, dass die beschriebene Vorrichtung allgemeine Bedeutung für das Laboratorium besitzt. Sie kann in allen Fällen verwendet werden, in welchen eine Behandlung von Chromatogrammen bzw. Elektropherogrammen mit gefährlichen oder kostspieligen Reagenzien ausgeführt werden soll.

ZUSAMMENFASSUNG

Eine Vorrichtung zur Behandlung von Chromatogrammen bzw. Elektropherogrammen mit radioaktiven, giftigen oder kostspieligen Lösungen wurde entwickelt. Sie besteht aus mehreren gleichen Plexiglas-Teilen, die zusammengeschraubt werden. Da die einzelnen Teile in geeigneter Weise gefräst wurden, entstehen zwischen ihnen schmale, lange Gefässe, mit einem Querschnitt eines flachen Sechseckes. Filterpapierstreifen werden entlang der grösseren Diagonale in diese Gefässe hineingeschoben und mit den entsprechenden Lösungen behandelt.

SUMMARY

A simple device for labelling or staining chromatograms or electropherograms is described. It may find application especially when radioactive, toxic or expensive solutions have to be used. This device consists of a number of identical units made of polymethacrylate. The individual units are hollowed out in such a way that when they are connected together, long narrow slits having a hexagonal cross-section are formed. The very small space of the slits reduces the volume of labelling solution necessary for treatment of the filter paper strips, to a minimum.

AN EVALUATION OF VENTED PROGRAMMED TEMPERATURE PRECOLUMNS IN GAS–LIQUID CHROMATOGRAPHY

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One of the principal problems encountered in the analysis of high molecular weight compounds by gas chromatography is satisfactory introduction of the sample onto the column. This was pointed out by RENSHAW AND BIRAN[1] who developed a relatively simple apparatus and technique whereby a dilute solution of methyl esters could be accurately measured into a "spoon", the solvent evaporated, and the "spoon" introduced into the heated portion of the chromatograph. The technique as described worked satisfactorily for C_{12} and higher fatty acid methyl esters. KIRKLAND[2] also developed a similar method in which the solvent was "evaporated" within the chromatograph as opposed to the external evaporation technique of RENSHAW AND BIRAN. In KIRKLAND'S method, the solution was injected into a flash vaporizer, carried into a cold column where condensation of high boiling components occurred, and the solvent vented to the atmosphere by means of a valve arrangement preceding a second (partition) column. After closing the vent valve, a traveling furnace moved the high boiling constituents through the first column and onto the second column where partition and subsequent separation occurred. A third method of sample introduction is possible with packed columns by utilizing temperature programming of the partition column. The solution can be vaporized, carried onto a "cold" partition column where the high boiling constituents are immobilized in the liquid phase while the solvent passes through the column. This method is the least satisfactory of the three because extreme solvent tailing generally occurs, particularly with low liquid phase loadings on the column.

When using packed columns and large solution injections, argon ionization detectors become overloaded with resulting electrode arcing while hydrogen flame detectors are often extinguished by the sudden pressure surge due to flash vaporization of the solvent. In addition, the solvent often removes liquid phase from the front of the column (exposing the support) and carries part of the column liquid phase into the detector with a subsequent unstable increase in background current and decreased sensitivity. Packed columns can handle large volumes of solvent, however, and this is undoubtedly one of the principal reasons they are preferred over the more efficient Golay columns which normally require a stream splitter and, effectively, more concentrated solutions for equivalent sensitivity.

KIRKLAND'S method and the temperature programming method both normally utilize heated injection ports which often result in sample decomposition, particu-

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larly with drugs, pesticides, and organo-metallics. Because of this, direct on-column injection techniques are again becoming popular, particularly in preparative separations[3].

Recently we have investigated a concept similar to that suggested by KIRKLAND. Basically the method consists of injecting a relatively large volume of solution into a short precolumn initially at low temperature and venting off the solvent at a point between this short column and the partition column (see Fig. 1). The vent is then

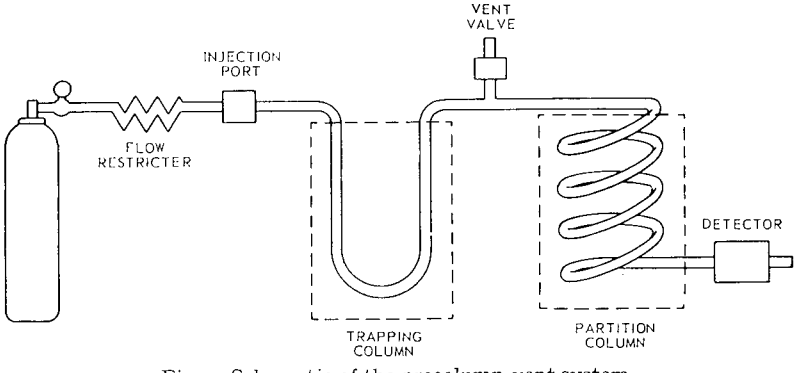

Fig. 1. Schematic of the precolumn-vent system.

closed and the precolumn temperature is programmed rapidly to transfer the higher boiling components onto the partition column. In this way, under optimum conditions, only traces of the solvent are introduced onto the partition column so that neither tailing nor detector overloading occurs. (This basic concept has recently been reported by KARMEN, WALKER AND BOWMAN[4] who eliminated the partition column to simply analyze for total lipids after venting off the low boiling solvent.)

EXPERIMENTAL

The first precolumn investigated was a $^1/_8$ in. O.D., $^1/_{16}$ in. I.D. by 8 in. stainless steel tube packed with uncoated $^{100}/_{120}$ mesh silicon carbide. This was used in series with a $^1/_{16}$ in. I.D. by 5 ft. partition column packed with 5 % SE 30 Silicone gum rubber (General Electric) on $^{160}/_{170}$ mesh Anakrom ABS (Analytical Engineering Laboratories). The partition column and an all-metal vent valve were maintained at 70°. The precolumn was wrapped with asbestos-covered nichrome heating wire. Flow rate was 24 ml/min at the detector and 30 ml/min at the vent outlet.

The second precolumn investigated was an uncoated $^1/_{16}$ in. O.D., 0.020 in. I.D. by 4 ft. length of stainless steel capillary tubing. The first 1.5 in. of the capillary was expanded to an internal diameter of approximately 0.035 in. to allow for insertion of Hamilton syringe needles. A modified Swagelok fitting was used with a Burrell silicone septum seal for needle insertion. This capillary precolumn was used in series with a 35 ft., 0.010-in. I.D. capillary column internally coated with SE 30 silicone rubber. The vent valve separating the precolumn from the partition column consisted of a 6 in. length of $^1/_{16}$ in. O.D. capillary tubing which led to a specially constructed, neoprene-sealed on-off toggle valve of minimum dead volume. The Golay column was maintained at 60°. The precolumn was electrically isolated at one

end to become a self-heating resistance element when connected to a 6 V transformer through an on-off switch. The flow rate was 12 ml/min at the detector and 25 ml/min at the vent outlet.

A conventional Beckman hydrogen flame ionization detector and electrometer amplifier were used in both cases for effluent detection.

In order to briefly investigate the possibility of using this system for high speed, high temperature chromatography, a special hydrogen flame unit was constructed. This consisted of a 1.5 in. diameter brass base around which 20 ft. of $^1/_{16}$ in. O.D., 0.020-in. I.D. stainless steel tubing was wrapped. The detector was machined into this base as were holes for air and hydrogen lines, a cartridge heater, a "Y" connector to join a capillary precolumn, vent line, and partition column, and a thermocouple. Four feet of $^1/_{16}$ in. O.D., 0.020-in. I.D. stainless steel tubing was used for the precolumn and a 2 in. piece of $^1/_{16}$ in. O.D., 0.020-in. I.D. tubing was used for the vent. All connections were designed to eliminate dead space and were silver-soldered. A 10 V transformer was used to heat the precolumn by isolation of one end to become a self-heating resistance element in the same manner as described above. The temperature rise was measured by attaching a thermocouple to the precolumn; at 10 V the precolumn heated from 25° to 250° in 15 sec with a helium flow of 40 ml/min (optimum flow rate for this system) passing through the precolumn. The partition column was coated in place with Versamid 900 (General Mills) and was maintained at 200°.

In all cases flow was limited by the use of a capillary flow restrictor and two-stage pressure regulator preceding the gas chromatographic systems. The flow difference between the vent and the detector with the first two systems was due to the flow resistance of the partition columns. In the third, high-speed system, flow rate was controlled almost entirely by the resistance of the capillary flow restrictor preceding the system so that the same flow rate was measured at the vent as at the detector. The flow resistance of the partition columns made possible the use of simple on-off valves for the vent. Helium was used in each case as the carrier gas.

RESULTS AND PERFORMANCE

To determine the maximum amount of solvent (diethyl ether) which could be injected into the precolumn without resulting in sample carry-over through the precolumn and out the vent, a series of dilutions were prepared of fatty acid methyl esters which differed in solute concentration by factors of 2. Increasing volumes of solution were then injected into the systems such that the sample weight remained constant. The determination of ester loss was then made in the case of the packed column by comparing the response for each ester injection with that of reference injections without venting. With the Golay column, this method of comparison was not possible because direct injection of the solvent into the column was not feasible. However, the responses for the first four dilutions using 2, 4, 8, and 16 μl injections were identical and as a result, it was assumed that no loss occurred.

It was found that with the $^1/_{16}$ in. I.D. by 8 in. precolumn packed with silicon carbide that up to 200 μl of solution could be injected without any loss of C_5 and C_6 fatty acid methyl esters when using a vent time of 1 min at a vent temperature of 25°. Larger injections resulted in some carry-over of the esters through the vent. The

1-min vent time was optimum for these relatively volatile esters with vent times shorter than 30 sec resulting in solvent being carried onto the partition column while vent times longer than 3 min resulted in some loss of these esters. It was estimated that more than 95 % of the solvent is vented within the first 10 sec. With the capillary precolumn using C_8 and C_9 fatty acid methyl esters as the chromatographic samples, up to 32 μl of solution could be injected using a 10-sec vent time (optimum) before a significant amount of solvent was carried onto the partition column. By using a longer vent time (up to 3 min) as much as 64 μl could be injected, but when larger solution volumes were used, serious nonreproducible losses of the esters through the vent occurred. The 32-μl to 64-μl injections used with the capillary injection system represented approximately $^1/_4$ to $^1/_3$ the internal volume of the tubing. Fig. 2 shows a typical chromatogram of the C_8 and C_9 fatty acid methyl esters chromatographed using this system.

In using the Golay system designed for high speed chromatography, it was found that after establishment of optimum flow rates, vent time, and precolumn heating

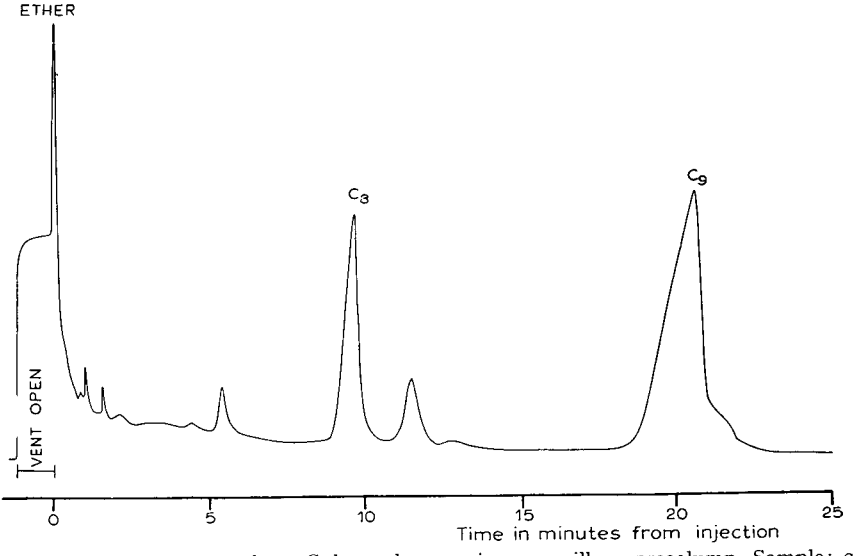

Fig. 2. Typical chromatogram from Golay column using a capillary precolumn. Sample: 20 μl injection containing 1.2 $\times$ 10^{-6} g methyl octanoate (b.p. 193°) and 2.5 $\cdot$ 10^{-6} g methyl nonanoate (b.p. 215°). Conditions described in text.

time, it was possible to reproducibly separate the NIH Metabolism Study Section Standard Mixture "C" of C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18} and C_{20} fatty acid methyl esters in 30 sec with a total analysis time of 1 min. The first four components were not completely separated, but were identifiable (see Fig. 3).

APPLICATION TO PROGRAMMED TEMPERATURE AND ISOTHERMAL COMMERCIAL CHROMATOGRAPHS

On the basis of the work discussed above, two commercial gas chromatographs were modified to enable the use of this system. In the first modified chromatograph

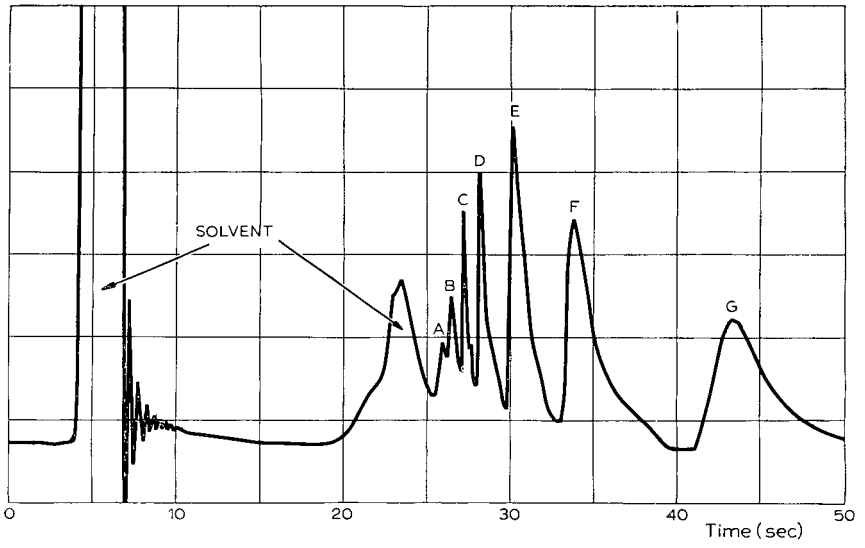

Fig. 3. Oscilloscope tracing of high speed methyl ester chromatography. Initial solvent peak is due to short vent time (13 sec). The second solvent peak is residual solvent held by condensed methyl esters. Sample size: 2 μl of 0.05 % ester mixture described in text.

(Beckman Thermotrac), the injection port was replaced with a 6-in. long precolumn filled with silicon carbide. The vent was placed in the feedthrough connector which joins the precolumn with the partition column. It was found that condensation of higher molecular weight esters occurred in this connector which was thermally attached to a heat sink (the oven wall). Subsequent slow vaporization of the esters from the connector as the column oven temperature was raised resulted in broad, unsymmetrical peaks as shown in Fig. 4. This was initially eliminated by maintaining the connector at the same temperature as the detector, however, it was found that when certain drugs and pesticides were chromatographed, decomposition occurred at this joint just as decomposition had occurred in conventional flash vaporizers. Consequently, a "floating" connection (one not physically or thermally connected to the oven wall) was used. In this case chromatograms exhibiting good symmetrical

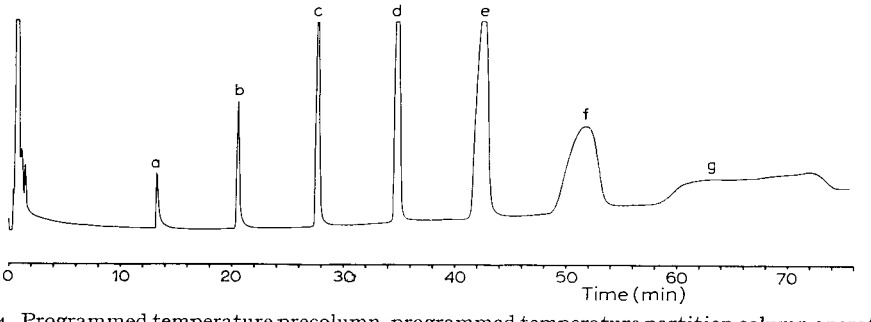

Fig. 4. Programmed temperature precolumn, programmed temperature partition column operation showing effect of cold junction between columns. Sample size: 100 μl of 0.05 % ester mixture described in text.

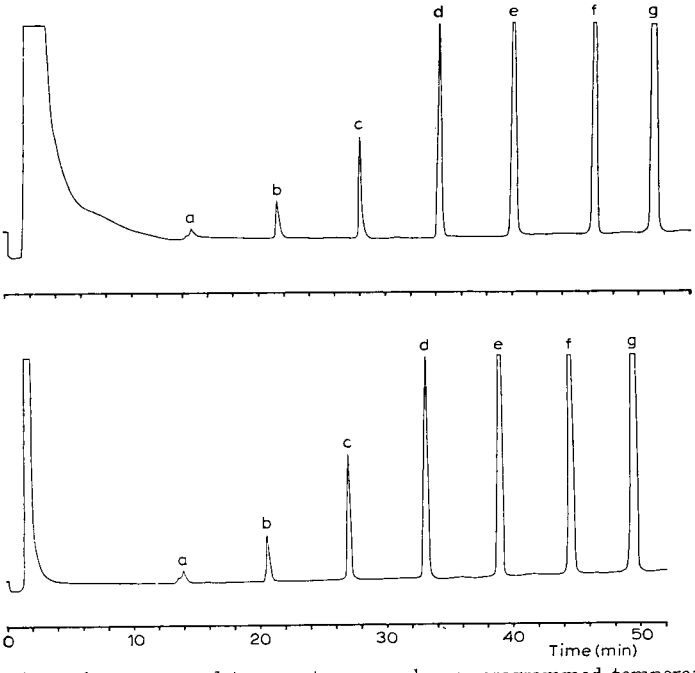

Fig. 5. Comparison of programmed temperature precolumn, programmed temperature partition column operation. Top: without solvent venting. Bottom: with solvent venting (10 sec vent). Sample same as in Fig. 4.

peaks were obtainable and no sample decomposition occurred. Fig. 5 illustrates chromatograms obtained through the use of this system; (a) with solvent venting and (b) without solvent venting for methyl ester analysis. No loss of C_8 and higher esters occurs with venting and much larger sample injections can be made without venting when the solvent is injected onto a cold precolumn than when injected into a hot vaporizer. In regard to this second factor, we had previously found that a 10-μl injection of diethyl ether into a flash vaporizer invariably resulted in extinction of the hydrogen flame. By comparison, since installation of this system, the flame has never been extinguished by injections of ether up to 100 μl in volume when using the same column and detector and the same carrier, hydrogen, and air flow rates.

Fig. 6 compares the isothermal partition column operation of a Barber Coleman model 61-c incorporating this system (a) with the precolumn maintained at 250°, (b) with venting and precolumn programming, (c) without venting but with programmed precolumn temperature. Effluent detection was by the Lovelock diode argon ionization detector. The improvement of chromatograms obtained with this system over conventional flash vaporization systems is readily apparent. Somewhat analogous to hydrogen flame detection, larger volumes of solvent can be injected without venting before electrode arcing occurs.

Although we have found no case where it is more desirable to use a hot (isothermal) flash vaporizer, an autotransformer does provide a means for isothermal operation and was used on the Barber Coleman to provide a means by which the effect of varying the heating rate of the precolumn could be investigated. The rate of precolumn

temperature rise is important, but is not critical within limits. If the rate is too slow, unsymmetrical peaks with shoulders occur for each component. We have found that 100-W cartridge heaters, operated at 115 V provide adequate heating rates when using carrier flow rates of less than 100 ml/min provided a low mass heat transfer block such as aluminum is used to provide even heat transfer from the cartridge heaters to the precolumn. We normally use flow rates of about 20 to 40 ml/min through $^1/_{16}$ in. I.D. columns and at these flow rates, no problems have been encountered.

With this system "instantaneous" injections are not required and indeed, are not desirable. Better results are obtained, particularly with solvent venting, if the

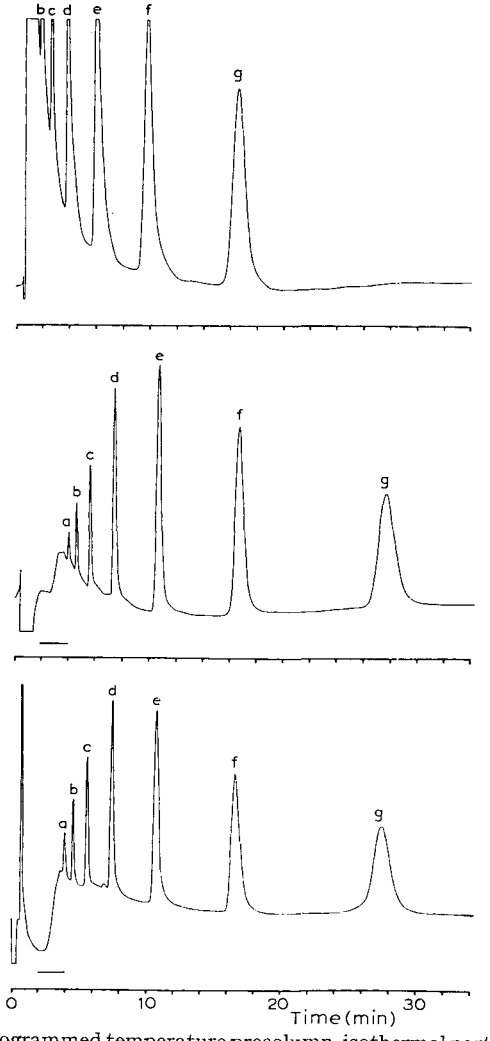

Fig. 6. Comparison of programmed temperature precolumn, isothermal partition column operation. Top: 5 μl of 0.05% ester solution injected onto hot precolumn without venting. Middle: 3 μl of 0.05% ester solution injected onto cold precolumn without venting followed by programmed precolumn. Bottom: 3 μl of 0.05% ester solution injected onto cold precolumn with 10 sec vent followed by programmed precolumn.

transfer of solution from the syringe to the precolumn is allowed to proceed over several seconds. With high boiling components it is even possible to make several repeat injections of dilute solutions before programming the precolumn, thereby effecting several-fold concentration of the sample.

ACKNOWLEDGEMENTS

The author wishes to thank Mr. WM. DABNEY for technical assistance in the construction, testing, an modification of each system. Portions of this work were conducted under contract to the U.S. Army Biological Laboratories, Ft. Detrick, Frederick, Md.

SUMMARY

The use of vented, programmed temperature precolumns in gas chromatography is described. The use of these precolumns facilitates the analysis of thermally unstable compounds, enables high speed chromatography of high molecular weight compounds, and aids in the analysis of trace amounts of high molecular weight materials in large volumes of low boiling solvents, particularly when using isothermal partition column operation.

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ANALYSIS OF FATTY ACID DERIVATIVES BY GAS–LIQUID CHROMATOGRAPHY*

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INTRODUCTION

During the course of our work on brain lipids[1] several oxygenated compounds of unknown structure were detected by gas–liquid chromatographic analysis. To characterize these compounds an effort was made to evaluate the influence of various structural groups, with particular reference to positional isomers, on relative retention times. It has been suggested that carbon number[2], carbon chain equivalent[3], R_{x9} units[4], and relative retention volumes[5] are useful for identification. With unknown mixtures containing a number of heterofunctional or isomeric components, the use of retention volume data alone for the identification of components by gas chromatography has serious limitations. The application of auxiliary chemical methods to the determination of the compound type, after collecting eluted chromatographic peaks, by means of functional group analysis[6] is limited by sample size and also is not always reliable. Wherever possible, other instrumental methods[7–10] have been employed for subsequent identification of the eluted peaks, but the supplementary instrumentation is expensive and the procedures time-consuming.

The systematic use of retention data from two or more columns having different liquid phases does provide a method for functional group classification[10], but it remains difficult to apply this in the identification of structural isomers. The purpose of this investigation is to show how conventional "log plots" and retention data from appropriately chosen columns can be used in a systematic way to provide a method for the identification of functional groups and also structural isomers.

EXPERIMENTAL

A F & M Model 500 gas chromatograph equipped with Model 1609 flame ionization attachment was used. The columns were 4 in. coils of borosilicate glass tube, 6 mm diameter and 8 ft. long packed with SE-30 siloxane polymer (2 parts) on acid–base washed Gas-Chrom P (80–100 mesh), or Apiezon M (APM) (20 parts) on acid–base washed Chromosorb W (80–100 mesh), or Carbowax 6000 (CW) (15 parts) on acid–base washed Chromosorb W treated with dimethyldichlorosilane (60–80 mesh), or a commercial (Applied Science Laboratories, Inc.) preparation of ethylene glycol succinate (EGS) (14 parts) on Chromosorb W (80–100 mesh). The column temperatures

* Supported in part by U.S.P.H.S. NB 03523-02.

were 160°, 165°, 175° and 190°, and the corresponding argon flow rates were 23, 20, 23 and 25 ml/min with EGS, Carbowax 6000, SE-30 and Apiezon M columns, respectively.

The compounds used in this work were either purified commercial materials or were prepared in this laboratory. These were either predominantly pure or had a component which was clearly identifiable (*e.g.* from a homologous series gas–liquid chromatography plot). At least three and usually five members of each homologous series were used in obtaining log plots.

Retention times were measured from the time of injection of sample to the time of appearance of the peak maxima on the recorder chart, and were corrected for calculated carrier gas front. Methyl esters of *n*-acids were chosen as standards for the determination of relative retention times. All the values included in this paper are relative to theoretical methyl *n*-octadecanoate. The relative retention times were plotted against carbon numbers on a two-cycle semi-log paper to obtain the log plots and the equivalent carbon numbers were calculated from these plots.

RESULTS AND DISCUSSION

All data presented in this study refer to the operating conditions stated above, and under these conditions the slopes of the log retention time *versus* carbon number plots of a given homologous series for the different columns were found to be unequal. Obviously, therefore, retention time ratios could not be used for the classification of the compound type. The slopes of retention time–carbon number graphs of various homologous series of compounds were determined relative to that of methyl *n*-esters as standard for the columns used. The relative slope and equivalent carbon number data for the different columns and compound types are given in Table I. It can be readily seen that, with a single column, even when the relative slope is unity, the differences in the calculated equivalent carbon numbers for many series of homologous compounds are not sufficiently large as to enable characterization of the compound type. However, a careful study of the data obtained from the different columns (Fig. 1 and Table I) would indicate that it is possible to identify the type of the compound. For example, both *n*-$C_{15}H_{31}CH(OH)C_2H_5$ and *n*-$C_{14}H_{29}CH(OH)C_3H_7$ have theoretical equivalent carbon numbers of 17.35 on the EGS column. They are readily distinguished on Carbowax 6000, however, by the values of 17.55 and 17.30, respectively.

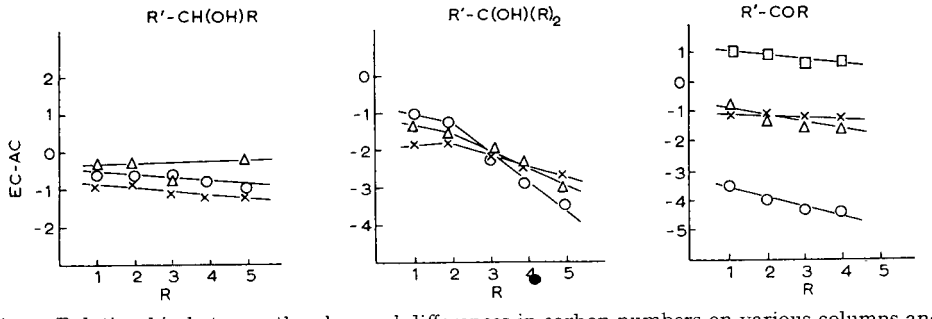

Fig. 1. Relationship between the observed differences in carbon numbers on various columns and the position of the functional group along the carbon chain. O—O EGS; △—△ CW 6000; ×—× SE-30; □—□ APM. EC = equivalent carbon number; AC = actual carbon number.

TABLE I

RELATIVE SLOPE AND EQUIVALENT CARBON NUMBER ON DIFFERENT COLUMNS

(Each compound has a basic 18-carbon chain)*

Compound type	EGS		Carbowax 6000		SE-30		APM	
	Rel. slope	Eq. C. No.	Rel. slope	Eq. C. No.	Rel. slope	Eq. C. No.	Rel. slope	Eq. C. No.
RCH_2OH	1.094	19.75	1.226	19.90	1.000	16.70		
$RCH(OH)CH_3$	1.000	17.35	0.926	17.50	1.000	17.00		
$RCH(OH)C_2H_5$	1.000	17.35	0.959	17.55	1.000	17.10		
$RCH(OH)C_3H_7$	1.000	17.35	0.990	17.30	1.000	16.85		
$RCH(OH)C_4H_9$	1.000	17.20	—	—	1.000	16.75		
$RCH(OH)C_5H_{11}$	1.000	17.00	1.170	17.70	1.000	16.80		
$RC(OH)(CH_3)_2$	1.000	17.00	1.000	16.75	1.000	16.20		
$RC(OH)(C_2H_5)_2$	1.000	16.80	1.000	16.57	1.000	16.30		
$RC(OH)(C_3H_7)_2$	1.000	15.85	1.000	15.90	1.000	15.85		
$RC(OH)(C_4H_9)_2$	1.000	15.15	1.000	15.70	1.000	15.60		
$RC(OH)(C_5H_{11})_2$	1.000	14.60	1.000	15.15	1.000	15.40		
RCHO	1.000	17.35	1.000	16.95	1.000	16.90	1.000	17.20
$RCOCH_3$	1.000	14.50	1.000	17.05	1.033	16.75	1.084	18.90
$RCOCH_2CH_3$	1.000	13.90	0.935	16.70	1.033	16.75	1.084	18.85
$RCOCH_2CH_2CH_3$	1.000	13.65	0.989	16.40	1.033	16.60	1.084	18.50
$RCOCH_2CH_2CH_2CH_3$	1.000	13.55	1.057	16.35	1.033	16.60	1.084	18.50
RCO_2CH_3	1.000	18.00	1.000	18.00	1.000	18.00	1.000	18.00
$MeCO_2RCO_2CH_3$	1.000	20.40	1.000	24.15	1.000	21.35	1.000	20.70
$RCH(Br)CO_2CH_3$	1.000	23.15	1.000	21.20	1.000	20.60	1.000	20.35
$RCH(OH)CO_2CH_3$	1.000	20.00	1.000	21.40	1.000	19.50	1.000	19.10
RCN	1.000	20.95	1.000	19.30	1.000	17.90	1.000	17.65
RCH(Br)CN	1.000	23.80	1.000	21.95	1.000	20.50	—	—
Retention time of C_{18} methyl ester in min	3.65		10.35		39.00		155	

* The methyl group in a methyl ester is not considered part of the basic carbon chain.

The use of many different columns is time-consuming and laborious. A few pairs of appropriately chosen columns should provide sufficient retention information to enable characterization. Combinations of column pairs were considered, and the differences in the theoretical equivalent carbon numbers of the various homologous series were calculated. The data presented in Table II show that EGS–Carbowax 6000 and EGS–SE-30 as column pairs provide, in general, the maximum numerical separation of equivalent carbon numbers, and most suitable of the combinations considered for distinguishing functional groups and isomers of the types included in this study.

Identification of important functional group types by means of retention volume constants reported by MERRITT AND WALSH[11] is rather limited in its application. The analysis described in this paper based on the equivalent carbon numbers permits not only functional group analysis but also characterization of positional isomers in the series studied.

ACKNOWLEDGEMENT

The technical assistance of Mrs. UNA BARNETT in the synthesis of many of the compounds used in this study is acknowledged.

TABLE II

COMPARISON OF COLUMNS FOR SELECTION OF COLUMN PAIRS
(Separation of equivalent carbon numbers)

Compound type	*EGS-CW*	*EGS-SE-30*	*EGS-APM*	*CW-SE-30*	*CW-APM*	*SE-30-APM*
RCH_2OH	—0.15	3.05		3.20		
$RCH(OH)CH_3$	—0.15	0.35				
$RCH(OH)C_2H_5$	—0.20	0.25		0.45		
$RCH(OH)C_3H_7$	0.05	0.50		0.45		
$RCH(OH)C_4H_9$	—	0.45		—		
$RCH(OH)C_5H_{11}$	—0.30	0.20		0.50		
$RC(OH)(CH_3)_2$	0.75	0.80		0.55		
$RC(OH)(C_2H_5)_2$	0.23	0.50		0.27		
$RC(OH)(C_3H_7)_2$	—0.05	0.00		0.05		
$RC(OH)(C_4H_9)_2$	—0.55	—0.45		0.10		
$RC(OH)(C_5H_{11})_2$	—0.55	—0.80		—0.25		
RCHO	0.40	0.45	0.15	0.05	—0.25	—0.30
$RCOCH_3$	—2.55	—2.25	—4.40	0.30	—1.85	—2.15
$RCOCH_2CH_3$	—2.80	—2.85	—4.95	—0.05	—2.15	—2.10
$RCOCH_2CH_2CH_3$	—2.75	—2.95	—4.85	—0.20	—2.10	—1.90
$RCOCH_2CH_2CH_2CH_3$	—2.80	—3.05	—4.95	—0.25	—2.15	—1.90
$CH_3CO_2RCO_2CH_3$	—3.75	—0.95	—0.30	2.80	3.45	0.65
$RCH(Br)CO_2CH_3$	1.95	2.55	2.80	0.60	0.85	0.25
$RCH(OH)CO_2CH_3$	—1.40	0.50	0.90	1.90	2.30	0.40
RCN	1.65	3.05	3.30	1.40	1.65	0.25
RCH(Br)CN	1.85	3.30	—	1.45	—	—

SUMMARY

A method for the classification of functional groups and positional isomers based on gas chromatographic retention data and theoretical equivalent carbon numbers is described. The columns used were ethylene glycol succinate (EGS), Carbowax 6000, SE-30 and Apiezon M. Combinations of EGS–Carbowax 6000 and EGS–SE-30 as column pairs appeared most suitable for the primary, secondary and tertiary alcohols, aldehydes, ketones and methyl esters of a few fatty acids and their derivatives studied. Operating conditions for the different columns are described.

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A PROTECTED RADIOACTIVE SOURCE FOR ELECTRON CAPTURE AND OTHER GAS CHROMATOGRAPHIC DETECTORS

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INTRODUCTION

In electron capture detectors so far described[1,2], tritium has been used as the primary source of ionisation. Tritium is chosen because the 0–8 kV β-particles emitted from a tritium source ionise the gas within the detector only through a range extending a few millimetres from the source. It is thus possible to make detectors in which ion pair formation occurs only within a small region close to the source, which is used as the cathode, and all the secondary electrons so formed travel the whole width of the detector before being collected at the anode. This situation is desirable in an electron capture detector because it combines maximum sensitivity with minimum secondary effects, *e.g.* peak inversion due to direct ionisation of the sample by radiation from the radioactive material.

However, the use of tritium sources has the disadvantage that the tritium-bearing compound used cannot be protected by sealing between metal foils without preventing the egress of β-particles. The radioactive material is thus open to attack from any reactive compound carried in the gas stream from the column. It is also susceptible to progressive deterioration due to the deposition of films of stationary phase, etc., stripped from the column. At temperatures above 200° there is loss of tritium from the source compound even in the absence of reactive compounds in the gas stream[3]. The use of detectors employing tritium sources is thus subject to certain limitations and some of these limitations apply also to other detectors in specific circumstances.

By using the technique described in this paper, the advantages normally associated with tritium can be obtained whilst using other, more stable sources and, in addition, the source material is protected from contact with the carrier gas stream from the chromatograph column. This is accomplished by enclosing the source within an additional chamber separated from the normal detector chamber. The source chamber is then maintained free from unwanted compounds by "scavenging" it with a stream of clean gas. The required ionisation in the detector chamber is secured by electrons passing from the source chamber into the main chamber under the influence of a suitable electrical field. Thus the two processes normally taking place in the detector, *viz.* the generation of an electron current by ionisation from the radioactive source and the subsequent electron impact processes leading to detection of the compounds carried from the column, are separated. This eliminates subsidiary effects resulting

from direct ionisation of the sample compound by primary radiation from the radioactive source, one of the causes of peak inversion in electron capture detectors. Moreover as the electron stream can be led into the main chamber in a specific region whatever radioactive material is used, there is a greater freedom of choice of radioactive material, and tritium need no longer be used.

APPLICATION TO ELECTRON CAPTURE DETECTORS

The application of this principle to an electron capture detector is illustrated in Fig. 1.

A stream of carrier gas from a chromatograph column is introduced into the detector chamber (B) through the gauze anode (A) in the normal way[1].

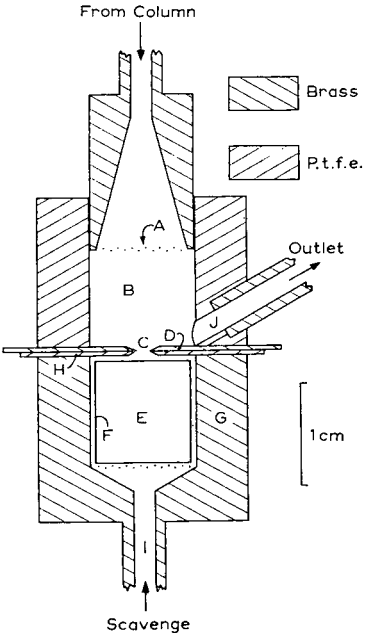

Fig. 1. Electron capture detector with an external radiation source.

Electrons generated in the cavity (E) by the radioactive source (F) diffuse through the hole (C) in the metal plate (D) under the influence of an electric field which is provided by a potential applied between the metal block (G) and the plate (D). The plate (D) is insulated from the block (G) by a thin P.T.F.E. sheet (H).

The electron current between the plate (D) and the anode (A) is recorded and used to indicate the presence of compounds in the carrier gas stream.

The source chamber (E) is kept free of contaminants by a separate flow of clean carrier gas entering via the pipe (I), which passes into the main chamber (B) through the hole (C). The exit from the detector chamber (B) is through the pipe (J).

EXPERIMENTAL

A detector was constructed as shown in Fig. 1, with an 18 μC ^{226}Ra source. For calibration purposes it was mounted in an oven at the output of a gas chromatograph employing a 4 ft. packed column of 4 mm I.D., and a gas density balance detector[4]. Provision was made for diluting the gas stream leaving the density balance before it entered the electron capture detector. The carrier gas used was nitrogen.

The variation of current through the detector with changing bias voltages applied to the source, between (G) and (D), is shown in Figs. 2a and 2b, which are

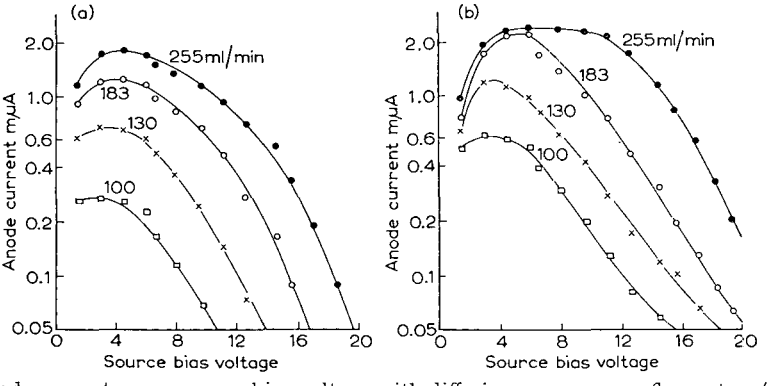

Fig. 2. Anode current *versus* source bias voltage with differing scavenge gas flow rates: (a) anode voltage = 4.5 V; (b) anode voltage = 9 V.

plotted with different collector voltages applied between the anode (A) and the cathode (D) of the detector chamber. The different curves in each figure were obtained with different scavenge gas flow rates.

Figs. 3a and 3b show the variation of electron current with changing collector voltage with two different rates of gas flow, and with various bias voltages applied to the source chamber.

The working conditions selected for stable operation were with the source bias

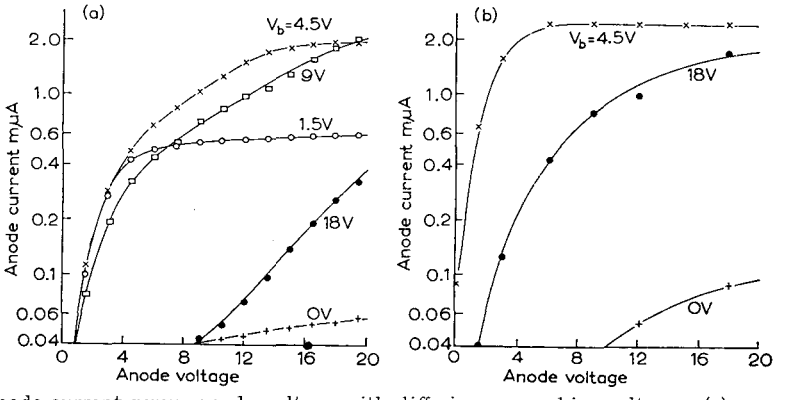

Fig. 3. Anode current *versus* anode voltage with differing source bias voltages: (a) scavenge gas flow = 130 ml/min; (b) scavenge gas flow = 255 ml/min.

voltage set at 6–9 V, the detector anode voltage in the range 3–15 V and a scavenge gas flow rate of 180–250 ml/min. For optimum conditions at a temperature of 245° the source and detector voltages used have both been 6 V, with a scavenge gas flow of 180 ml/min and a carrier gas input from the column of 60 ml/min. A representative sample concentration *versus* detector current curve is given in Fig. 4. The sample concentrations plotted in the curve were derived from the gas density balance output,

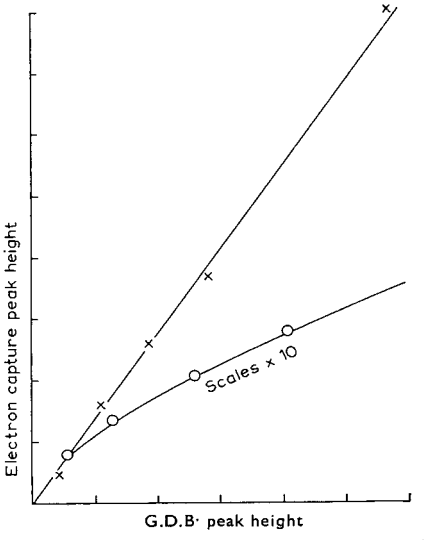

Fig. 4. Detector linearity. Electron capture peak height *versus* gas density balance peak height. Detector temperature: 67°; scavenge gas flow: 180 ml/min; total gas flow through detector outlet: 210 ml/min; anode voltage: 6 V; source bias voltage: 6 V; inter-detector dilution ratio = 1,500:1.

the dilution factor between the two detectors being of the order of 1,500*. The response is approximately linear with peak current changes of the order of 20 % of the standing detector current. In analytical work, where peak areas are of more interest than the electron capture at peak sample concentration, the detector calibration in terms of area is linear to the same degree over the same range of peak heights.

DISCUSSION

The curves of Fig. 3 show that as expected, electron flow from the source chamber (E) into the detector chamber (B) is controlled by the direct effect of the applied field in the detector chamber as well as the bias voltage applied between the plate (D) and the source chamber (G). The source chamber (G) may be regarded as a cathode and the plate (D) as a subsidiary anode competing with the detector anode (A) for the available electrons. The curves also show that the gas flow rate has a strong influence on the electron current. As electron drift velocities are orders of magnitude greater than the gas flow velocity (see *e.g.* ref. 5), gas flow cannot influence electron flow directly. This implies that the electron flow is largely influenced by the presence of relatively slowly moving ions in the gas stream. The common assumption that current

* A method for making large, stable dilution ratios is to be published.

in such detectors is carried by free electrons rather than by negative ions has been confirmed in this laboratory (unpublished work). Thus it is probable that the effect of gas flow rate is concerned with movement of the positive ions which are created in the source chamber by the radiation from the radioactive source.

This movement of positive ions can influence the electron current in two ways, (i) by dispersing the positive ion space charge left in the source chamber by the withdrawal of electrons and (ii) by a process of "ambipolar diffusion" (see *e.g.* ref. 6), *i.e.* positive ions are carried by the gas stream from the source chamber into, or towards, the detector chamber (B) against the applied field. As electrons are attracted towards the positive ions their movement from the source into the detector is facilitated by the flow of scavenge gas. The process may well influence the relative collection efficiencies of the two "anodes" (A) and (D).

In short, the electron current through the detector depends on the two applied voltages and upon the gas flow rate and the actual current observed is the result of a balance between a number of different processes. It can be seen, however, that it is possible to choose a working point at which the current is not critically dependent on any one variable. Thus stable operation can be secured.

The mechanism of the external source is obviously rather complex and, in its present form the gas flow rates necessary for its successful operation are larger than the carrier gas flow through average analytical columns. They are not, however, so large as to dilute unduly the sample entering the detector, when working with packed columns. Indeed, it has been found convenient to use additional dilution between the column and the detector and it is probable that when working with specific compounds for which electron capture detectors have high sensitivity, it could be used in its present form with capillary columns.

It is possible that a considerable reduction in the scavenge gas flow could be made if the source chamber were redesigned in such a way as to increase the effectiveness of the source bias voltage; perhaps an additional "pusher" electrode could be used. Also little attention has been paid to the size of the hole (C) through which the electrons are transferred, and to the thicknesses of the plate (D) and its insulator (H). These factors may each be expected to influence the efficiency of transfer of electrons from the source chamber to the main chamber and the dependence of electron current on gas flow rate.

With small collecting voltages applied to the anode, the linearity of the detector is of the same order as that of other electron capture detectors, and the use of these small collecting voltages ensures that the small fields in the detector under these conditions do not materially increase the energy of the electrons in the chamber. Thus one factor which can introduce anomalies in detector sensitivities is eliminated.

When using higher collecting voltages the detector response may be anomalous at low sample concentrations. The use of pulsed voltages has shown that the anomalous response is a function of mean current through the chamber rather than of the applied voltage, and that time constants of the order of seconds are involved. This implies that space charges, or surface charges, are of great importance in the operation of the detector. Different and possibly better performance may therefore be expected with a source of size different from the 18 μC adopted here, and with detectors designed to minimise the effects of surface charges.

APPLICATION TO ARGON DETECTORS

The same type of external source may be used with a normal argon detector anode[1], *e.g.* as in Fig. 5. One source can in fact be used with interchangeable anodes to form a dual purpose detector.

The advantage of an external source used in this way with an argon detector is the relative immunity of the radioactive source to deterioration resulting from contact with corrosive samples or stationary phase bleeding from the column.

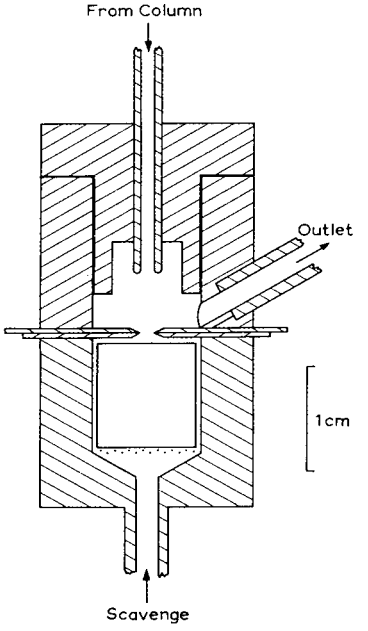

Fig. 5. Argon detector with an external ion source.

Such a detector was tried, and was found to produce an apparently normal response, but no data as to detector linearity was obtained. As the source size is significant in the performance of argon detectors, and as the source characteristics are not the same with argon as with nitrogen as a scavenge gas, further development work would be required to produce an argon detector of optimum performance.

CONCLUSION

The technique described for isolating the radioactive source in ionisation detectors has been shown to be practicable in the case of electron capture detectors, and to make possible the safe use of such detectors at higher temperatures than would be reasonable when using the more usual tritium sources. The upper limit of temperature is now set by the constructional materials used; the detector described has operated satisfactorily for some months at 250°.

Indication has also been obtained that the technique could be applied successfully to argon detectors. The radiation sources in these detectors can thus be protected

from the adverse action of compounds leaving the column. The technique makes practicable a valuable extension of the application of ionisation detectors in gas chromatography.

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SUMMARY

By use of the technique described, ionisation detectors can be constructed in which there is no contact between the radioactive source and compounds eluted from the chromatograph column. This has advantages:

1. In electron capture detectors where it makes the use of radioactive materials other than tritium convenient. Higher operating temperatures may therefore be used.

2. In situations where eluted compounds might otherwise lead to deterioration of the radioactive source.

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A GAS DILUTION SYSTEM FOR CONTROL OF DETECTOR RESPONSE IN GAS CHROMATOGRAPHY

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INTRODUCTION

Detectors used in gas chromatography have sensitivities which vary according to the compound being detected, and only seldom, *e.g.* for the gas density balance[1], is it possible to predict the nature of the variation from readily available independent data. The form of the variation is often of considerable interest and a correlation between sensitivity and chemical structure may provide information regarding molecular structure as well as clarifying aspects of the mechanism of detection[2-4]. This is particularly true of the electron capture detector[5]; relative electron capture coefficients, sometimes loosely called "electron affinities", of many compounds are being studied with the aim of correlating electron capture with chemical reactivity and physiological properties[6,7].

In such a study it is necessary to make comparative sensitivity measurements, and there are two general experimental methods. Thus detector output readings can be determined for known loads of sample applied to the column. For this purpose the compounds to be tested must be available in measurable quantities and in a state of reasonable purity. A more widely applicable method is to correlate the detector output directly with the concentration of sample leaving the column as indicated by a second detector of known quantitative response.

Difficulties arise when the sensitivities of the two detectors differ widely and in some circumstances their working ranges do not overlap at all. As it is impractical in such cases to pass the sample through both detectors at the same concentration, samples leaving the column at sufficiently high concentrations for the one detector must be diluted before entering the other. Small dilution factors can be achieved by adding gas to the total effluent from the column but, where the required factor is large, this leads to excessive gas flows. A novel technique has therefore been developed for taking a small sample of controllable size from the column outflow and adding it to an independently chosen flow of diluting gas.

EXPERIMENTAL

An electron capture detector[8] was mounted in a small oven and connected to the output of an existing gas chromatograph (Fig. 1) in which the detector was a gas density balance[9]. Between the two detectors was placed a gas flow splitting device, shown in more detail in Fig. 2. This was connected initially as shown by the broken line in Fig. 1, *i.e.* the dilution flow did not pass through the "pulsator".

In adjusting the apparatus the pump speed was set, by the needle valve (NV 1), to give a convenient gas flow (E, Fig. 2) into the detector; *e.g.* with the scavenge flow set at 180 ml/min[8] and a pumping rate (P) of 210 ml/min the flow (E) into the detector would be 30 ml/min. The dilution flow (D) was then adjusted by the second needle valve (NV 2) to be nearly equal to this amount, leaving a small quantity (δC) to be

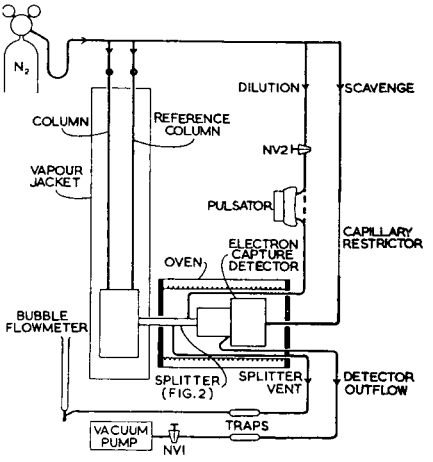


Fig. 1. The gas flow splitter interposed between a gas density balance and a high-temperature electron capture detector.

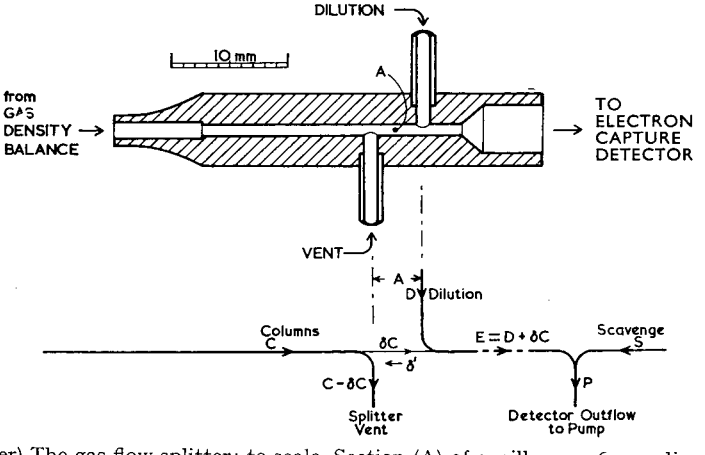


Fig. 2. (Upper) The gas flow splitter; to scale. Section (A) of capillary 1.06 mm diameter, 4.4 mm long. Dilution input pipe 1.4 mm I.D., 140 mm long. (Lower) Gas flow paths. Sample passing to detector = δC, $\delta C = P - (D + S)$ and is set by adjustment of P and D. δ' shows reversed direction of mean flow when using pulsator.

drawn in from the columns. The remainder of the column flow passed through a trap (Fig. 1) and a flowmeter to waste. Thus, by suitable adjustment of NV 2, δC could be chosen to be *e.g.* 1.5 ml/min and, with the figures quoted, the gas entering the electron capture detector would contain sample at 5 % of the concentration leaving the gas

density balance. The proportion of the sample leaving the column which eventually entered the electron capture detector was determined by the ratio between δC and C, the total gas flow leaving the gas density balance; *e.g.* with a sample column passing 100 ml/min and a reference column passing 50 ml/min, and the other settings as above, only 1 % of the sample would enter the detector. Flow rates measured at the splitter vent were used to calculate approximately the dilution ratio at any setting of the apparatus, although absolute figures were not required for the present purpose.

In developing this method of dilution the following points were considered important:

(1) It was economical in gas, only a small proportion of the sample being actually diluted.

(2) At no point was a fine capillary tube carrying sample, the section at A being sufficiently wide not to block.

(3) The dilution, or splitting, factor was controlled by large pressure drops across elements outside the oven and not carrying sample in significant concentration. The factor was therefore stable with respect to small changes in back pressure, changes in detector temperature and sample concentration.

(4) Only at one part of the system, in the capillary (A), was the gas flow rate small and the volume of tubing here was sufficiently small to cause no significant time delay.

(5) Adjustments could be made without changing gas flow rates in the detector.

It was found necessary to fit the needle valves used (Griffin and George S11–100) with worm drives (Meccano) for convenience in making the adjustments, and to replace the rubber gland washers from the valves with similar ones turned from polytetrafluoroethylene before satisfactory stability could be attained. It was then possible to work conveniently with dilution ratios of the order of 100:1 at room temperature.

At higher dilution ratios, or at higher temperatures, drift proved to be troublesome and stable results could be obtained only over periods of a few hours. The system was therefore modified, as shown by the full lines of Fig. 1, by the inclusion of a "pulsator", a device for making transient reductions in the rate of flow of gas from needle valve (NV 2) into the flow splitter.

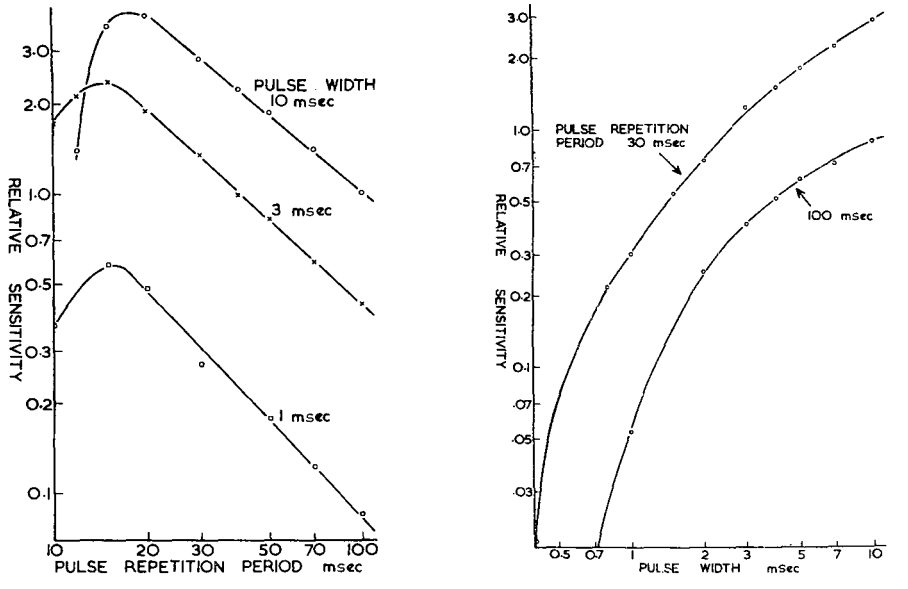
The pulsator was constructed by connecting pipes into a headphone earpiece with epoxy resin, and sealing the diaphragm with a polythene gasket and, externally, with silastomer, thus forming a gas-tight cavity in the dilution gas stream, with the diaphragm as one wall. When an electrical pulse was fed to the magnets of the headphone the movement of the diaphragm caused a transient reduction of pressure in the cavity and hence a transient reduction in the gas flow rate to the flow splitter. This in turn caused a compensating increase in flow through the section (A) of the capillary, so maintaining the constancy of flow (E) to the detector.

The needle valve (NV 2) was now readjusted to make the mean flow in the dilution line (*i.e.* through NV 2) slightly greater than that taken by the pump, thus reversing the mean flow through the capillary (A). Sample from the column flow entered the electron capture detector gas flow system only during the pressure pulses provided by the electrical system. As these were short, well spaced, rectangular pulses, *e.g.* with a width of the order of 3 msec and a repetition period of 100 msec, sample was flowing into the detector for only a small fraction of the time, and splitting was achieved in proportions that depended very largely on the characteristics of the electrical pulses rather than on a delicate balance of gas flow rates.

The gas flowed through a sufficiently large cavity (about 0.3 ml) between the splitter and the active volume of the detector to ensure that considerable mixing took place. It could thus be regarded as a continuous sample, rather than a series of pulses, as it entered the detector.

RESULTS

The way in which the detector response was controllable by electrical adjustment of the pulse width and pulse repetition frequency is demonstrated by Figs. 3 and 4, in which the sensitivity of the detector and splitter, regarded as a unit, is compared with an arbitrary standard setting; a relative sensitivity of unity corresponds to a dilution factor of approximately 1.6 %. Smooth control was, in fact, achieved over a range of sensitivities of rather greater than 100:1, *i.e.* from about 6 % to below 0.06 %. In this range the dilution ratio was not critically sensitive to the value of pulse voltage (Fig. 5) provided that this was sufficiently large. The range of dilution factors could be extended



Figs. 3 and 4. Effective sensitivity as a function of pulse width and pulse repetition period. Square pulses of peak height 100 V. Dilution factor at relative sensitivity of 1 = 1.6 %.

in either direction by changing the pump speed (NV 1) and making corresponding changes in dilution flow (NV 2), thus altering the gas flow to which the sample was added.

The flexibility of the system is demonstrated by the chromatograms of Fig. 6. Curve (a) is a chromatogram of a mixture of chlorinated methanes taken from the gas density balance record. Curve (b) is the corresponding simultaneous chromatogram from the electron capture detector operated, in this case, with a constant dilution roughly appropriate for comparison of the chloroform peaks in the two records. The other peaks in the electron capture record are not accurately measurable. On repeating

the chromatogram, with an identical load but this time with a progressive increase of dilution, the electron capture chromatogram (c) was obtained. All of the peaks were now measurable.

The dilution factor achieved at any one setting has been sufficiently stable for

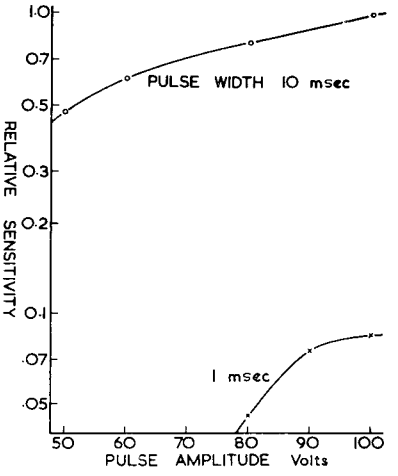


Fig. 5. Effective sensitivity as a function of pulse voltage. Pulse repetition period = 100 msec.

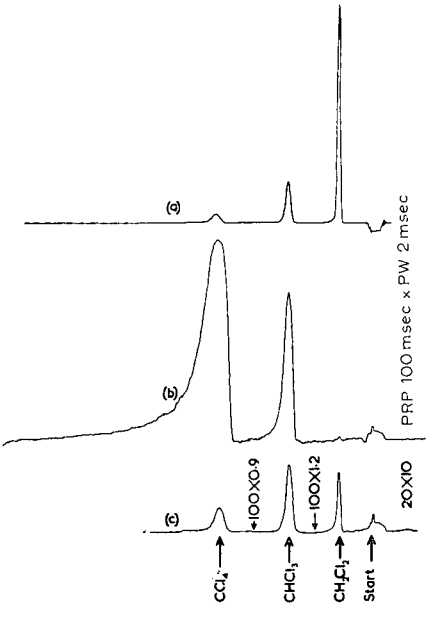


Fig. 6. Chromatograms demonstrating that suitable conditions can be chosen for measurement of a wide range of compounds. (a) Gas density balance chromatogram of mixture CH_2Cl_2–$CHCl_3$–CCl_4 approx. (20:5:1, v/v). Total load 0.25 μl. (b) Simultaneous electron capture record at constant dilution. Detector temperature 69°. PRP = pulse repetition period; PW = pulse width, both in msec. (c) Repeated electron capture record with progressively adjusted dilution.

spontaneous changes to be masked by detector sensitivity changes due directly to variations in temperature, and detector calibration curves have been prepared using many test substances, *e.g.* chloroform[8].

DISCUSSION

The practical value of the technique lies primarily in that it provides a dilution system which can be used to reduce the sensitivity of a detector by a stable factor. By combining the two methods described above, *viz.* sampling the column effluent using the vacuum pump with or without the pulse system, the factor can be chosen to be anywhere within the range 1:1 to 1:2000.

It is demonstrated (Fig. 3) that the reduction in sensitivity is inversely proportional to pulse repetition period (PRP), as would be expected, provided that the PRP is greater than about 25 msec. The effect of a change in PRP in this range is therefore calculable. Fig. 4 shows, however, that the observed sensitivity varies non-linearly with respect to pulse width, changing more rapidly than pulse width when this is less than 1–2 msec, and less rapidly when it is more than 3–4 msec. This, again, is to be expected as the square pulse applied to the diaphragm can result in a square pressure pulse in the headphone cavity only when the pulse is short compared with the time required for the gas flow to compensate the depression caused by the diaphragm displacement. There is thus an upper limit to the pneumatic pulse width which can be generated in the earpiece. Once a pressure change has been generated in the headphone cavity, it takes a definite time to reach the flow splitter, and short pulses are further shortened and attenuated in the process of their propagation to the splitter. The sensitivity of the detector–splitter combination thus falls more rapidly than the electrical pulse width. With the apparatus constructed the two effects overlap and there is no region in which the effective pneumatic pulse in the splitter is of the same duration as the electrical pulse applied to the diaphragm, and therefore no region where the dilution factor can be calculated from the applied pulse. All measurements can, however, be made relative to a standard sample, or the apparatus can be calibrated at selected settings of the pulse width control.

It is probable that a wider range of control, with a more linear relationship between dilution factor and electrical pulse width, could be achieved with apparatus designed specifically for pulsed gas flow, with a wider, shorter tube from the pulsator to the splitter, without rubber connections, and with a high acoustic impedance at the gas input side of the pulsator. A shorter length of capillary bore at A might also be an advantage. The lower limit of the pulsator range might be extended by the same means and possibly also by using a moving coil transducer, instead of the moving iron headphone, and driving it electrically with ramp waveforms instead of square waves. In its present form, however, the apparatus has been proved suitable for studies of groups of compounds of widely differing electron capture properties by comparative methods.

The facility with which the detector sensitivity can be changed, simply by switching the pulse width or repetition frequency, makes it convenient to measure constituents of widely different electron capture coefficients in a mixed sample, *e.g.* mono- and dichlorobiphenyls occurring in the same reaction mixture can be studied in one chromatogram. This method has the advantage over the alternative procedure, *viz.*

control of amplifier gain, that load size can be chosen to make peaks due to inactive compounds large enough, compared with noise level, to be measurable without overloading the detector by more active compounds; but calibration of the splitter is, of course, necessary at each of the settings used.

The same method may be useful in other applications, *e.g.* detection of trace constituents using conventional detectors, where similar difficulties occur, and a further application is in preparative chromatography. Here it is not always desirable to pass the whole sample leaving the column through the detector, and various sampling systems have been tried. The present system has advantages in that large split ratios can be achieved without the use of fine capillaries and the ratio should not be critically dependent on back pressure caused by the collector. It is also expected to be a suitable system for sampling process streams for chromatographic analysis.

ACKNOWLEDGEMENT

I am indebted to MR. A. DUNLOP for carrying out much of the technical work in proving this technique.

SUMMARY

A technique is described for controlling detector response by variable splitting of the gas flow leaving a chromatograph column. This gives, in effect, a detector of variable sensitivity and allows direct comparison to be made between a highly sensitive electron capture detector and a gas density balance of lower sensitivity but with a known response.

The pneumatic pulse technique devised should also be applicable to other problems, *e.g.* trace analysis, preparative chromatography or process stream sampling.

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GAS–SOLID ADSORPTION CHROMATOGRAPHY OF SOME AROMATIC HYDROCARBONS AND NITROGEN HETEROCYCLES ON ALUMINA

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In previous papers[1,2] we proposed that aromatic hydrocarbons are adsorbed preferentially flatwise, by means of the π-electron system, on to an alumina surface while aromatic nitrogen heterocycles are adsorbed preferentially edgewise or tilted, by means of the non-bonding (n-)electrons of the nitrogen atom, on to such surface. If substituents on the aromatic ring(s) or the general geometry of the ring system were to cause sufficient steric hindrance to such manners of adsorption adsorbability would be decreased. In the case of a parent aromatic nitrogen heterocycle, in particular, insertion of appropriately located substituents might cause a change from n-type adsorption to π-type or to πn-hybrid type adsorption. From studies on linear elution adsorption chromatography SNYDER[3] has reached similar conclusions on the geometry of adsorption of arenes though he does not, in general, believe that *bona fide* π-complexation is involved. In addition, he has proposed that localization of the anchoring site on the molecule occurs in aromatic nitrogen heterocycles which are, nonetheless, also held flatwise. GILES AND MCKAY[4], on the other hand, proposed that arenes are adsorbed in small clusters, stacked face-to-face and edge-on to the surface, for the alumina which they used. The present research was conducted in an effort to gain further insight into these questions of geometry of adsorption, but using gas–solid adsorption elution chromatography instead of liquid–solid adsorption chromatography as used in the previous studies. Compounds investigated were largely confined to the benzene, naphthalene, pyridine, and quinoline systems.

EXPERIMENTAL

Naphthalene, acenaphthene, and the isomeric dimethylnaphthalenes were available in analytically pure form from other studies[5]. Quinuclidine was synthesized by a five-step procedure. Thus, isonicotinic acid (Distillation Products Industries) was converted to ethyl isonicotinate by refluxing with absolute ethanol into which anhydrous hydrogen chloride was passed. Following the procedure of STERNBACH AND KAISER[6], except that catalytic hydrogenation of the intermediate 1-carboethoxymethyl-4-carboethoxypyridinium bromide was conducted at 2–5 atm pressure instead of at 70 atm as reported, this ester was transformed into 3-quinuclidone. 3-Quinuclidone was reduced to quinuclidine (isolated as the hydrochloride and purified

via the picrate) by the HUANG-MINLON reaction[7]. Other compounds used were available from commercial sources and were fractionally distilled before use.

Chromatography was conducted by means of an F and M model 202 programmed gas chromatograph using copper spiral columns (64 mm internal diameter) packed with F and M 30–60 mesh alumina as adsorbent and helium (passing at the rate of 40 ml/min, as measured at 25° and an inlet pressure of 3.0 ± 0.2 atm absolute) as eluting carrier gas. A thermal conductivity cell was employed as the detector. During each run the column was maintained at a constant temperature (between 173° and 367°) by means of a cylindrical oven surrounding the column. Prior to starting a series of runs at some particular temperature the column was maintained at a temperature at least 25° higher than that of the runs for 3 h or longer. The column temperature as read from the instrument panel was corrected by use of a calibration chart, wherein the true temperature was taken as that determined potentiometrically under steady-state operating conditions and sensed by means of a thermocouple placed at the geometric center of the column spiral. In general, liquid samples were introduced neat by means of a calibrated syringe. Solid samples and some of the less volatile liquids were introduced as solutions in benzene or cyclohexane.

Chromatograms exhibited sharp leading and broad trailing boundaries as are characteristic of gas–solid adsorption chromatography[8]. Since in certain cases maxima were ill-defined, the measured retention time (uncorrected), t_R, was taken as the time elapsed between the initial rise of the air peak and the initial rise of the chromatogram proper. Values of t_R for a variety of cyclic compounds investigated in an exploratory manner are given in Table I. In cases studied more extensively t_R was determined as a function of sample size (0–9 μl for neat liquids, 0–80 μl for solutions) for each compound at each temperature. Except for smaller samples (0–2 μl and 0–20 μl, respectively), which showed positive deviations in t_R, t_R increased linearly with decreasing sample size[9]. The linear portion of the curve was extrapolated to zero sample-size to yield the intercept, $t_R'°$. The slope of this linear portion was found to vary with the compound used and to increase algebraically with increasing temperature of the column. Compared to curves for hydrocarbons, 2,6-lutidine (the only nitrogen heterocycle so studied) showed exceptionally large negative slopes at all temperatures. Although t_R was easily measured to an accuracy of ± 0.05 min, the long extrapolation to zero sample-size decreased the estimated accuracy in $t_R'°$ to ± 0.2 min for neat liquid hydrocarbons, ± 0.4 min for 2,6-lutidine, and ± 0.8 min for solutions. Using 5 % (w/v) solutions in cyclohexane, a column length of 61 cm, and a temperature of 362° acenaphthene and all of the ten possible dimethylnaphthalenes gave experimentally indistinguishable values of 8.0 ± 1.0 min for $t_R'°$, as compared to a value of 3.0 min for naphthalene itself.

Where extrapolated retention times were to be compared at different column temperatures correction was made for the change in rate of flow of the carrier gas due to thermal expansion upon its entering the column. Arbitrarily taking 500° K as a standard of comparison the corrected extrapolated retention time, $t_R°$, was calculated by means of the relationship

$$t_R° = \left[\frac{T}{500}\right] t_R'°,$$

where T is the column temperature in °K. No correction to $t_R°$ was made for pressure

drop in the column. In Tables II and III are presented data on $t_R°$ for benzene, substituted benzenes, naphthalene, and 2,6-lutidine at various column temperatures and using the regular F and M alumina as well as this alumina pre-treated with quinuclidine. Figs. 1 and 2 show plots of log $t_R°$ *versus* $1000/T$, made partially from data given in Tables II and III.

DISCUSSION

It was originally noted by JAMES AND MARTIN[10] that the retention time for a substrate in gas–liquid partition chromatography should increase with increasing molecular weight (*i.e.* with decreasing volatility or increasing boiling point) of the substrate and with increasing interaction (attraction) between the substrate and the stationary phase. For gas–solid adsorption chromatography a similar relationship should hold.

TABLE I

MISCELLANEOUS EXPLORATORY CHROMATOGRAPHIC RUNS

Compounds listed together in one group were all run on the same column in close succession, although not necessarily in the order given. Strongly retained compounds were removed by temperature programming before subsequent compounds in the group were investigated. All columns were 122 cm long. Boiling points are taken from standard references.

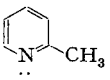
Compound	*B.p.* (°C)	*Column temperature* (°C)	*Sample size* (μl)	t_R* (*min*)
p-Xylene	138	227	12.5	5.6
Ethylbenzene	136	227	12.5	5.5
2-Ethylthiophene	134	227	11.5	5.5
2,5-Dimethylthiophene	137	227	11.5	5.6
m-Xylene	139	254	25	5.4
Thiophene	84	254	16	1.5
3-Methylthiophene	115	254	19.2	3.0
2-Ethylthiophene	134	254	22.5	5.1
2,5-Dimethylthiophene	137	254	27.5	4.7
2-Picoline	129	254	19.6	> 90
2,6-Lutidine	143	254	22.8	16.1
Pyridine	116	362	16	> 180
2-Picoline	129	362	19.5	13.9
2-Ethylpyridine	149	362	22	11.5
2,6-Lutidine	143	362	22	1.1
3,5-Lutidine	171	362	22	> 180
Quinoline	238	362	23.5**	> 85
8-Methylquinoline	249	362	27**	7.4
1-Methylisoquinoline	248	362	27**	> 120
2,4-Dimethylquinoline	265	362	29**	36.5
2,6-Dimethylquinoline	267	362	29**	32.0
2,8-Dimethylquinoline	252	362	26**	8.2
2-Methylbenzoxazole	201	362	25.5**	> 48
Quinuclidine***	—	308–394	36	> 180

* For those compounds for which indefinite values are given for t_R, timing was stopped at the t_R shown and the compound was removed from the column by subsequent temperature programming.

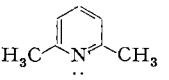
** Solution, 25% (w/v) in benzene.

*** Solution, 10% (w/v) in benzene. Run with temperature programming. The evolution of quinuclidine started after more than 2 h at 394°.

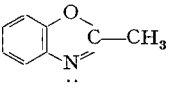
Observed discrepancies between the order of increasing boiling points and the order of increasing retention time in gas–solid chromatography might then be ascribable to variations in the strengths of adsorption of the substrate molecules on to the solid adsorbent. Examination of Table I shows that several nitrogen heterocycles exhibit unusually large values for t_R. For the series pyridine, 2-picoline, 2,6-lutidine, in particular, the order of increasing t_R is a marked inversion of the order of increasing boiling point.



2-Picoline



2,6-Lutidine



2-Methylbenzoxazole

Quinuclidine

Also 8-methylquinoline and the three dimethylquinolines studied have t_R less than for quinoline while all of the dimethylnaphthalenes are more strongly retained than is naphthalene. Quinuclidine, moreover, is strongly retained in spite of the facts that its molecular weight approximates that of ethylbenzene and that it sublimes readily on being heated in air. These results are consistent with the group adsorption (geometric) factors noted by SNYDER[3] and with our previous proposal (based on liquid–solid chromatography on alumina) that azacyclic compounds are adsorbed on alumina preferentially by means of the n-electrons on the nitrogen atom. In the pyridine molecule steric hindrance to adsorption is provided by alkyl groups in an α-position but not by ones in a β-position. For quinoline, steric hindrance to adsorption is largest for an alkyl group in the 8-position (where the C_{Ar}–C_{Al} bond is directed parallel to the spatial direction of the n-electrons) and smaller in the 2-position (where corresponding directions make an angle of about 120°). 1-Methylisoquinoline and 2-methylbenzoxazole have even less hindered n-electrons than does 2-methylquinoline. For quinuclidine the alternative possible formation of a π-complex rather than of an n-complex is not present. In contrast to the nitrogen heterocycles the thiophenes studied showed little irregularity (compared to one another or to arenes of similar boiling point) in t_R, consistent with previous observations[2,3,11].

PINES *et al.*[12] proposed that catalytically active alumina contains two kinds of Lewis acid sites, strong and weak, as determined by the ability or lack of ability, respectively, of the site to effect either skeletal rearrangement of certain alcohols during the process of dehydration or double bond migration in the initially formed alkene. Treatment of the alumina with ammonia or amines served to effect dehydration without skeletal rearrangement, probably because the nitrogen compounds were tenaciously adsorbed to the strong sites, while some weak sites were still available for the dehydration process. In a preliminary effort to investigate the possibility that arenes and azacyclic compounds might be adsorbed on different sites on the alumina surface we have determined values of $t_R°$ at various temperatures for benzene, the alkylbenzenes ØR (where R = Me, Et, Pr, and Bu), *m*-xylene, naphthalene, and 2,6-lutidine using columns containing, first, plain alumina and then, later, the same alumina after treatment with non-eluting quinuclidine.

Typical data obtained are presented in Tables II and III and plotted in Figs. 1 and 2. Unfortunately, at the upper limit of column temperatures, T, available with our apparatus retention times for all of the aromatic nitrogen heterocycles (other than 2,6-lutidine) of interest were too large to allow suitable determinations of $t_R°$ *versus* T.

TABLE II

GAS ADSORPTION CHROMATOGRAPHY OF BENZENE AND MONOALKYLBENZENES ON ALUMINA

Variables in run / *Compound B.p., °C***	*Column temp °C*		243			259			276			292		
	*Column**	*A3 bare*	*A3 Qt*	*A4 bare*	*A3 bare*	*A3 Qt*	*A4 bare*	*A3 bare*	*A3 Qt*	*A4 bare*	*A3 bare*	*A3 Qt*	*A4 bare*
	Corrected extrapolated retention time, $t_R°$ (in min)												
Benzene	80.1	1.3	1.2	1.5									
Toluene	110.6	2.9	2.3	3.4	2.0	1.7	2.4		1.3			1.0	
Ethylbenzene	136.2	5.3	5.0	6.2	3.5	3.0	4.6	2.4	2.1	3.2	1.9	1.7	2.4
Isopropylbenzene	152.4	8.1	6.2		5.0	4.2	6.7	3.7	3.3	4.8	2.6	2.3	3.5
n-Propylbenzene	159.2	9.4	7.7		5.8	5.4	8.5	4.5	3.8	6.1	3.3	2.9	4.2
tert.-Butylbenzene	169.1				7.6	6.5		5.3	4.6		3.7	3.1	
sec.-Butylbenzene	173.3				7.9	6.9		5.4	5.2		4.1	3.8	
Isobutylbenzene	172.8	14.2			9.9	8.4		6.4	5.7		4.9	4.1	
n-Butylbenzene	183.3	18.7			11.7	9.8		7.6	6.9	10.2	5.5	4.6	7.3

* Columns A3 and A4 had the same dimensions (61 cm long) and contained the same weights of alumina (11 g). Column A3 QT resulted from passing 95 μl of a 5% (w/v) solution of quinuclidine in dioxane onto column A3 at 389° and maintaining this temperature for several hours.

** Boiling points were obtained from *Selected Values of Properties of Hydrocarbons*, Am. Petroleum Inst., Research Project 44.

Examination of Tables II and III shows that for each compound $t_R°$ decreases with increasing T. Also at any particular T, the compound is adsorbed less tenaciously on quinuclidine-treated alumina than on plain (or bare) alumina. Although columns A3 and A4 were prepared in the same manner, $t_R°$ values on them were somewhat different. From Table II one notes that, in general, for a particular column and temperature $t_R°$ increases with increasing boiling point of the alkylbenzene used. The pair isobutylbenzene–*sec.*-butylbenzene, however, provides an exception to this trend. The inverted order for this pair may be ascribed to a larger steric hindrance by the *sec.*-butyl group, $-CH(CH_3)C_2H_5$ (as compared to the isobutyl group, $-CH_2CH(CH_3)_2$), toward flatwise adsorption of the benzene ring. The magnitude of such steric hindrance would be largely dependent upon the relative sizes of the substituents on the carbon atom adjacent to the ring. From Table III one notes that at the common temperature of 292° 2,6-lutidine is retained 2–5 times as long as *m*-xylene despite the facts that these two substrates have the same symmetry and nearly equal boiling points and molecular shapes.

TABLE III

GAS ADSORPTION CHROMATOGRAPHY OF THREE COMPOUNDS ON ALUMINA

Compound	*B.p.** (°C)	*Column temp.* (°C)	*Corrected extrapolated retention time, $t_R°$ (min)*			
			*Column A3***		*Column A4*	
			bare	*quinuclidine-treated*	*bare***	*quinuclidine-treated****
m-Xylene	139	243	5.6	4.3	6.2	3.6
		259		3.2	4.3	2.5
		276		2.2	3.6	1.8
		292		1.7	2.3	1.5
2,6-Lutidine	143	292	11.9	7.9	9.6	4.0
		308	6.8	4.4	6.0	2.6
		324	5.1	3.1	4.2	1.8
		340	3.6	2.2	3.1	1.4
Naphthalene	218	292				7.5
		308			12.0	5.5
		324			7.7	3.9
		340			6.5	3.1

* Obtained from standard sources.
** See footnote* to Table II.
*** Prepared from column A4 in a manner similar to that used in obtaining column A3 QT.

Following the method of GREENE AND PUST[13] the heats of adsorption, H_{ads}, of the compounds given in Tables II and III were obtained from the slopes of the linear plots in Figs. 1 and 2. It is apparent from a glance at these figures that H_{ads} does not differ greatly from one compound to another. Thus, benzene and the alkylbenzenes give H_{ads}-values of 12.2 ± 2.2 kcal/mole on bare alumina (for both columns used) and of 11.5 ± 2.1 kcal/mole on quinuclidine-treated alumina (for column A3 only). For column A4 the corresponding data are: *m*-xylene (12.3, 10.8); naphthalene (14.5, 12.7); and 2,6-lutidine (16.0, 13.7)—with an estimated accuracy of ± 0.7 kcal/mole in each value and an average change in H_{ads} on going from bare to quinuclidine-treated alumina of —1.9 kcal/mole. The similarities in the data for 2,6-lutidine and

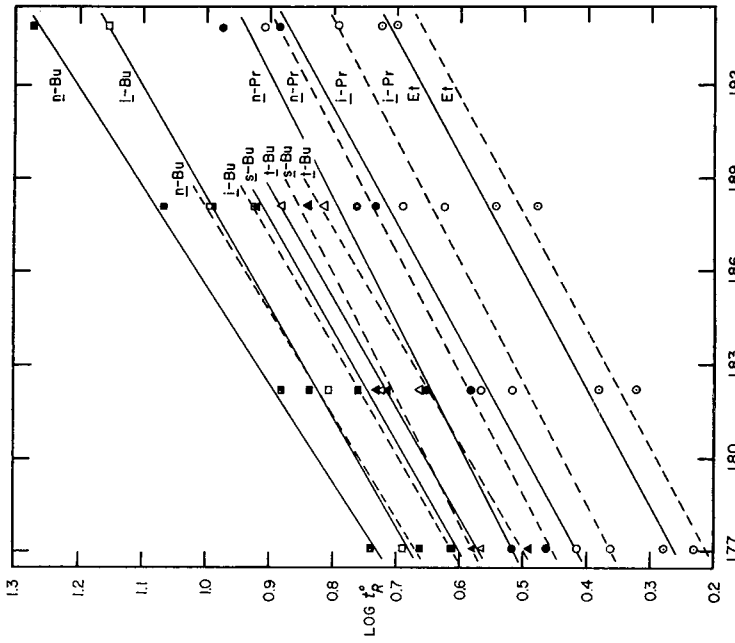


Fig. 2. Plots of $\log t_R^\circ$ *versus* $1000/T$ using column A 3. Broken lines refer to quinuclidine-treated column; solid lines, to the bare column for monoalkylbenzenes.

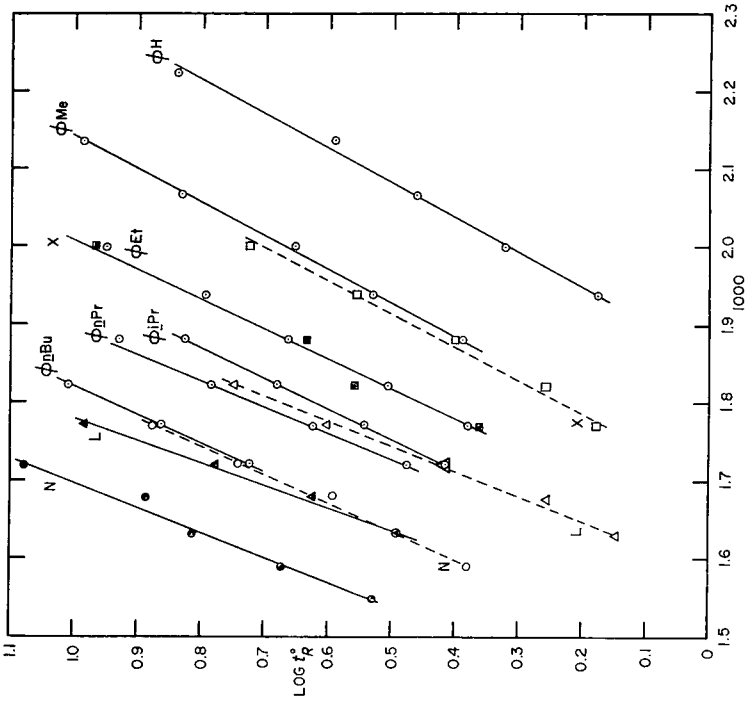


Fig. 1. Plots of $\log t_R^\circ$ *versus* $1000/T$ using column A 4. Broken lines refer to quinuclidine-treated column; solid lines, to the bare column. N = naphthalene; L = 2,6-lutidine; X = *m*-xylene.

m-xylene lead us to believe that both of these compounds are adsorbed preferentially flatwise and that the somewhat larger values in $t_R°$ and H_{ads} for 2,6-lutidine may be ascribed to the greater polarity of the pyridine ring system as compared to the benzene ring system. Further studies with other azacyclic compounds at higher column temperatures will be needed in order to check on this possibility. Meanwhile, however, it should be noted that our measurements are concerned with those molecules of substrate which traverse the column most rapidly and, therefore, probably are adsorbed only the smallest number of times on the energetically weakest adsorption sites. It is conceivable that the geometry of adsorption of an azacyclic compound may vary with the strength (or type) of adsorption site which it occupies on the surface.

ACKNOWLEDGEMENT

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SUMMARY

By means of gas-solid adsorption elution chromatography on alumina, either plain or pre-treated with non-eluting quinuclidine, retention times and heats of adsorption were determined for benzene, eight monoalkylbenzenes, *m*-xylene, naphthalene, and 2,6-lutidine. In general both retention time and heat of adsorption decreased on impregnating the alumina with quinuclidine. For plain alumina various pyridines and quinolines were very strongly retained so long as the non-bonding electrons on the nitrogen atom were not sterically hindered by ring substituents. Results are consistent with data on adsorbabilities obtained earlier from solid–liquid chromatography.

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CHROMATOGRAPHIC STUDIES ON ORGANO-TIN COMPOUNDS
PART I. THE GAS CHROMATOGRAPHY OF ALKYL-TIN COMPOUNDS

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Although much study of organo-tin compounds has been made[1], little data is available concerning the chromatographic behaviour of organo-tin compounds. KAESZ *et al.*[2,3] have observed the possibility of eluting perfluoroalkyl, and perfluorovinyl tin compounds on polyethylene glycol column. ABEL, NICKLESS AND POLLARD[4] have published separations of the tetra-methyls of the Group IV B elements, using Apiezon L columns. Gas chromatography studies on organo-tin compounds have also been reported by MATSUDA AND MATSUDA[5], while FRANC, WURST AND MAUNDRY[6] have studied these compounds using paper and gas chromatography.

The two factors which render the gas chromatography of σ- and π-bonded organo-metallic compounds difficult as compared to organic compounds are:

(i) chemical reactivity, *e.g.* instability towards oxygen or moisture, and

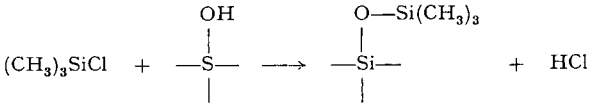
(ii) thermal instability.

The column support used in gas–liquid chromatography, should be solid-particles which are inert; but often this is not the case. If a support is insufficiently covered by the stationary liquid phase, *e.g.* 2–5 %, adsorption on the exposed siliceous sites becomes significant with polar solutes, and trailing occurs. As a consequence, retention volumes are no longer just directly proportional to the weight of solvent, and hence specific retention volumes can only be measured with columns containing a high proportion of stationary phase. Where organo-metallic solutes are involved, this adsorption becomes very important, the bond spreading is so bad that squalane columns can hardly be used for analysis of mixtures of such materials[7].

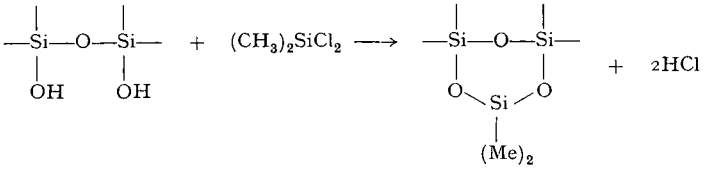
Chemical instability gives rise to chemical change as the compound passes through the chromatographic column. This usually occurs through formation of bonds between the compound and reactive groups either on the column support (*e.g.* acid sites), or the stationary phase (*e.g.* hydroxy-groups as in polyethylene glycol). This phenomenon is termed trans-esterification[8], and was observed in the organo-tin hydrides, chloro-silanes, and amino-compounds, *e.g.* hexamethyldisilazane.

The initial approaches to pre-treatment of support to remove this activity was to add small amounts of highly polar and involatile liquids to the support[8,9], or to acid and then alkali wash the support[10–12]. More recently there have been attempts to deposit solids such as silver on the support surface[13], but unfortunately, this method cannot be used in the presence of thio-compounds, *e.g.* silyl thioethers[7]. The alternative method is to treat the active sites of the support, (which are presumed to be hydroxyl groups (-Si-O-H), and replace these by groups which should yield at least a weakly

adsorbing site. Both trimethylchlorosilane[14,15] and dimethyldichlorosilane[16,17], have now been used successfully to reduce the activity, the surface reaction is presumed to be of the type:



or:



When the hydroxyl groups are not adjacent, then a chlorosilyl ether $\geqslant SiOSi(CH_3)_2Cl$ may be left, which is not beneficial since it will be as reactive as the grouping replaced, because of the chlorine grouping.

As an alternative to this treatment, hexamethyldisilazane has been used since it reacts quantitatively with hydroxyl groups[18,19], and was used by BOHEMEN, LANGER, PERRETT AND PURNELL[8], and has now been used to treat all the common solid supports[20]. Many advantages have been claimed for hexamethyldisilazane, but it is expensive and gives a surface similar to the trimethylchlorosilane.

Much thought must be given to the detector and its design since often when a compound is eluted from a column, decomposition occurs in the detector, invalidating the elution process.

When such decomposition occurs, the metal is deposited on the wires or filaments of the katharometer, or on the collector plates of a flame ionization gauge. Recently the formation of tarry, and finally carbonaceous deposits which foul the katharometer filaments, has been reported[21], when operating columns containing polar materials such as polyesters. Recommended treatment in such cases is regular flushing of the detector block, with both polar and non-polar solvents. Although such treatment was beneficial, in the course of time, however, the tarry deposits would carbonize, leading to permanent changes in the katharometer resistance. The partial contacts of carbon deposits between helices of the coiled filament, presumably were responsible for the increase in recorder base line noise. When finely-divided powder metal is deposited in the katharometer, especially on the filaments, a similar situation arises, but the bridge becomes permanently out of balance, since unfortunately no cleaning procedure can be used. A similar situation is found with the flame ionisation detector, especially the conventional types where the collector electrode plate is vertically above the flame. A modified detector is required, and even when detection can occur, attention must be paid to saturation limits, since non-linearity of signal response, and the inversion effect as reported by NOVÁK AND JANÁK[22] has been observed.

EXPERIMENTAL

Trans-esterification was overcome by treatment of the supporting phase[8]. Celite 545 (36–60 mesh B.S.S.) was baked at 300° for 5 h, acid- and alkali-washed, dried at 50°, and treated with trimethylchlorosilane.

After such treatment it was possible to chromatograph and separate the methylchlorosilanes[23] and organo-tin hydrides, but as indicated later, the choice of stationary phase is important for this type of compound.

The gas–liquid chromatography of thermally unstable organo-metallic compounds was carried out using separation techniques at normal temperatures (20–100°) followed by combustion in a conventional micro-analytical furnace, absorption of water, and detection of the carbon dioxide with a Stuvé katharometer[24]. The metal (as oxide) deposited in the furnace gradually poisons the copper oxide furnace packing, and has to be replaced frequently.

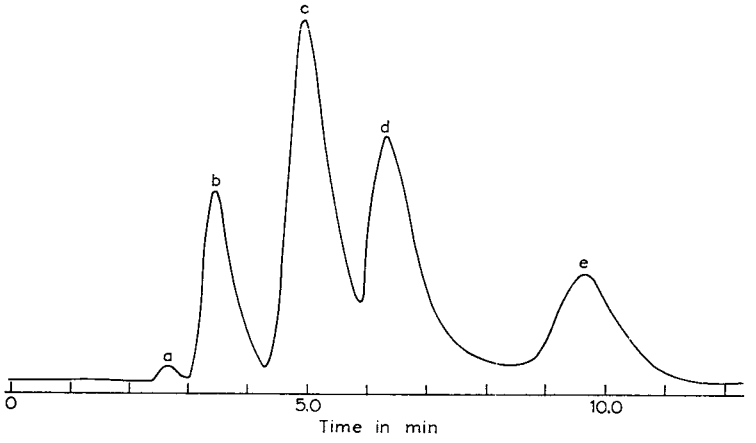


Fig. 1. Separation of alkylsiloxanes. (a) Diethyl ether; (b) trimethylsilyl methyl ether; (c) trimethylsilyl ethyl ether; (d) hexamethyldisiloxane; (e) trimethylsilyl *n*-propyl ether.

The unit gave linear calibration curves for aliphatic and aromatic hydrocarbons, ketones, and alkylsiloxanes (for separation see Fig. 1), and a separation of nickel and iron carbonyls is shown in Fig. 2. The column was 3 ft. of 25 % w/v di-2-ethylhexyl sebacate on Celite (36–60 mesh) at 56°. Carrier gas was oxygen free nitrogen at 50 ml/min.

However, it was found by subsequent work that the tin tetra-alkyls and related compounds could be detected by thermal conductivity cell, a modified flame ionization gauge[23], and a commercial gas-density balance unit[25] (Griffin and George D6 unit, Wembley, London). The latter unit has many advantages for such work, not

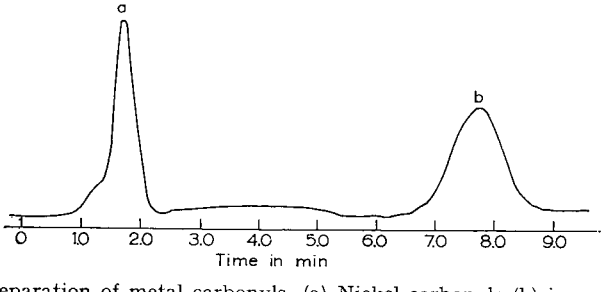


Fig. 2. Separation of metal carbonyls. (a) Nickel carbonyl; (b) iron carbonyl.

the least of these being that the sample is not subjected to a temperature greater than the column temperature; presumably a temperature at which the compound is stable. Results comparing these three detectors, with tetramethyl-tin with the appropriate conditions are given below:

Thermal conductivity detector (Fig. 3)

Column 6 ft. of 25 % w/w Apiezon M on treated Silicel 36–60 mesh.
Column temperature 140°. Detector temperature 150°.
Hydrogen carrier gas 30.0 ml per min. Recorder 5 mV F.S.D.

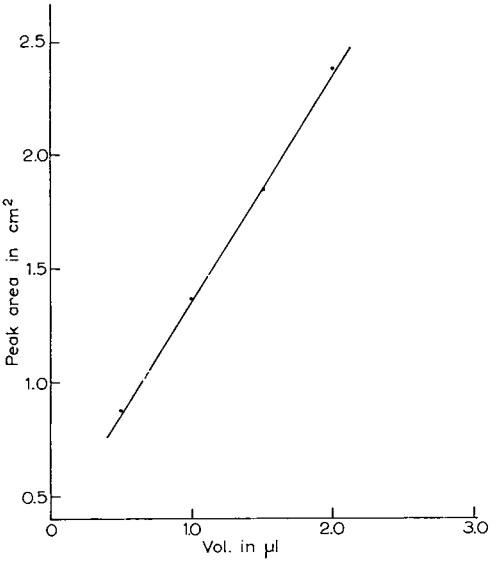


Fig. 3. Calibration graph for tetramethyltin using a thermal conductivity detector.

From Fig. 3, it is obvious that the graph is linear for low sample volumes, but above 2.5 µl, the thermal conductivity response is no longer linear. The relative detector responses to a number of compounds are given in Table I, using the nomenclature due to JAMIESON[26].

TABLE I

Compound	*Response per mole relative to tetramethyltin (= 100)*
Tetramethyltin	100
n-Hexane	98
Cyclohexane	92
n-Heptane	87
Benzene	116

Flame ionization detector

Column 6 ft. Apiezon M on Silocel (36–60 mesh).
Column temperature 140°. Bleed off 98 %;

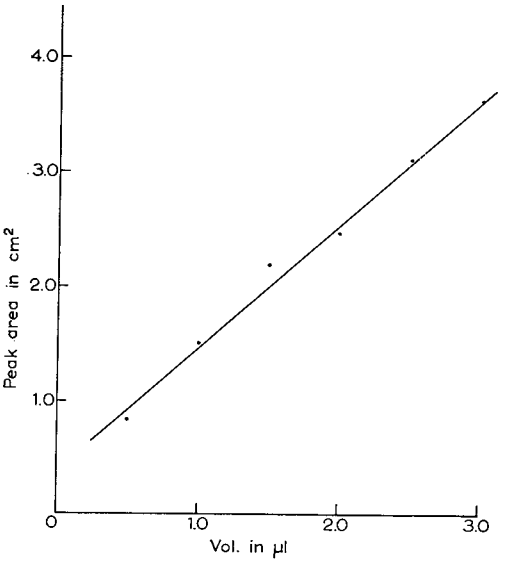


Fig. 4. Calibration graph for tetramethyltin using a flame ionization detector.

Flow rate of nitrogen through column 7 ml/min.
Hydrogen flow rate 30 ml/min.
Air flow rate 400 ml/min.

The response results are shown in Fig. 4, while it can be seen the graph is again linear over the lower ranges, while Fig. 5 shows the saturation phenomena at 4.0 μl sample size or above, compared with the shape of the elution peak at 3.0 μl sample size.

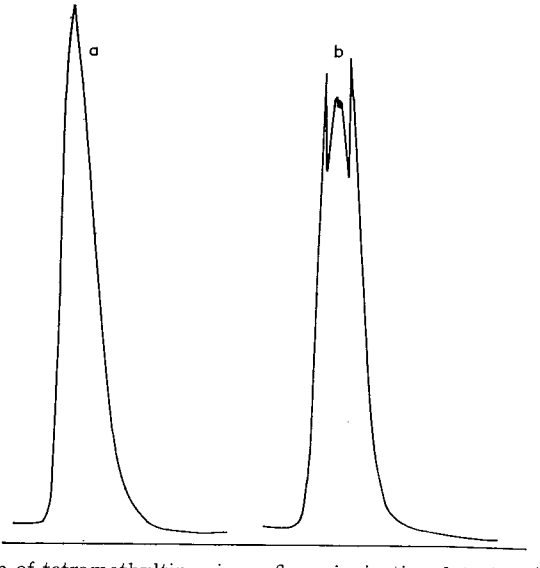


Fig. 5. Elution pattern of tetramethyltin using a flame ionization detector. (a) Normal response; (b) saturation response.

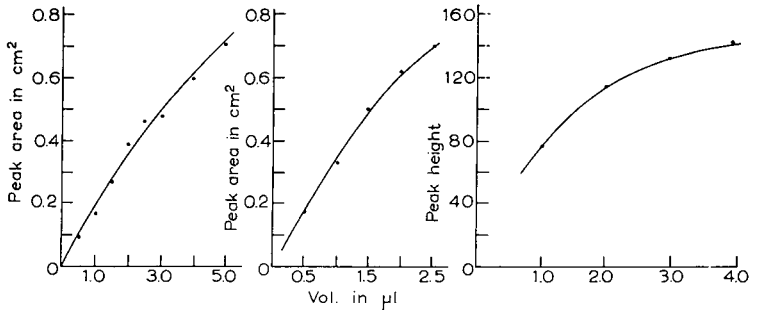


Fig. 6. Calibration graphs for tetramethyltin using a gas density detector.

Gas-density balance detector

Column 6 ft. 15 % w/w silicone oil E301 on Celite 545 (36–60 mesh).
Column and balance temperature 100°. Flow rate 30.0 ml of nitrogen per ml.

Due to non-linearity of signal response, the tetramethyltin was diluted with *n*-heptane, in the ratios 1:1, 1:5, and 1:10, and the results are shown in Fig. 6 (a), (b) and (c). Relative gas density balance responses for the compounds tested in the thermal conductivity experiments are given in Table II.:

TABLE II

Compound	*Response per mole relative to tetramethyltin (= 100)*
Tetramethyltin	100
n-Hexane	51
Cyclohexane	73
n-Heptane	78
Benzene	73

The relative sensitivities of the three detectors is:

Thermal conductivity:	Gas-density balance:	Flame ionization gauge
1	10	1.700

During a study of the addition reactions of alkyltin mono- and dihydrides with unsaturated hydrocarbons, it was found necessary to find conditions under which such hydrides could be detected without breakdown. The gas-density balance proved to be excellent for this purpose, although the compounds were also detected using the flame ionization gauge. A separation of trimethyltin hydride, tetramethyltin and dipropyltin dihydride is given in Fig. 7. The conditions were identical for those given in Fig. 6, except the column temperature was 80°. It was possible to elute trimethyltin hydride through Silicone E301, Apiezon L, and dinonyl phthalate phases, but when attempts to elute down a squalane (hexamethyltetracosane) column were made, decomposition occurred, and an elution pattern of the type shown in Fig. 8 was obtained. Several batches of squalane were used but all gave similar results. The shape of the peak is extremely interesting, because the negative flat portion is obviously

hydrogen formed by decomposition. The flat portion is interpreted as saturation of the gas-density balance, and the small positive peak is identified as tetramethyltin. This shows some rearrangement reaction must take place in or on the stationary phase

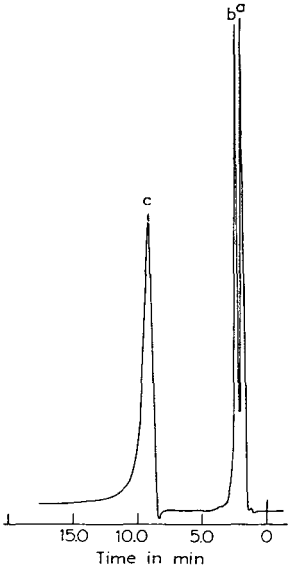


Fig. 7. Elution pattern of alkyltin hydrides. (a) Trimethyltin hydride; (b) tetramethyltin; (c) dipropyltin dihydride.

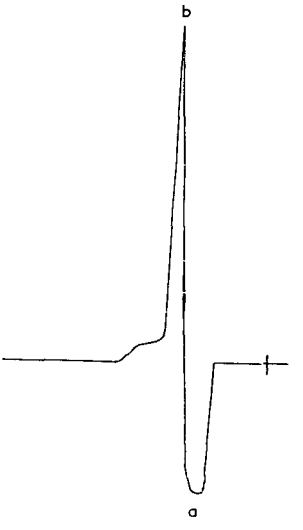


Fig. 8. Elution pattern of trimethyltin hydrides on squalane. (a) Hydrogen; (b) tetramethyltin.

because the trimethyltin hydride was identified as pure before injection on to the column. The reaction of the hydride with the phase was not unexpected, since the hydride readily adds to a triple bond[27], and less easily to a double bond. If some unsaturation was present in the squalane, then the reaction:

$$R - C \equiv CH + Me_3SnH \longrightarrow R - CH \equiv CHSnMe_3$$

is possible. However, this reaction does not produce hydrogen, or tetramethyltin. In a radical-mechanism, which is favoured by NEUMANN, NIERMANN AND SOMMER[28], it is possible that a reaction of the type:

$$Me_3SnH + R - C \equiv CH \longrightarrow R - CH \equiv CSnMe_3 + H$$

can take place. The hydrogen atom formed may then abstract hydrogen from further trimethyltin hydride forming hydrogen molecules and the radical Me_3Sn. Such a radical could again react with trimethyltin hydride, abstracting a methyl group, and so forming the tetramethyltin. Exchange reactions of this type between differing tetra-alkyl-tins are being studied, in an attempt to elucidate these points. Compounds of the type $R - CH = CHSnMe_3$ are perfectly stable and can be chromatographed without decomposition, when the molecular weight is low, *i.e.* up to $R = C_7$ (alkyl).

ACKNOWLEDGEMENTS

The authors wish to thank the Gas Council for the award of a Gas Council Research Scholarship to one of them (D.J.C.) during the tenure of which this work was carried out. They also wish to acknowledge financial help given to them by Unilever Ltd., for the purchase of the gas-density balance unit, and the Associated Octel Company for the potentiometric recorder.

SUMMARY

Conditions are given for the gas chromatographic elution of tetra-alkyl-tin, alkyl tin mono- and dihydrides, using thermal conductivity, gas density balance, and flame ionization detectors.

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AN APPROACH TO KINETIC STUDIES USING RADIOCHROMATOGRAPHY ON THIN LAYERS

PART I.

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(Received May 9th, 1963)

INTRODUCTION

A common technique used in biosynthetic studies, is that of counting radioactive samples at "infinite thickness". When obtained by counting at infinite thickness, the activities of samples derived from a common parent compound, can be related to one another and the parent compound with the minimum of correction[1].

It occurred to us that the same technique applied to thin-layer chromatography should offer considerable advantages in semi-quantitative kinetic studies of complex reactions. One would require that:

(i) the adsorbent layers be uniformly thin, but at infinite thickness to the radiation being emitted,

(ii) the compounds be uniformly distributed in depth through the thin layer before and after chromatography, and

(iii) all the reaction products be well separated by the chromatographic procedure, so that they do not interfere with one another when they are counted. The separation necessary would depend on the resolution of the scanner.

In order to test the above hypothesis, we required a scanning unit sufficiently sensitive to detect ^{14}C and ^{35}S radiation* emitted from a thin-layer chromatogram. The unit described was suitable for this purpose.

It soon became apparent in preliminary studies, that the technique could be improved by using more uniform thin layers. Better results were also obtained when the thickness of the adsorbing layer was adjusted to give a weight of adsorbent of 22–25 mg/cm^2. This was probably due to difficulties in obtaining uniform distribution of sample when spotting on thicker layers. We therefore designed the second unit described herein, which is a modification of a spreader described in the literature[2] and commercially available**. Both units are simply constructed and inexpensive. Results of kinetic studies using this new technique, will be described elsewhere.

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* The technique is uniquely suited to studies with these isotopes, (and others emitting β particles of similar energy), since *ca.* 22 mg/cm^2 represents infinite thickness of an absorber for these isotopes, and this thickness is suitable for thin-layer chromatography.

** From Camag, Chemie Erzeugnisse und Adsorptions Technik, A.G., Hombergerstr. 14, Muttenz B.L., Switzerland.

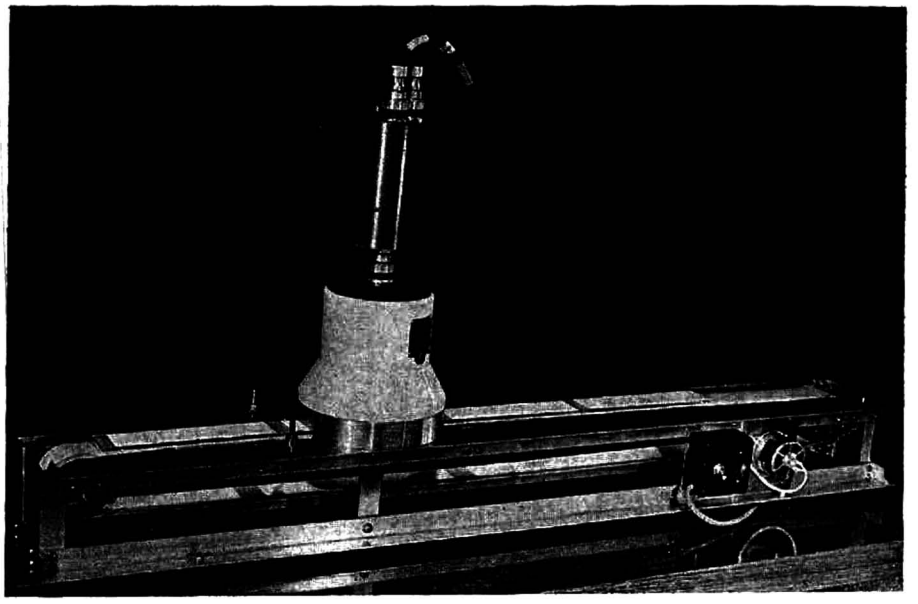

Fig. 1. Scanner unit in operation. Thin-layer chromatoplates are centered and pass from left to right under a thin window G.M. tube mounted in the EKCO castle head.

DESCRIPTION OF APPARATUS

Scanning unit

The scanning unit was built to utilise the head of an EKCO G.M. counting unit. Other G.M. castle units could be similarly modified. It consists of a continuous belt driven by a synchronous motor, and an adaptor to mount the EKCO head unit on the framework enclosing the belt and drive mechanism, and in close proximity to the belt. The unit in operation, is pictured in Fig. 1, and the adaptor, dimensioned for our EKCO head, in Fig. 2.

The scanner support assembly is shown in Fig. 3, and consists of three main parts. There is the belt running on adjustable rollers, a synchronous motor which can be readily interchanged when a change of scanning speed is required, and a smooth flat platform whose height can be adjusted, and above which is placed two adjustable guides for centering the plates on the belt. Dimensioning of this unit would depend on the requirements of the users. Our unit will handle over three hours counting without requiring attention, if run-on is provided at one end and a run-off at the other end of the belt. A continuous automatic device for handling the plates after counting would allow overnight operation. The unit is not as efficient as the windowless unit recently described[3], but is suitable for studies of isotopes other than tritium, and does not require any special ancillary equipment.

Thin-layer applicator

The modification to our existing applicator was suggested by the aforementioned experiences and by an observation that it should be possible to trim dry chromatoplates by repassing them under the applicator blade. Accurate setting of this blade would give a layer of excellent uniformity, free of variations that can arise during

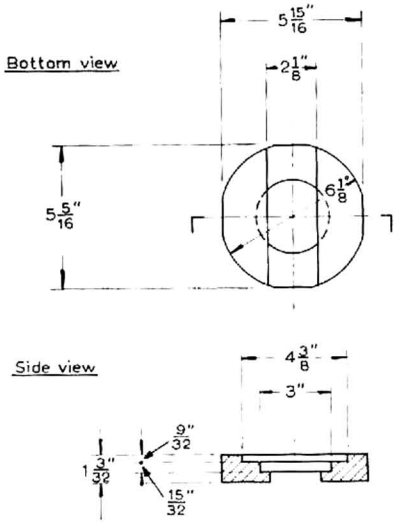

Fig. 2. Adaptor. Bottom view and side view showing dimensions in inches.

preparation and drying. Two simple micrometer depth gauges were fitted to the applicator blade of our unit to serve this purpose. They were located outside the adsorber well to prevent rusting, and the method of mounting is clearly shown in Fig. 4.

Accurate setting of both the micrometers was very simply achieved. Glass chromatographic plates were placed under the applicator blade, which was lowered onto them with the micrometers wound back. The micrometers were next wound down

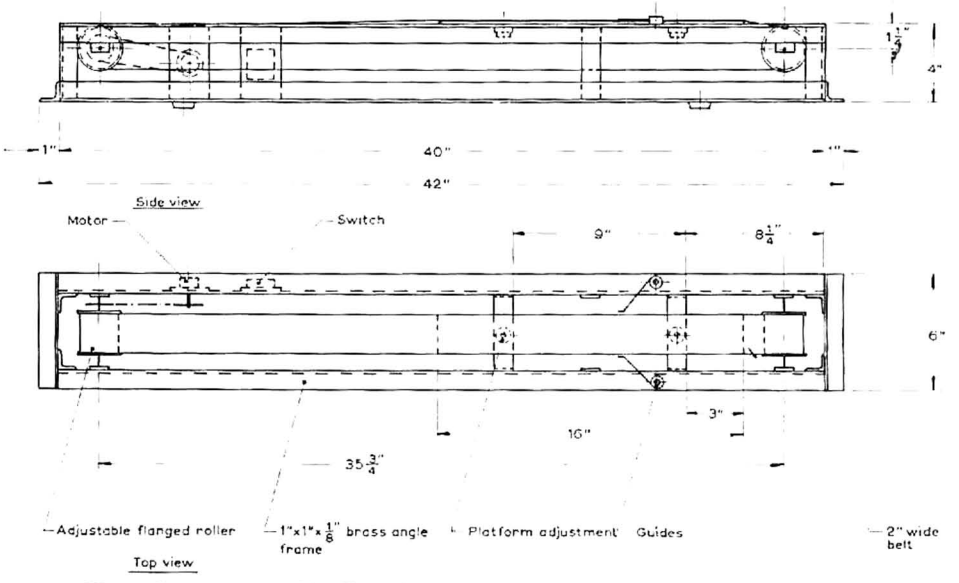

Fig. 3. Scanner assembly. Top view and side view showing dimensions in inches.

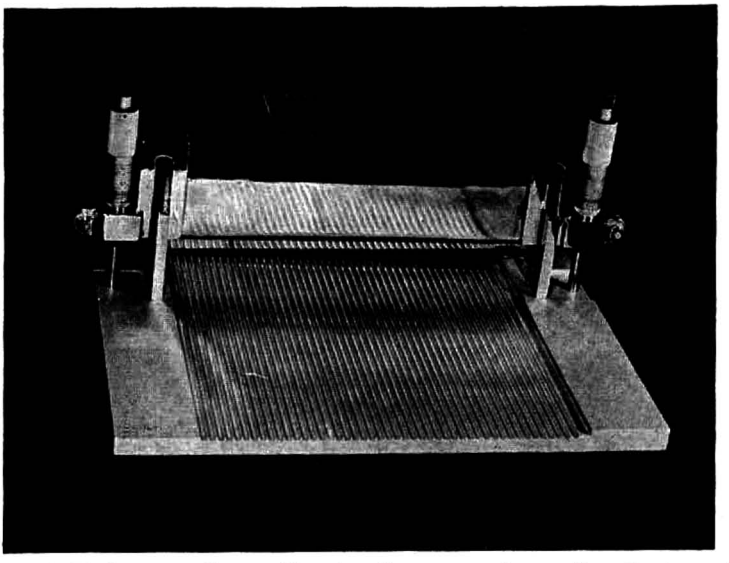

Fig. 4. Improved thin-layer applicator. Showing the means of mounting the two micrometers outside the adsorbent trough.

by their ratchet stop knobs, while pressure was applied to the blade, until they were set on the base plate. Both micrometers were now adjusted to the desired height by screwing the barrel in the normal way.

EXPERIMENTAL

We have recorded in Table I, the results of an investigation into the uniformity of the thin layers obtained by the above procedure. Three plates (cut from polished plate glass) were prepared in the one operation. Two of the plates, plates 2 and 3 (Table I) were repassed under the blade after drying, and all plates were divided into 1 cm^2 areas by marking with a razor blade. The individual square centimeters were taken from the plates and weighed. The results show clearly that the uniformity of the thin layers on the chromatoplates was very much improved. The results in Table I were obtained using aluminium oxide G adsorbent (40 g aluminium oxide, 60 ml water, stirred for 90 sec) and a similar improvement was obtained with Silica Gel G (30 g silica gel, 60 ml water, stirred for 90 sec). The plates were thoroughly dried at 100° in an oven in the approved way and then equilibrated overnight in the laboratory atmosphere before the weighings were commenced. The variation shown in measurements on the untreated plate is probably greater than one would normally obtain, as the knife setting was 1 mm; but regardless of the uniformity of the untreated plate it is evident that an excellent plate can be prepared from it by the "dry trimming" technique.

The error in the result, 25.3 ± 1.5 mg/cm^2, is thought to be largely due to the difficulty experienced in accurately marking the plates in square centimeters and then removing the adsorbent from these squares. The uniformity of the plates was checked by taking six micrometer readings along the length and in the middle of the plates.

TABLE I

RESULTS OF THE MEASUREMENT OF ADSORBER UNIFORMITY

	Plate 1			*Plate 2*			*Plate 3*		
	Weight/cm² of surface area (mg/cm²)			*Weight/cm² of surface area (mg/cm²)*			*Weight/cm² of surface area (mg/cm²)*		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
1	75.8	73.8	72.3	60.0	61.0	61.0	25.5	23.7	23.9
2	78.0	73.6	69.6	64.0	63.0	60.1	25.5	24.6	25.0
3	86.2	82.4	79.1	64.1	66.2	62.7	25.7	25.6	25.4
4	109.6	104.8	100.4	62.7	60.0	61.2	25.3	24.4	25.0
5	112.4	112.5	107.8	58.8	58.7	56.9	25.3	24.5	25.0
6	109.4	105.5	106.8	63.3	58.6	60.2	26.8	25.4	26.7
7	103.2	103.2	107.2	55.1	55.9	55.7	25.6	24.8	25.6
8	88.2	88.4	89.4	56.0	53.9	53.2	26.5	25.2	25.8
9	81.6	80.1	83.2	55.5	56.7	56.6	24.6	24.0	25.0
10	75.1	74.7	78.7	52.0	50.7	52.6	26.0	25.2	26.3
11	67.2	67.2	70.6	55.3	56.2	56.8	25.6	24.4	25.5
12	61.5	62.3	62.3	56.9	54.8	58.9	26.2	24.5	25.8
Column total	1048.2	1028.5	1027.4	703.7	695.7	695.9	308.6	296.3	305.0
Number	12	12	12	12	12	12	12	12	12
Column average	87.3	85.7	85.6	58.6	58.0	58.0	25.7	24.7	25.4
Plate average	86.2 ± 26.3 mg/cm²			58.2 ± 8.0 mg/cm²			25.3 ± 1.5 mg/cm²		
Plate particulars	Plate uniformity = 3.827 ± 0.009 mm (six measurements along centre) Size = 18 cm × 4 cm Knife set = 1 mm (Untouched after plating)			Plate uniformity = 3.827 ± 0.012 mm (six measurements along centre) Size = 18 cm × 4 cm Knife set = 0.75 mm (One pass under blade)			Plate uniformity = 3.826 ± 0.004 mm (six measurements along centre) Size = 18 cm × 4 cm Knife set = 0.35 mm (Reduced from 1 mm in steps—five passes under blade)		

This would not detect all the slight surface irregularities, which would further contribute to the error.

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SUMMARY

The construction of two instruments, a simple scanning unit for thin-layer chromatograms and a modified spreader for obtaining very uniform thin layers, is described.

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THE APPLICATION OF SPECTRAL REFLECTANCE TO THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Although the thin-layer chromatographic technique offers many distinct advantages, its analytical utility is restricted by two shortcomings. In the first instance, the difficulty experienced in obtaining reproducible R_F values with thin plates usually makes it necessary to run standards alongside the samples for comparison purposes. Secondly, the quantitative removal and extraction of individual spots from plates is a tedious process which often cannot be accomplished without decomposition occurring. Both of these operations would become superfluous if it were possible to effect the *in situ* identification and determination of chemical species separated on thin plates.

The use of spectral reflectance for these purposes was suggested by various studies[1–4] which demonstrated its utility with respect to paper chromatography. Furthermore, it has been shown that the reflectance spectra of substances concentrated on particulate adsorbents can be used for their identification,[5] and that spectral reflectance can be employed to determine the concentration of dyes scavanged from solution by the batchwise addition of starch[6]. A critical evaluation of the application of reflectance measurements to the direct analysis of solid mixtures has established the fact that analytically useful data can usually be obtained with samples in powdered form[7]. In view of these results, it was decided to investigate the analytical applications of spectral reflectance to thin-layer chromatography using, as a pilot system, water-soluble dyes and aluminum oxide plates prepared according to a method devised by MOTTIER[8]. This system, in that it is stable and absorbs in the visible portion of the spectrum, lent itself most conveniently to the purpose at hand.

EXPERIMENTAL

Stock solutions containing 50 mg of the dyes studied (aniline blue, eosine B, basic fuchsin, malachite green, naphthol yellow S, and rhodamine B) per 100 ml of solvent were applied as spots by means of a 10 μl Hamilton microsyringe. Except for the aqueous eosine B, the solvent used was 95% ethanol. The 10 × 7 × 0.15 cm plates were cut from ordinary window glass and were coated with adsorbent by distributing the adsorbent–water mixture with a glass rod which rested on one thickness of masking tape affixed to the ends of the plates. This technique gave a uniform coating 0.2–0.3 mm thick. The plates were dried at 180° for 2 h and stored in a desiccator. Merck aluminum oxide G and silica gel G were used as adsorbents.

The dyes were chromatographed in *n*-butanol–ethanol–water (80:20:10 by volume) by the ascending technique according to MOTTIER[8], and the plates were then dried at 110° for 15 min. Direct spectral examination of these plates was accomplished by covering them with a clean glass plate of identical dimensions, fixing the ends together with masking tape, and then introducing them into the reflectance attachment of the Beckman Model DK-2 Spectrophotometer employed for this purpose. A sheet of paper, resembling in color the adsorbent material being used as a reference standard, was inserted behind the plate to serve as a reflecting background. The reference standard was prepared by grinding some of the adsorbent from the plate under examination and packing it into the cell described by BARNES *et al.*[9].

A Beckman Model DU Spectrophotometer fitted with a standard attachment for the measurement of diffuse reflectance was employed to examine spots scraped off chromatographic plates. The cells used to hold both sample and reference material consisted of white paper, of a size that permitted its introduction into the sample holder of the reflectance attachment, to which a microscope cover glass had been affixed by two pieces of tape. Fifty milligrams of material were carefully compressed between the cover glass and the paper until a thin layer having an approximate thickness of 0.3 mm and an approximate diameter of 1.8 cm was obtained. This last was necessary, since the impinging beam of light had an approximate diameter of 1.4 cm. As before, the reference standard consisted of adsorbent from the plate under examination.

RESULTS AND DISCUSSION

Direct examination of chromatographic plates

As indicated in Fig. 1, which contrasts the transmittance spectrum of an aqueous solution of eosine B with the reflectance spectra of $2.5 \cdot 10^{-3}$ mg of the dye adsorbed on

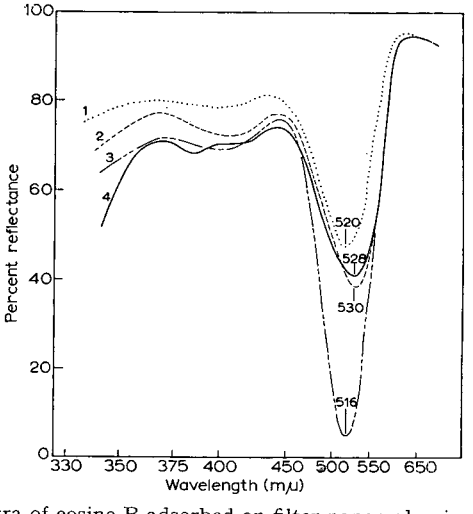

Fig. 1. Reflectance spectra of eosine B adsorbed on filter paper, alumina, and silica gel compared with the transmittance spectrum of an aqueous solution of the dye. (1) Silica gel (Merck thin-layer chromatography grade). (2) Filter paper (Whatman No. 42). (3) Alumina (Merck thin-layer chromatography grade). (4) Transmittance spectrum.

filter paper, alumina and silica gel, the spectra obtained for the different dyes were influenced by the nature of the adsorbent employed. The positions of the absorption maxima obtained for the dyes under various experimental conditions are summarized in Table I. In all cases the absorption maxima obtained for transmittance shifted to

TABLE I

ABSORPTION MAXIMA OF TRANSMITTANCE AND REFLECTANCE SPECTRA OF DYES

Dye	Transmittance*		Reflectance*		
	H_2O	EtOH	Filter paper	Alumina	Silica gel
Aniline blue	—	600	615	594	592
Eosine B	516	524	530	528	520
Basic fuchsin	—	549	553	550	540
Malachite green	—	620	628	615	615
Naphthol yellow S	—	435/389	443/394	436/391	431/390
Rhodamine B	—	546	550	550	547

* Readings are given as mμ.

higher wave-lengths when the reflectance spectra of the dyes adsorbed on Whatman No. 42 filter paper were determined. These results agree substantially with those of YAMAGUCHI *et al.*[2]. That no such general trend is observed in the case of the alumina or the silica gel is probably attributable to the larger number of experimental variables introduced by their employment. For example, ZEITLIN AND NIIMOTO[10] have shown that spectral displacements depend, in part, upon the extent of subdivision of the adsorbent. For these reasons, the standardized procedure described above was employed for the preparation and development of the plates. There was no discernible change in the reflectance of dyes separated on plates which had been stored in a desiccator over silica gel for periods up to three days following their development. It might also be noted that a complete inhibition of the fluorescence exhibited by eosine B and rhodamine B in transmittance measurements occurs when the dyes are adsorbed on filter paper, alumina or silica gel.

With proper precautions it is possible by direct examination of chromatographic plates to obtain spectra suitable for identification purposes as shown in Fig. 2a and 2b, which depict the reflectance spectra for the various dyes adsorbed on alumina. Spots having diameters as small as 5 mm could be centered by using the red portion of the visible spectrum and measured with ease. In the resolution of complex dye mixtures, a spot separation of one centimeter sufficed to permit spectral identification of the component dyes.

The quantitative potential of the technique was demonstrated by examining plates spotted with dilution series of the dyes. Fig. 3, which shows the reflectance spectra of various concentrations of eosine B adsorbed on alumina, typifies the data obtained during this study. The solutions were added in 5 μl increments to give spots whose diameters approximated 1 cm. As indicated above, the thickness and particle size of the adsorbent and the drying temperature of the plates were kept fairly constant. It was found that excessive tailing decreased the precision of measurements, and an effort was made to keep it to a minimum. Precision was likewise affected by the im-

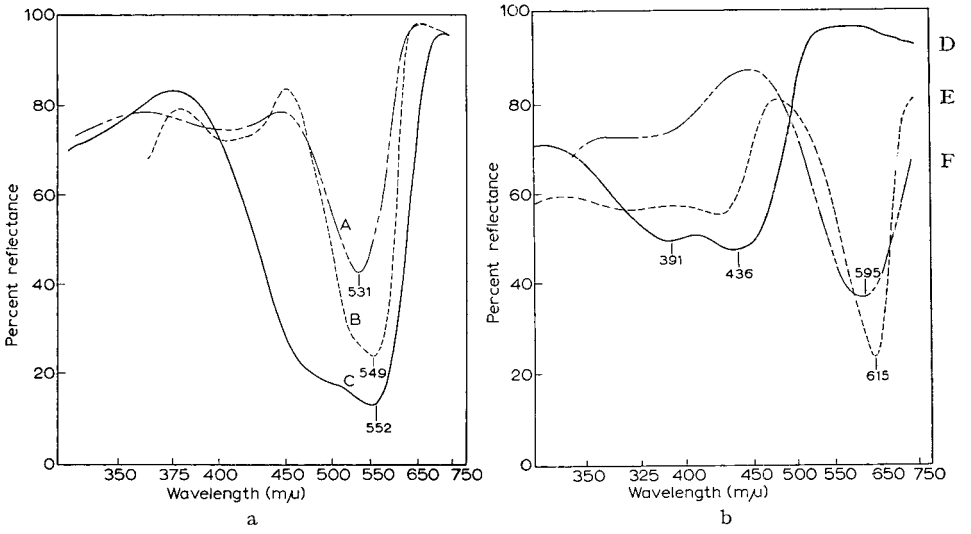

Fig. 2. (a) Reflectance spectra of dyes adsorbed on alumina. (A) Eosine B. (B) Rhodamine B. (C) Fuchsin. (b) Reflectance spectra of dyes adsorbed on alumina. (D) Naphthol yellow S. (E) Malachite green. (F) Aniline blue.

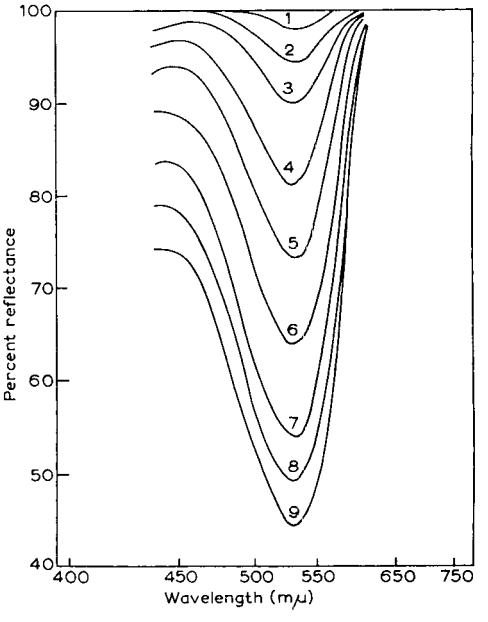

Fig. 3. Reflectance spectra of various concentrations of eosine B adsorbed on alumina. Concentrations in mg/100 ml: (1) 0.78; (2) 1.56; (3) 3.12; (4) 6.25; (5) 12.5; (6) 25.0; (7) 50.0; (8) 75.0; (9) 100.0.

proper positioning of the spot in the impinging light beam, and care was taken to center it in the manner described previously. No significant changes in reflectance were observed over a period of a week when the plates were stored in a desiccator in the dark. When these precautions were taken, the largest difference in reflectance found between any two members of thirteen sets of triplicate samples of eosine B was 4.0 units on the 100-unit reflectance scale. These samples represented a concentration range of $5 \cdot 10^{-3}$ to $4 \cdot 10^{-5}$ mg of added dye. The data for this study are presented in Table II, which indicates the reproducibility that can be expected for reflectance

TABLE II

REPRODUCIBILITY OF REFLECTANCE READINGS AT 530 mμ OBTAINED FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF EOSINE B ADSORBED ON ALUMINA

Concentrations of dilution series (mg dye/100 ml soln.)	*Trial*		
	1 (%R)	*2 (%R)*	*3 (%R)*
0.78	98.0	98.5	98.4
1.56	94.5	93.8	93.5
3.12	89.5	91.0	91.0
4.30	87.0	88.0	87.3
6.25	83.0	82.0	81.5
8.50	79.0	77.5	78.5
12.50	76.5	74.0	73.5
18.00	71.0	71.5	69.0
25.00	66.5	62.5	63.5
37.50	60.0	59.3	59.5
50.00	55.0	55.5	54.0
75.00	49.0	50.0	49.0
100.00	44.5	47.0	45.0

readings obtained for different spots of the same concentration. A consideration of Fig. 4, in which these data are plotted in the form 2—log % R *versus* concentration, reveals that Beer's law holds only as a limiting law for reflectance and that a linear relationship obtains only for concentrations less than 5 mg of dye/100 ml of solution. As may also be seen in Fig. 4, plotting the same data in the form 2—log % R *versus* the square root of the concentration extends this linear relationship ten-fold to an upper limit of 50 mg/100 ml. The same relation between reflectance and concentration was found to exist for rhodamine B. These results are in agreement with those obtained by YAMAGUCHI *et al.*[3] for different food dyes adsorbed on filter paper.

Examination of spots removed from chromatographic plates

The precision attained in the determination of the adsorbed dyes by the direct examination of the chromatographic plates was improved by scraping the spots off the plates and measuring the reflectance of this material with the cell described above. Such a device proved to be necessary, as the amount of material removed from the plates was insufficient to fill the cells available for this purpose. The addition of more alumina was undesirable, since it introduced a considerable dilution factor that decreased the sensitivity of the method. Using this improved cell and taking precautions to insure a homogeneous sample of relatively uniform particle size, it was possible to

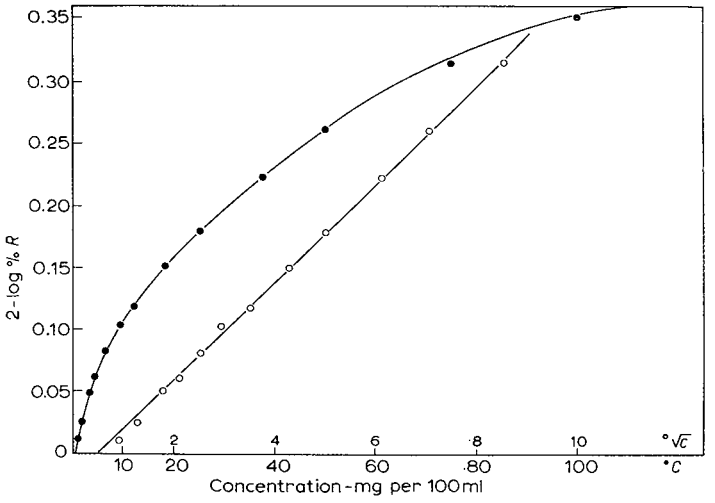

Fig. 4. 2—log % reflectance at 530 mμ of eosine B adsorbed on alumina as a function of concentration. ●——● C; ○——○ $C^{\frac{1}{2}}$.

get reflectance readings for replicate samples which differed by no more than 0.6 reflectance units. This is indicated in Table III, which lists readings obtained at various wave lengths for three different samples of eosine B of identical concentration.

The procedure employed was identical with that followed in the determination of the dyes by direct examination up to the point the sample material was removed from the plate. The 50 mg comprising the sample were weighed to $\pm$ 0.2 mg and then ground in a small agate mortar for two periods of 15 sec each to insure homogeneity and uniform

TABLE III

REPRODUCIBILITY OBTAINED FOR DIFFERENT SAMPLES OF IDENTICAL CONCENTRATIONS OF EOSINE B ADSORBED ON ALUMINA

Wave length (mμ)	*Sample I (%R)*	*Sample II (%R)*	*Sample III (%R)*
540	85.7	86.1	86.3
530	85.2	85.4	85.7
520	85.9	86.3	86.4

particle size. The grinding procedure was standardized, as it was found that a measurable difference in reflectance was produced by varying the operation. LERMOND AND ROGERS[7] reported similar results for the screening of sample materials. The greatest difficulty encountered was the attainment of reproducible packing of the sample in the cell. To assure the degree of precision achieved in the test of reproducibility which is summarized in Table IV, it is essential that the samples have approximately the same diameter and thickness and possess a uniformly smooth surface. The largest observed difference for any pair of readings obtained for the same sample repacked in the same holder was 0.7 reflectance units. When one considers that these differences are of the same order as the ones obtained for the replicate samples listed in Table III, it becomes

TABLE IV

TEST OF REPRODUCIBILITY OF PACKING REFLECTANCE CELL. EOSINE B ADSORBED ON ALUMINA

Wave length (mμ)	*1st packing (%R)*	*2nd packing (%R)*	*3rd packing (%R)*
620	98.4	98.4	99.0
600	97.2	96.8	97.2
580	93.3	93.7	93.8
560	89.0	89.1	89.5
540	86.9	87.2	86.5
530	86.7	86.8	86.3
520	87.2	87.2	86.9
500	89.6	89.6	89.6
480	91.8	91.9	91.9
460	93.4	93.7	93.8

apparent that the precision of the technique is limited by the reproducibility of packing the sample.

The relationship between reflectance and the concentration of eosine B adsorbed on alumina was investigated again, this time by means of the spot removal technique. A plot of the data obtained in the form 2—log % *R versus* concentration gave the same type of smooth curve as was obtained by direct examination and depicted in Fig. 4. The only notable difference was the upward extension of the linear relationship to a dye concentration of 20 mg/100 ml of solution. It is possible to extend this upward even further to 40 mg/100 ml if, as is done in Fig. 5, the concentration is plotted

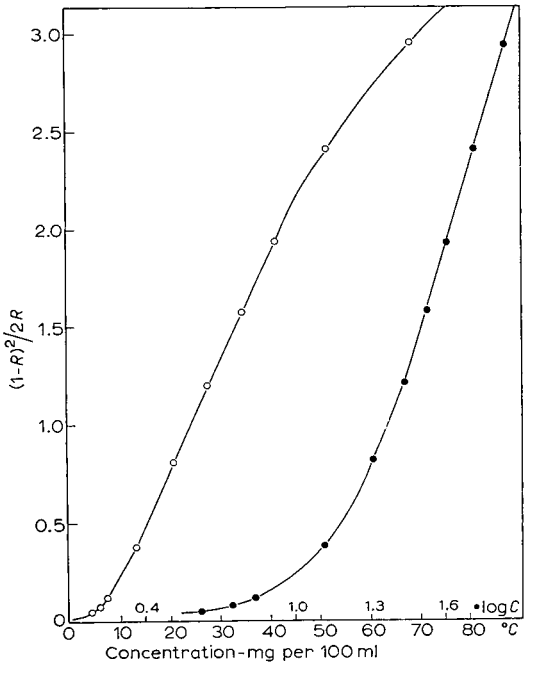

Fig. 5. Kubelka-Munk values at 530 mμ for eosine B adsorbed on alumina as a function of concentration. O——O C'; ●——● log C'.

against $(I-R)^2/2R$, the form of the Kubelka-Munk expression most often used[11]. Beyond this point, the curve is so smooth that it can serve a quantitative function. By plotting the logarithm of the concentration *versus* $(I-R)^2/2R$, as shown in Fig. 5, it is possible, if this is desired, to expand the linear relationship to the highest concentration studied, 350 mg/100 ml.

Finally, some measurements were made on a dilution series using the cell without the glass cover, which increased readings as much as 6.0 reflectance units at 530 mμ. For samples having a smooth surface, the precision was unaffected by the removal of the glass. Utilization of the cell without the glass cover might be desirable when dealing with samples of low reflectance or with spectral regions requiring a quartz plate.

The above results show that the components of complex mixtures of dyes separated on thin-layer plates can be identified rapidly, without recourse to R_F values, by direct examination of the plates by spectral reflectance. The same operation is capable of providing quantitative data having a precision of approximately ± 5%. A degree of precision identical to that afforded by transmittance is attained if the reflectance measurements are carried out on spots removed from the chromatographic plates and packed in an appropriate cell. By applying the Kubelka-Munk function, linear reflectance-concentration relationships can be obtained for concentration ranges of interest to the analytical chemist. Although the present study was restricted to the visible portion of the spectrum, other regions can be used as well[12] if the cell is provided with a quartz plate or employed without a cover. In general, one can say that the application of spectral reflectance to thin-layer chromatography would enhance its utility greatly by simplifying and expediting the analysis of complex mixtures.

ACKNOWLEDGEMENT

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SUMMARY

The components of dye mixtures resolved on thin-layer plates were identified by direct examination of the plates by spectral reflectance The amounts of adsorbed dye were determined at the same time with a precision of approximately ± 5%. Reflectance measurements carried out on spots removed from the plates and packed in an appropriate cell afforded a degree of precision identical to that attained with transmittance.

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THE THIN LAYER CHROMATOGRAPHIC CHARACTERIZATION OF SOME PHENOLIC COMPOUNDS RELATED TO THE TOCOPHEROLS AND THEIR OXIDATION PRODUCTS

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In connection with other work concerning the oxidation products and metabolites of the tocopherols which is underway in this laboratory, it was necessary to investigate the chromatographic behavior of a number of model compounds. The silica gel thin-layer system was particularly well suited for the separation of these materials; the three best solvents used are shown in Table I, which summarizes the R_F values for 27 hydroquinones, hydroquinone diacetates, chromanols, dichromans, and various oxidation products of the α-tocopherol model compound (2,2,5,7,8-pentamethyl-6-hydroxychroman).

Three different spray reagents were used for development of the finished chromatograms; 60% sulfuric acid, followed by heating in an oven at 150°, as a general reagent which shows the location of most organic compounds, 5% potassium ferricyanide followed by 5% ferric chloride which shows compounds oxidized by ferricyanide ion with formation of Turnbull's blue[1], and neutral silver nitrate in acetone which generally detects the free phenolic hydroxyl compounds[2]. The color reactions with these reagents are also shown in Table I.

The melting points are given for all compounds along with a reference to the literature melting point. In cases where there is a disagreement with the literature melting points, some other evidence is given to substantiate the compound's authenticity, *e.g.*, elementary analysis, nuclear magnetic resonance (NMR), infra red spectra, or derivatization. Several of the compounds have not previously been reported.

EXPERIMENTAL

The thin layer plates (8 × 8 in.) were prepared from "Silica-Gel G. according to Stahl," (Brinkmann Instruments Co., Great Neck, Long Island, N. Y.) by mixing 30 g of the dry powder with 60 ml of distilled water and applying to the glass plates with a 250 μ spreader. After air drying the plates were baked in an oven for 1 h at 110°.

All chromatograms were prepared by the ascending method at 20° in a solvent saturated atmosphere. The compounds (2γ from ethanol solution) were spotted 2 cm from the bottom of the thin layer plate. The plate was submerged in solvent to a depth of 3 to 5 mm and the solvent allowed to run a distance of 15 cm. The running times for the 15 cm of solvent travel for the respective solvents were: chloroform, 47 min; benzene, 35 min; cyclohexane–tetrahydrofuran (THF) (1:1), 53 min.

TABLE I

CHARACTERIZATION OF SOME PHENOLIC COMPOUNDS RELATED TO TOCOPHEROLS AND THEIR OXIDATION PRODUCTS

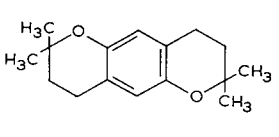
Compound	Reference	Melting points		R_F			Spray reagents		
		Literature	Found	Chloroform	Benzene	Cyclohexane THF (1:1)	H_2SO_4	$K_3Fe(CN)_6$	$AgNO_3$
Hydroquinones									
Unsubstituted	4	172.3	172–174	00–04	00–04	43–47	tan	blue	grey
2-(CH_3)	5	124–125	127–129	00–05	00–04	45–50	tan	blue	grey
2,3-(CH_3)$_2$	6	221	225–226	02–06	00–04	49–54	grey	blue	grey
2,6-(CH_3)$_2$	7	149–151	145–147	05–08	02–06	51–56	yellow	blue	grey
2,5-(CH_3)$_2$	8	212	219–220	05–09	02–05	53–58	tan	blue	grey
2,3,5-(CH_3)$_3$	4	170	170–173	00–25	03–06	—	yellow	blue	grey
2,3,5,6,-(CH_3)$_4$	9	220	229–230	00–30	03–05	—	yellow	blue	grey
Hydroquinone diacetates[a]									
Unsubstituted	10	121–122	123–124	48–54	05–08	52–56	tan	—	—
2-(CH_3)	11	43–44	35–36	52–57	05–08	56–62	tan	—	—
2,3-(CH_3)$_2$	12	105–106	105–106	50–55	03–06	56–62	yellow	—	—
2,6-(CH_3)$_2$	13	92–93	85–88	52–56	04–07	55–61	tan	—	—
2,5-(CH_3)$_2$	—	—	133–135	50–55	03–07	55–60	yellow	—	—
2,3,5-(CH_3)$_3$	14	112	109–110	48–54	01–05	54–58	yellow	—	—
2,3,5,6-(CH_3)$_4$	15	202–203	207–208	37–41	04–07	—	yellow	—	—

2,2-Dimethylchromans									
5,7-$(CH_3)_2$-6-(OH)	16	92.5–93.5	98–99	45–51	24–31	55–64	yellow	blue	grey
5,8-$(CH_3)_2$-6-(OH)[b]	16	77–78	91–92	27–34	14–21	54–63	yellow	blue	grey
5,7,8-$(CH_3)_3$-6-(OH)	17	94–94.5	93–94	45–55	20–26	68–73	yellow	blue	grey
5,7,8-$(CH_3)_3$-6-(CO_2CH_3)	—	—	92–93	64–68	22–28	64–75	yellow	—	—
Dichromans									
Unsubstituted (I)	—	—	159–161	65–71	41–48	72–79	tan	blue	—
o-$(CH_3)_2$ (II)[b]	18, 16	101–102	97–100	75–79	63–70	74–80	yellow	blue	—
p-$(CH_3)_2$ (III)	16	193–196	191–193	74–78	64–71	77–84	yellow	blue	—
Oxidation products									
Red (IV)[c]	19	109–110	—	22–27	00–04	54–59	brown	blue	—
Purple (V)[c]	16	142–143.5	—	00–04	00–03	05–08	brown	blue	grey
Quinone (VI)[c]	20	62	—	09–15	00–03	52–57	tan	blue	—
Yellow dimer (VII)[b]	3, 21, 22	126–127	77–79	63–68	17–25	72–76	green	blue	grey[d]
Dihydroxy dimer (VIII)	21	—	185–188	45–51	12–17	66–75	yellow	blue	grey
Trimer (IX)	3	—	227–228	73–78	28–35	77–83	brown	blue	—

[a] All of the diacetates, hydroxychromans, and dichromans were prepared from their respective hydroquinones.
[b] The NMR spectrum and elementary analysis were consistent with the structure proposed
[c] The IR spectra of these compounds were identical with authentic samples.
[d] After one hour.

Preparation of 2,2,7,7-tetramethyl-3,8-dihydrobenzo[1,2-b:4,5-b']dipyran (I)

This compound was prepared in good yield by treating hydroquinone with excess isoprene in refluxing acetic acid containing zinc chloride as described previously[18,19]. After several recrystallizations from alcohol–water mixtures and finally vacuum sublimation, the compound melted at 159–161° and showed no OH stretching bands in the infrared. The NMR spectra showed a singlet at 8.75τ; two triplets at 8.32τ and 7.35τ and a singlet at 3.70τ which stood in the area ratio of 6:2: 2:1 respectively.



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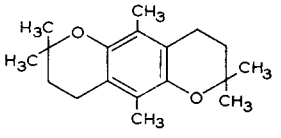

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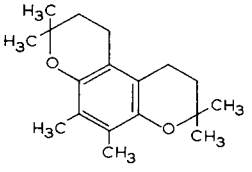

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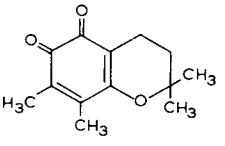

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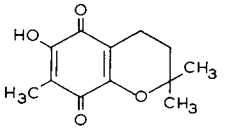

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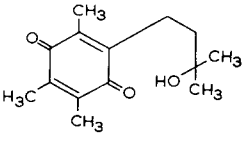

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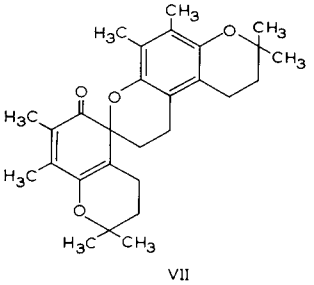

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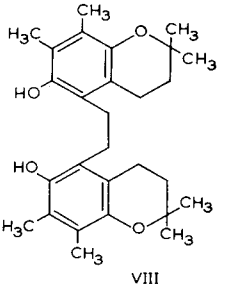

VIII

Trimer of unknown structure[3]

IX

A comparison of this NMR spectra with that of 3,3,5,6,8,8-hexamethyl-2,9-dihydrobenzo[1,2-*b*:4,3-*b'*]dipyran (III), which showed triplets at 8.27τ and 7.48τ tends to support the assignment of structure I to this compound as indicated by the up field shift of its more shielded methylenic protons.

Analysis. Calculated for $C_{16}H_{22}O_2$: C, 78.0; H, 8.94. Found: C, 78.0; H, 9.09.

Preparation of 2,5-dimethylhydroquinone diacetate

The diacetate of 2,5-dimethylhydroquinone was prepared by refluxing the hydroquinone in excess acetic anhydride–pyridine mixture for 1 h. The mixture was poured into cold water and the white crystalline compound obtained in nearly quantitative yield. It was recrystallized once from ethanol and then sublimed in a vacuum, m.p., 133–135°.

Analysis. Calculated for $C_{12}H_{14}O_4$: C, 64.9; H, 6.31. Found: C, 65.5; H, 6.82.

Preparation of 2,2,5,7,8-pentamethyl-6-acetoxychroman

This compound was prepared exactly as described above for the 2,5-dimethylhydroquinone diacetate except that the compound was not sublimed. The recrystallized material, shining plates, was dried in a vacuum at room temperature, m.p., 92–93°.

Analysis. Calculated for $C_{16}H_{22}O_3$: C, 73.3; H, 8.39. Found: C, 72.8; H, 8.46.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to WILLIAM ANDERSON and S. A. FUQUA for their help with the NMR spectra and to the Public Health Service of the Department of Health, Education and Welfare for support of this work by a grant (A-5552).

SUMMARY

Characterization of a number of phenolic compounds related to the tocopherols and their oxidation products by the use of thin-layer chromatography on silica gel is described.

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THIN-LAYER CHROMATOGRAPHY OF RESIN ACID METHYL ESTERS

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INTRODUCTION

The problem of separating and identifying resin acids has been the subject of considerable research effort. A partition chromatographic system has been employed but is rather tedious and complete separations are not obtained[1]. Partition paper chromatography has been applied with limited success[2,3]. Gas chromatography has been used with partial success[4]; in the Forest Products Laboratory, resin acid methyl esters have been gas chromatographed without isomerization of the sensitive abietadienic acid esters[5]. Mass spectroscopy has been used successfully[6]. However, a much simpler method is desirable.

DANIELS AND ENZELL[7] have successfully applied a method, developed by WICKBERG[8] for the separation of unsaturated hydrocarbons by partition of their silver π-complexes between hexadecane and aqueous methanol, to the separation of resin acid methyl esters. It seemed quite possible from this work that a more convenient system could be developed having the silver salt in the stationary phase. Indeed, thin-layer chromatography using silver nitrate impregnated silica gel has been successfully applied to some difficult separations of unsaturated fatty acid esters[9] and triglycerides[10]. The acetic acid containing solvent system of BARRETT, DALLAS AND PADLEY[10] was ruled out because of the probability of acid isomerization of the resin acid esters. The ethyl ether–hexane system of MORRIS[9], however, offered promise.

EXPERIMENTAL

Glass plates (200 mm × 200 mm) were coated with a slurry of alumina (aluminum oxide G with binder, Research Specialties Co.) and silver nitrate solution using a spreader set for a thickness of 250 μ. A solution of 12 g silver nitrate in 20 ml water was diluted with 40 ml methanol and the resulting solution added to 30 g alumina and mixed by shaking. The plates prepared with this coating were allowed to air dry overnight. Before use, the plates were dried at 110° for 30 min.

The resin acid methyl esters were prepared using a freshly distilled ethyl ether solution of diazomethane in a similar manner to that of SCHLENK AND GELLERMAN[11]. The diazomethane solution was added dropwise to 1% solutions of pure or mixed resin acids in 9:1 (v/v) ethyl ether–methanol at 0° until a slight excess of diazome-

* Maintained at Madison, Wis. (U.S.A.), in cooperation with the University of Wisconsin.

thane solution was discernible against a white background. The solution was then concentrated under a stream of purified nitrogen. A mixture of methyl stearate, oleate and linoleate (Applied Science Laboratories, State College, Pa.) was run on all plates for comparison. The methyl esters of commercial pine gum rosin and resin acids obtained unaltered from longleaf pine oleoresin by amino-cellulose ion exchange[12] were dissolved in hexane and filtered through alumina (Woelm, neutral, activity I) to remove oxidized materials.

Best results were obtained when 25 % by volume peroxide-free anhydrous ethyl ether in petroleum ether (b.p. 30–60°) was used as the developing solvent. It was important that the developing solvent be added to the tank just before inserting the plate. The thin-layer plates were allowed to develop (ascendingly) the length of the plates (180 mm) in a constant temperature room held at 30°. Development time ranged from 80 to 90 min.

The developed plates were sprayed with 1:4 (v/v) conc. sulfuric acid–ethyl ether solution. Sulfuric acid diluted with ether has the additional advantage of being easier to spray than conc. sulfuric acid alone. After heating in an oven at 110° for 15 min, all spots except the fatty acid and saturated resin acid esters could be readily seen under ultraviolet light. All spots could be observed by charring at 200° for 1 h.

RESULTS

Fig. 1 shows the results that were obtained with the described chromatographic system. Plates 200 mm × 500 mm with descending development were also used for

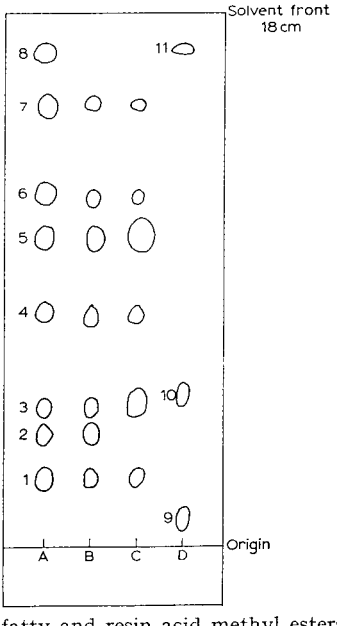

Fig. 1. Thin-layer chromatogram of fatty and resin acid methyl esters. Methyl (1) isopimarate, (2) levopimarate, (3) palustrate, (4) pimarate, (5) abietate, (6) neoabietate, (7) dehydroabietate (8) dihydro and tetrahydro resin acids, (9) linoleate, (10) oleate, and (11) stearate. (A) Resin acid methyl ester standard mixture prepared from pure resin acids, (B) resin acid methyl esters from oleoresin, (C) resin acid methyl esters from gum rosin, and (D) fatty acid methyl esters.

the separation of methyl dihydroisopimarate or methyl dihydrolevopimarate from methyl tetrahydroabietate (prepared by hydrogenation of levopimaric acid with Adams catalyst in acetic acid). The use of a less polar solvent, *e.g.*, petroleum ether alone, on standard plates also gave good separation of the dihydro from the tetrahydro resin acid esters, although some of the other resin acid esters were no longer resolved under these conditions.

The developing solvent should be placed in the tank just before use. Successful separation apparently depends on a combination of gradient development and an extended development resulting from the evaporation of solvent from the plate during the development. Attempts to operate under a saturated solvent atmosphere by the use of blotters did not approach the separation attained under the described conditions even though various solvent components and proportions were tried.

The use of hazardous and expensive silver fluoroborate as per DANIELS AND ENZELL[7] gave no improvement over the use of silver nitrate. Doubling the silver nitrate content did not improve separations. Incorporation of ethylene glycol in the stationary phase to effect a liquid–liquid partition chromatographic system gave inferior results.

Silica with calcium sulfate binder was tried as a support for the silver nitrate, but the binder was not effective in the presence of silver nitrate and, consequently, it was difficult to keep the layer intact while spraying and handling these plates. The alumina–silver nitrate plates, on the other hand, can be sprayed without special precautions and are easily handled.

The maximum loading of each resin acid ester is about 10 μg per spot. Minimum detection limits of the sulfuric acid–ether spray reagent for unsaturated acid esters when heated and observed under ultraviolet light are from 0.5 to 1 μg, whereas these minimum limits are from 1 to 2 μg when charring at 200° is used as a means of detection. Both of these methods are far more sensitive than using antimony pentachloride or any of the standard lipid reagents such as 2′,7′-dichlorofluorescein or rhodamine B. Permanent records of the plates were made with a Xerox 914 copying machine.

Methyl abietate–neoabietate and methyl palustrate–levopimarate are the most difficult pairs to separate, especially when too large a sample size is used. The methyl palustrate–levopimarate separation can be specifically improved by increasing the amount of ethyl ether in the solvent system to 40 %. Separation of methyl abietate–neoabietate when one of these esters is present to a much larger extent than the other is more difficult. A longer plate may be required under some circumstances.

Since the R_F or R_{solute} values vary somewhat from plate to plate, it is advisable to run a set of standards when identifying the components of a mixture. However, the order to elution remains as shown in Fig. 1. This order is somewhat different from that of the partition system of DANIELS AND ENZELL[7].

Oxidized resin acid esters can interfere with the identification. This is particularly true of the methyl abietate or neoabietate oxidation products which make it difficult to establish the presence of methyl isopimarate, levopimarate, or palustrate.

Application of this method to methylated resin acids from gum rosin and oleoresin reveals the presence of the primary resin acids in oleoresin and the expected absence of methyl levopimarate among the resin acids of gum rosin.

Confirmation of the identity of resin acid esters has been achieved by recovery

and spectral examination of the esters. A solution of the unknown was streaked onto the plate by an adaptation of the streaking apparatus of MCKIBBINS *et al.*[13] and developed. The plate was then masked and several 1 mm-wide strips parallel to the direction of development sprayed with the sulfuric acid–ether reagent. The zones on the unsprayed portions of the plates corresponding to the spots on the sprayed areas were removed from the plate and the esters eluted in a microapparatus developed at the Forest Products Laboratory[14]. The esters were then additionally characterized by infrared and ultraviolet spectroscopy.

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SUMMARY

A procedure has been developed in which all of the common pine resin acids may be cleanly separated by thin-layer chromatography of their methyl esters on alumina impregnated with silver nitrate.

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NOTE ADDED IN PROOF

We are grateful to T. NORIN and L. WESTFELT for sending us a copy of their paper, "Thin-layer, column, and gas-liquid chromatography of resin acid esters and related terpenes," *Acta Chem. Scand.* (in press) in which they describe the use of silver nitrate on silica gel plates and a benzene–ether solvent system for the separation of resin acid esters.

IDENTIFICATION OF IMPURITIES IN α-TRINITROTOLUENE BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

2,4,6-Trinitrotoluene (α-TNT) is a common military explosive, prepared commercially by the three-stage nitration of toluene, and purified by the sellite process[1]. The final product is relatively pure; however, various by-products do remain in the purified TNT to alter its physical properties. In this laboratory we have sought for a qualitative scheme to separate and identify the impurities present in production grade TNT.

A review of the literature reveals that very few, if any, identification methods for α-TNT impurities are reported. Although quantitative methods utilizing infrared[2] and gas chromatography[3] are cited in the literature, application of these methods for identification work leaves something to be desired. Infrared lacks sensitivity unless some preliminary concentration step such as column chromatography is used[4]. Only the mono- and di-nitrotoluene isomers have been successfully separated and determined by gas chromatography. Paper chromatography, as reported by ETTEL *et al.*[5], can be used for separating some of the nitroaromatic compounds. With the advent of thin-layer chromatography (TLC) according to KIRCHNER[6,7] and STAHL[8], a simple, efficient method of separation has been presented. TLC is often effective for the separation of materials that cannot be resolved by other techniques.

This article describes a two-dimensional TLC method for the separation and identification of α-TNT impurities, including some oxidation–reduction products of decomposition as well as common production grade impurities. In addition, a unique detection method is described in which the reductor of the developing reagent is directly incorporated in the thin layer.

APPARATUS AND REAGENTS

Applicator, chromatojar, silica gel G, and glass plates (200 × 200 mm), purchased from Brinkmann Instruments, Inc.

Zn metal dust, AR grade, from Mallinckrodt Chemical Works.

All solvents were reagent grade, and further purification was not necessary. Petroleum ether with a boiling range of 30–60° was used.

The spray reagent consisted of 0.25 % *p*-diethylaminobenzaldehyde (*p*-DEAB), 0.25 *N* HCl in absolute ethanol. Concentrated HCl was used to prepare the reagent.

An Agla micrometer syringe was used to deliver aliquots of sample.

EXPERIMENTAL

Preparation of thin layer

STAHL'S procedure for preparation of thin layers was modified in order to incorporate zinc dust as reductor for the developing reagent. The proportions were 30 g of silica gel G and 3 g of zinc dust added to 65 cc of rapidly stirred distilled water. Glass plates (200 × 200 mm) were coated by pulling the applicator at a constant speed with a Bodine speed reducer motor. Silica gel G/Zn plates were activated at 110° for 1–2 h before use.

R_G measurements

Approximately fifteen solvent systems were tested on activated silica gel G/Zn plates, using a three component mixture of nitro-, 2,4-dinitro-, and 2,4,6-trinitrotoluenes as the sample. Based on the separation efficiency, two solvent systems were selected: (1) a 15:85 ethyl acetate–petroleum ether mixture (solvent I), and (2) a 25:75 1,2-dichlorethane–petroleum ether mixture (solvent II). When more complex mixtures were studied, solvent I proved to be superior to solvent II. It also became evident that no single solvent or mixed solvent system could resolve all of the fourteen impurities sought; therefore, it was decided to utilize the two best solvents in a two-dimensional method to gain full advantage of each.

To evaluate the efficiency of the two selected solvent systems, R_G values were measured for each of the fourteen components, using α-TNT as the reference compound. Chromatographic conditions and the graphic plot of R_G values in solvents I and II are presented in Fig. 1.

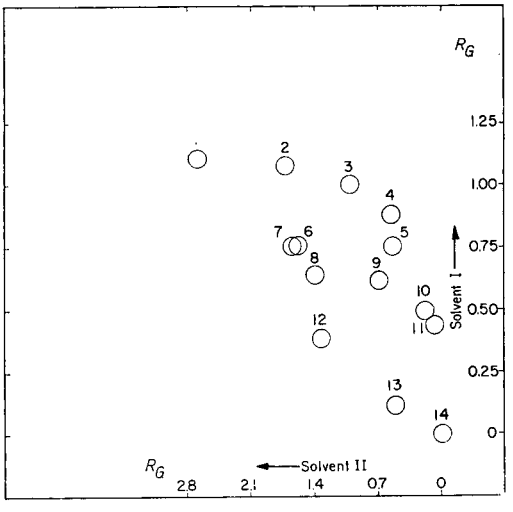

Fig. 1. Graphic plot of R_G values. Chromatographic conditions: Silica gel G/Zn plate, T = 25°. Reference compound: α-TNT. Length of chromatographing (a) Solvent I = 1.1 h (b) Solvent II = 1.0 h. Spray reagent: *p*-diethylaminobenzaldehyde. Compounds: (1) *m*-Nitrotoluene (MNT), (2) 2,5-Dinitrotoluene (2,5-DNT), (3) 2,4,6-Trinitrotoluene (α-TNT), (4) 1,3,5-Trinitrobenzene (TNB), (5) 4,6-Dinitroanthranil (4,6-DNA), (6) 3,5-Dinitrotoluene (3,5-DNT), (7) 2,6-Dinitrotoluene (2,6-DNT), (8) 2,4-Dinitrotoluene (2,4-DNT), (9) 2,4,5-Trinitrotoluene (γ-TNT), (10) 2,4,6-Trinitrobenzaldehyde (TNB_{al}), (11) 2,4,6-Trinitrobenzylalcohol (TNB_{OH}), (12) 3,4-Dinitrotoluene (3,4-DNT), (13) 2,3,4-Trinitrotoluene (β-TNT), (14) 2,4,6-Trinitrobenzoic acid (TNB_{acid}).

Procedure

An aliquot containing 0.5 to 1.0 mg of α-TNT in chloroform is placed about 1.5 in. from the lower right hand corner of an activated silica gel G/Zn plate. The diameter of the spot is kept at about 0.5 in. to minimize broadening of the spot as the sample is chromatographed. The plate is placed in a chromatojar containing 200 cc of solvent I.

Using an ascending technique, the sample is chromatographed for 1.1 h. The plate is exposed to the atmosphere for 3 min. Solvent I is replaced with an equal amount of solvent II, and the sample is rechromatographed at an angle of 90° to the direction of flow of solvent I for an additional hour. The separated impurities are located by spraying *p*-DEAB reagent on the plate. Yellow, brown and red spots develop immediately.

RESULTS AND DISCUSSION

From the graphic plot of R_G measurements, it can be seen that: (1) solvent I will partially or completely separate about nine of the fourteen components, (2) solvent II will tend to group the components according to the degree of nitration, and (3) the combined properties of both solvents will resolve twelve components.

To test the validity of the R_G data (Fig. 1) a synthetic sample containing isomers of nitro-, dinitro-, and trinitrotoluenes and several oxidation–reduction products of α-TNT was chromatographed by the described procedure. Fig. 2 shows the locations of

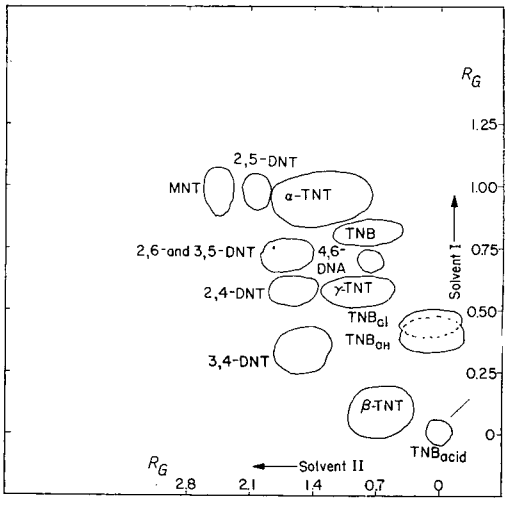

Fig. 2. Two-dimensional separation of TNT impurities.

developed spots on the two-dimensional TLC plate. Only the 2,6- and 3,5-DNT spots are not resolved, as predicted in Fig. 1. Partial separation of trinitrobenzyl alcohol and trinitrobenzaldehyde is obtained; however, the difference in color of the spots helps distinguish the two compounds. Both spot location and color provide evidence to be used to identify components.

Since it is unlikely that all fourteen impurities will be present together in TNT samples, several production grade TNT samples were examined by the TLC procedure. Table I lists the impurities found in different lots of TNT.

From Table I it can be seen that the common impurities found in α-TNT are dinitro- and trinitrotoluenes of which 2,4-DNT is the predominant impurity.

TABLE I

IMPURITIES IN PRODUCTION GRADE α-TNT

Sample	*Impurities found*
A	2,4-DNT, γ-TNT
B	2,4-DNT, γ-TNT
C	2,4-DNT, γ-TNT, β-TNT
D	2,4-DNT, 2,6- or 3,5-DNT, γ-TNT, β-TNT
E	2,4-DNT, 2,6- or 3,5-DNT, 2,5-DNT, γ-TNT

Zone refined α-TNT samples were also analyzed by this chromatographic procedure. Two samples were obtained: (1) the "purified" TNT, and (2) the red-brown zone of accumulated impurities. The latter sample was found to contain three additional impurities not observed in the original sample: traces of 2,5-DNT, trinitrobenzene, and an appreciable amount of an immobile impurity. The zone of "pure" TNT showed traces of 2,4-DNT, γ-TNT, and an immobile impurity.

In making a tentative identification of the immobile impurity it was noted that, although trinitrobenzoic acid (TNB-acid) does not migrate, the color and behavior of the immobile spot does not resemble that of TNB-acid. TNB-acid placed on a silica gel G/Zn plate apparently reacts with the zinc dust in the thin layer and becomes fixed in a concentrated spot.

The immobile impurity from the zone refined TNT sample was isolated from TLC plates in sufficient quantity to obtain an infrared spectrum. Very few characteristic bands could be identified; however, the very low intensity of the carbonyl band would suggest that TNB-acid was not a major component.

The possibility that the immobile impurity was α-nitroTNT was investigated. A solution of α-nitroTNT in toluene, of uncertain age and doubtful purity, was the only authentic sample available. Chromatography of the solution indicated two major components. One of the components was identified as trinitrobenzyl alcohol. The other component, an immobile material, was assumed to be α-nitroTNT. It was found that the infrared spectrum of the immobile component and the original immobile component of α-TNT were identical. It was, therefore, concluded that the immobile impurity observed in various TNT samples must be α-nitroTNT.

The possibility of generating additional impurities in TNT by heating near the melting point for 1 to 5 days, or during zone refining, was investigated. For this reason, some of the likely oxidation–reduction products of α-TNT were included in this work, *e.g.*, trinitrobenzene, 4,6-dinitroanthranil, 2,4,6-trinitrobenzyl alcohol, 2,4,6-trinitrobenzaldehyde, and 2,4,6-trinitrobenzoic acid. It was found that two different grades of TNT showed no appreciable change; however, there may be a gradual increase in the component tentatively identified as α-nitroTNT.

The chemistry of the condensation reaction between nitroaromatics and *p*-DEAB was studied. It was found that one or more nitro groups are reduced to the amine, followed by the condensation of the amine with *p*-DEAB to form a Schiff's base. Although the position and/or number of the nitro groups involved in the reaction is not known conclusively, it is our belief that one nitro group is reduced and involved in the final condensation product. Supporting evidence is as follows: (1) C, H and N analyses of the condensation product with α-TNT extracted from TLC plates are very

close to being correct for a 1:1 ratio of α-TNT to *p*-DEAB; and, (2) direct condensation products formed between *m*- and *p*-nitroaniline and *p*-DEAB are yellow-orange and red, respectively, as are the products formed with 3,5- and 2,5-DNT on TLC plates.

The developing reagent, *p*-DEAB, is similar to the Ehrlich's reagent, *p*-dimethylaminobenzaldehyde (*p*-DMAB); however, with aromatic nitro compounds the nitro group must be reduced to the amine before condensation can occur. Stannous chloride is commonly used as the reductor with *p*-DMAB[5]. It has been our experience that zinc dust can be incorporated directly in the thin layer, giving an efficient, uniform reducing medium. Nitroaromatic compounds recovered from silica gel G/Zn plates without use of the spray reagent showed no change in physical properties as compared with the authentic sample.

The *p*-DEAB/zinc detection system is quite sensitive. One microgram of nitro-, dinitro- and trinitrotoluenes can readily be detected. Approximately 5 μg of the oxidation–reduction products is required to obtain positive test.

Several authentic samples were synthesized for this work, since they were not available commercially or were too expensive to be purchased. The 2,5-DNT and α-TNT were isolated from nitration products. The trinitrobenzyl alcohol was obtained through the hydrolysis of its corresponding trinitrobenzyl bromide. The 4,6-DNA was prepared according to the procedure of SPLITTER AND CALVIN[9]. In all cases, melting point, infrared, and/or C, H and N data were used to confirm the identity of the product.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to H. E. UNGNADE for preparation of certain nitroaromatic compounds, to M. J. NARANJO for C, H and N analyses, and to H. H. CADY for providing zone refined TNT samples. This work was done under the auspices of the U. S. Atomic Energy Commission.

SUMMARY

A two-dimensional thin-layer chromatography method, capable of separating and identifying the impurities in α-trinitrotoluene (α-TNT), is described. A unique developing method is used in which a zinc reductor is incorporated directly in the thin layer. *p*-Diethylaminobenzaldehyde is condensed with the reduced product to obtain the color-developed spot. The impurities commonly occurring in TNT are readily identified by means of this procedure and are found to be the dinitro- and trinitrotoluenes. In addition, an unexpected impurity, α-nitroTNT, was found in one sample of TNT.

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SEPARATION OF 3-METHYLHISTIDINE FROM HISTIDINE BY THIN-LAYER CHROMATOGRAPHY

A RAPID METHOD FOR DETECTING WHALE EXTRACT IN SOUP PRODUCTS

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During our studies on soup products, it was necessary to devise a method for the detection of whale meat extract in these products.

On assessing the β-alanylhistidine dipeptides by published methods[1,2] a marked difference was observed between whale and beef extracts in that whale extract had a much higher total dipeptide content than beef extract.

We have recently received information that POCCHIARI *et al.*[3] have found a substantial difference not only quantitative, but also qualitative, between the dipeptides of whale and beef extracts. While beef extract shows a predominance of carnosine (β-alanylhistidine) and a small amount of anserine, (β-alanyl-1-methylhistidine) whale extract contains not only carnosine, but also a considerable proportion of β-alanyl-3-methylhistidine.

It was therefore necessary to devise a system capable of separating 3-methylhistidine from histidine and a rapid method based on thin-layer chromatography has been adopted.

EXPERIMENTAL

Chromatoplates and solvents

The chromatoplates were prepared according to the method described by LEES AND DE MURIA[4]. The one-dimensional plates (24 × 8.5 cm) were covered with an 0.2 mm layer of Silicagel G (E. Merck, A. G., Darmstadt) and the two-dimensional (15 × 15 cm) with an 0.15 mm layer. They were dried in air for 2 h, without activation in the oven.

Solvent No. 1, methanol–pyridine–water–glacial acetic acid (6:6:4:1 v/v), was used for the one-dimensional run.

Solvent No. 2, phenol–ethanol–water–ammonia (3:1:1:0.1 v/v), was used for the first direction in the two-dimensional run, while solvent No. 1 was used for the second direction.

Developers

A: Polychromatic developer proposed by MOFFAT AND LYTLE[5].

B: Solution containing 0.2 g of ninhydrin and 0.02 g of hydrindantin in absolute ethyl alcohol–glacial acetic acid (80:20 v/v).

0.5 ml of pyridine was added to 100 ml of the reagent prepared as above.

The hydrindantin was prepared according to the method of MOORE AND STEIN[6].

Preparation of 3-methylhistidine

Pure 3-methylhistidine was prepared as follows. Histidine was converted to phthaloyl-histidine by the method of SHEEHAN AND FRANK[7], as these authors applied it in the preparation of phthaloyl-DL-phenylalanine. The phthaloyl-histidine was methylated as described by TALLAN *et al.*[8].

3-Methylhistidine was separated from the solution containing histidine, 1-methyl-histidine and 3-methylhistidine on a column of Amberlite resin IR 120 C.G. fraction C

Fig. 1. Thin-layer chromatography on Silicagel "G". 15 cm run; solvent No. 1. A = tablet containing 15% beef extract; B = 3-methylhistidine; C = tablet containing 12% whale extract.

(50 cm high, 0.9 cm diam.) by the method of MOORE *et al.*[9] for the separation of basic amino acids. The combined fractions containing 3-methylhistidine were desalted on a column (14 cm high, 0.9 cm diam.) of Amberlite resin IR 120 C.G. fraction E (H form).

The resin was washed with 80 ml of distilled water and the 3-methylhistidine was eluted with 50 ml of 1.5 *N* NH_4OH. The flow rate was 60 ml/h.

Preparation of the solutions under examination and chromatographic procedure

The amount of total dipeptide contained in the product under test was determined by the proposed method[2]. A quantity equivalent to 0.075 g of dipeptides was dissolved in

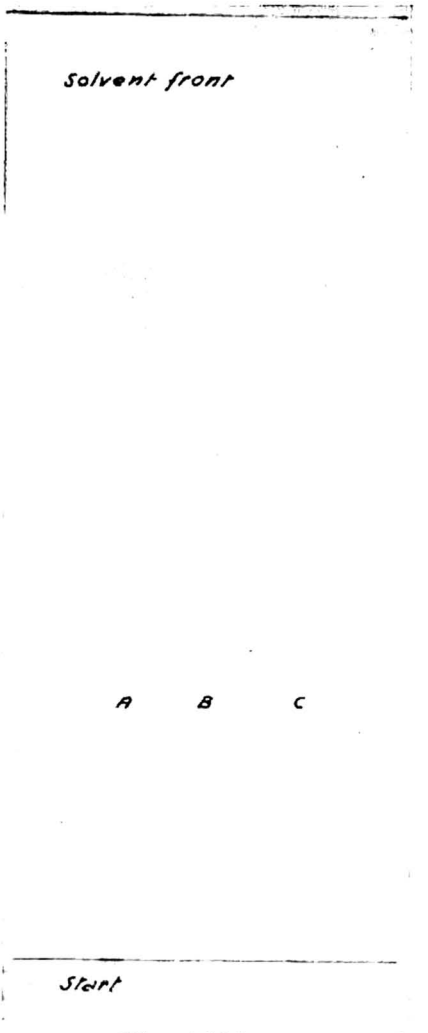

Fig. 2. Thin-layer chromatography on Silicagel "G". 15 cm run; solvent No. 1. A = tablet containing 15% beef extract; B = 3-methylhistidine; C = tablet A + 5% whale extract.

25–30 ml of hot water and then left to cool under running water until the fat had solidified. The liquid was filtered through glass wool into a 100 ml flask containing 5 g of trichloracetic acid dissolved in a little water. The solution was diluted to 100 ml

and, after shaking, allowed to stand for 15 min. All the solution was transferred to an Erlenmeyer flask with a ground glass neck containing 2 g of kieselguhr, shaken for 2 min, and then filtered through a Whatman No. 40 filter paper.

5 ml of the resulting filtered solution was transferred to a 20 ml vial with thick walls, and 5 ml of concentrated HCl was added. The whole was heated on a waterbath for 5 min after which the vial was sealed in a flame. The vial was then placed in a 250 ml flask having a ground glass neck and containing 150–200 ml of methylcellosolve (ethylene glycol monomethyl ether) and refluxed for 2 h. The flask was cooled and the vial opened. The contents were poured into a 100 ml beaker and dried on a boiling water bath until all the HCl was completely eliminated; the residue was taken up in 10 ml of distilled water. The histidines present in the hydrolysate (histidine, 3-methylhistidine, 1-methylhistidine) were precipitated, according to the β-alanylhistidine dipeptide precipitation method[2]. The precipitate, after centrifuging, was dissolved in concentrated HCl. The resulting solution was saturated with H_2S, filtered through a Whatman No. 41 filter paper, and dried on a boiling water bath until all the HCl was completely eliminated. The residue was taken up in 3 ml of distilled water and 3 μl of the solution placed on a one-dimensional chromatoplate at a distance of 1.5 cm from one of the shorter ends. A few μl of a solution containing a suitable concentration of pure 3-methylhistidine were placed on the same plate. The chromatoplate was placed vertically in a suitably sized museum jar containing solvent No. 1. The solvent front was allowed to reach exactly 15 cm from the starting point and the plate was placed in an oven with forced air circulation, at 85° for 30 min. The plate was allowed to cool, the developer was carefully sprayed on to the surface, and the plate was then returned to the oven for the time required by the developer employed (see Table I).

TABLE I

TIMES AND TEMPERATURES USED FOR DETECTION WITH DEVELOPERS A AND B

Developer	*Time (min)*	*Temperature °C*
A	10	110
B	5	85

For two-dimensional chromatography 9 μl of the test solution was placed in the lower left-hand corner of the plate (15 × 15 cm) at 1.5 cm from the two edges. Two lines, crossing at right angles, are drawn in pencil at a distance of 11.5 cm from the lower edge and from the left edge. These lines, which cross the whole adsorbent surface from left to right and from top to bottom respectively, represent the limit of run of the solvents in the two directions and form two thin adsorbent channels. Two spots of standard 3-methylhistidine solution are placed on these channels, at the same height as the unknown sample. Each of the spots, running in a single solvent, will indicate the position reached by 3-methylhistidine in that solvent (see Fig. 4). The chromatoplate was placed vertically in a suitably sized museum jar containing solvent No. 2 for the first run. When the solvent front reached the limiting line, the plate was placed in an oven with forced air circulation at 55° for 45 min. After cooling, the plate, rotated through 90° was placed in another similar museum jar containing solvent No. 1 for the second run. When this solvent also had reached the limiting line, the plate was re-

moved and dried in an oven at 85° for 30 min. After cooling, the whole silica gel surface was sprayed with the developer and then returned to the oven for the time required by the developer used.

RESULTS AND DISCUSSION

During the present work we observed that the histidine spot in one-dimensional chromatography is often accompanied by other spots having only a slightly different R_F, but it is easily recognisable by the violet colour at the edges and the reddish centre with a yellowish halo when the chromatogram is developed with freshly prepared developer A.

Fig. 3. Thin-layer chromatography on Silicagel "G". 15 cm run; solvent No. 1. A = tablet containing 14% beef extract + 1% whale extract; B = 3-methylhistidine; C = tablet containing 15% beef extract.

TABLE II

R_{His} VALUES OF HISTIDINE, 3-METHYLHISTIDINE AND 1-METHYLHISTIDINE

Amino acid	$R_{His} \times 100$	
	in solvent No. 2	*in solvent No. 1*
Histidine	100	100
3-Methylhistidine	153	84
1-Methylhistidine	156	86

The values represent the average of 6 runs.

Still referring to one-dimensional chromatography, 3-methylhistidine together with 1-methylhistidine, if present, is found below histidine (see Table II). However, 3-methylhistidine can readily be distinguished by the intense blue-violet colour, almost bordering on black, and the yellowish halo it assumes when developer A is used. Under these conditions, 1-methylhistidine assumes a light yellow colour, sometimes tinged with green, and thus does not interfere with 3-methylhistidine.

If, however, there are any doubts as to the nature of the methylhistidine separated by this method from histidine, a second chromatogram can be developed with

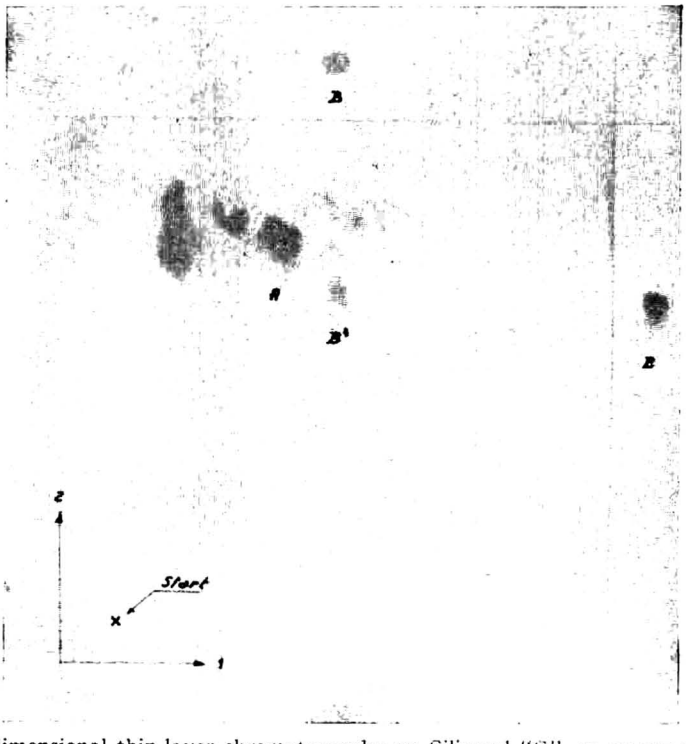

Fig. 4. Two-dimensional thin-layer chromatography on Silicagel "G". 10 cm run; 1st direction solvent No. 2, 2nd direction solvent No. 1. Tablet containing 14% beef extract + 1% whale extract with 3-methylhistidine control. Loading 9 μl. A = histidine; B = 3-methylhistidine (control); B^1 = 3-methylhistidine from whale meat extract.

developer B. In this case, 3-methylhistidine gives a red-brown spot, and 1-methylhistidine a bluish-grey colour tinged with violet.

Relatively large amounts of histidine interfere with the detection of small amounts of 3-methylhistidine by one-dimensional chromatography because of the tail produced by the former when run in solvent No. 1 (see Fig. 3). In such cases, two-dimensional chromatography should be used to separate the 3-methylhistidine from the histidine tail (see Fig. 4). By this means we can detect 1 μg of 3-methylhistidine associated with 15 μg of histidine. Comparison of R_F values and colour reaction with the pure compound will assist identification.

SUMMARY

A rapid method for separating 3-methylhistidine (and possibly 1-methylhistidine) from histidine by means of thin-layer chromatography is described. It can be used to detect 1 μg of 3-methylhistidine associated with 15 μg of histidine. The R_F values relative to histidine (R_{His}), of 3-methylhistidine and 1-methylhistidine in the solvents used, are also given.

The method has been devised in order to detect whale meat extract in soup products, even when it is mixed with beef extract.

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ZUR CHEMISCHEN KLASSIFIZIERUNG VON PFLANZEN

XXIV. UNTERSUCHUNG VON HASCHISCH-INHALTSSTOFFEN DURCH DÜNNSCHICHTCHROMATOGRAPHIE*

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(Eingegangen den 24. Mai 1963)

Kürzlich gelang uns die Trennung der Haschisch-Inhaltsstoffe Cannabidiol (CBD), Cannabinol (CBN) und Tetrahydrocannabinol (THC) durch Verteilungschromatographie an Silikon-imprägniertem Papier[1]. Wir konnten jedoch durch eine Craig-Analyse säulenchromatographisch gereinigter Cannabisextrakte nachweisen, dass papierchromatographisch einheitliches Tetrahydrocannabinol aus mehreren Komponenten besteht[2]. Von anderen Autoren[3,4] wurden noch weitere unbekannte phenolische Substanzen aufgefunden, die eine Verwandtschaft mit den Cannabinolverbindungen vermuten liessen. Ein eingehendes Studium dieser Zusammenhänge ermöglichte uns die Dünnschichtchromatographie, die sich neben anderen inzwischen bekanntgewordenen Analysenverfahren auf der Grundlage der Papierchromatographie[5–7], Säulenchromatographie[5] und Gaschromatographie[8] durch grosse Trennspezifität auszeichnete. Ein wesentlicher Vorteil ist der geringe Zeitbedarf, der die Methode auch für forensische Untersuchungen und Routineanalysen besonders geeignet erscheinen lässt.

Bei der Suche nach einem Chromatographiesystem konnten wir uns auf Literaturerfahrungen stützen. KOLSEK und Mitarb.[4] trennten Cannabisextrakte verteilungschromatographisch mit Benzol an Formamid-imprägniertem Papier, DE ROPP[5] mit Cyclohexan an N,N-Dimethylformamid-gesättigtem Papier. Für die Dünnschichtchromatographie bewährte sich am besten als Träger der stationären Phase Kieselgel G Merck, imprägniert mit N,N-Dimethylformamid und als mobile Phase Cyclohexan. Die Laufgeschwindigkeiten der Substanzen und der Trenneffekt hängen in starkem Masse vom Grad der Imprägnierung ab. Eine gleichmässige Imprägnierung der Adsorptionsschicht wurde durch eine spezielle Arbeitstechnik erreicht, die im experimentellen Teil ausführlich beschrieben ist. Die R_F-Werte der als Gemisch chromatographierten Verbindungen Cannabidiolsäure (CBDS), CBD, CBN und einem synthetischen Isomeren des THC vom Schmp. 62–63° sind Fig. 1 zu entnehmen.

Zur Vergrösserung der Laufstrecke in der Nähe des Startpunktes zurückbleibender Substanzen kann es sich als zweckmässig erweisen, das Fliessmittel mehrmals hintereinander über die Adsorptionsschicht laufen zu lassen (siehe Fig. 1).

Zum Sichtbarmachen der Substanzen auf dem Chromatogramm bewährte sich von den "klassischen" Cannabinolreagentien (siehe Tabelle I, Nr. 1–5) nur das

* XXIII. Mitteilung: H. SIEPER, R. LONGO UND F. KORTE, *Arch. Pharm.*, 296 (1963) 403.

Reagens nach BEAM für den spezifischen Nachweis von CBDS und CBD. Die besten Ergebnisse lieferte Echtblausalz B Merck. Mit diesem Reagens färben sich die Cannabinolverbindungen so empfindlich und differenziert an, dass sie auch ohne die Hilfe

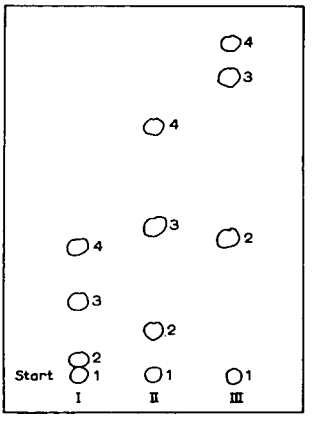

Fig. 1. Dünnschichtchromatogramme der Cannabinolverbindungen nach ein- und mehrmaligem Entwickeln mit Cyclohexan. (1) Cannabidiolsäure; (2) Cannabidiol; (3) Cannabinol; (4) synthetisches Tetrahydrocannabinol, Schmelzpunkt 62–63°. (I) 1 × Cyclohexan; (II) 2 × Cyclohexan; (III) 3 × Cyclohexan.

von Vergleichssubstanzen sicher identifiziert werden können. Die Farben sind sowohl auf dem Chromatogramm als auch nach ihrer Elution vom Adsorbens über Monate ohne sichtbare Veränderung haltbar. Über eine quantitative Bestimmungsmethode auf dieser Grundlage berichten wir an gleicher Stelle.

DÜNNSCHICHTCHROMATOGRAPHISCHE UNTERSUCHUNGEN VON TETRAHYDROCANNABINOLEN

CBDS, CBD und CBN sind definierte kristalline Verbindungen, dagegen fällt Tetrahydrocannabinol bei allen Isolierversuchen als viskoses Öl an. Fast alle bisher synthetisch oder halbsynthetisch dargestellten Tetrahydrocannabinole sind ebenfalls ölige Produkte; ihre Einheitlichkeit muss daher in Frage gestellt werden. Tatsächlich sind Doppelbindungs-, *cis-trans*- und bei genuinen Tetrahydrocannabinolen auch Diastereo-Isomere denkbar. Es schien uns daher von grossem Interesse, alle uns zugänglichen natürlichen und synthetischen Tetrahydrocannabinole dünnschichtchromatographisch auf ihre Einheitlichkeit zu prüfen und untereinander zu vergleichen.

Das Lösungsmittelsystem N,N-Dimethylformamid–Cyclohexan bewährte sich auch für die Trennung der Tetrahydrocannabinole. Eine Anfärbung der Chromatogramme mit Echtblausalz B gestattete die Unterscheidung R_F-identischer Verbindungen. Da geringe Unterschiede in den Farbnuancen schlecht beschrieben werden können, wurden die farbigen Umsetzungsprodukte vom Chromatogramm eluiert und in Lösung spektrophotometriert. Die Wellenlängenmaxima sind in Fig. 2 eingetragen.

Eine Analyse der Chromatogramme in Fig. 2 ergibt:

TABELLE I

FARBREAKTIONEN VON CANNABINOLVERBINDUNGEN NACH CHROMATOGRAPHIE AN KIESELGEL DÜNNSCHICHTEN

Nr.	*Reagens*	*Farbe und Sichtbarkeitsgrenze (µg)*			*Bemerkungen*
		CBD	*CBN*	*THC*	
1	BEAM Reagens[9] (5% äthanol. KOH)	blau 0.5	keine Farbe	keine Farbe	Färbung erst nach 5 Min. Erhitzen auf 105°
2	GIBBS Reagens[10] (2,6-Dibromchinon-4-chlorimid–Isopropylamin)	blaugrün 1	blaugrün 1	grauviolett 1	Farben werden nach 5 Min. Erhitzen auf 105° grau
3	GHAMRAWY Reagens[11] (*p*-Dimethylaminobenzaldehyd–H_2SO_4)	braunrot 0.5	braunrot 1	braunrot 1	Färbung erst nach 15 Min. Erhitzen auf 105°
4	DUQUENOIS Reagens[12] (Vanillin–Acetaldehyd–HCl)	violett 0.5	violett 10	violett 1	
5	BLACKIE Reagens[13] (Benzaldehyd–*sek.*-Butanol)	keine Farbe	keine Farbe	keine Farbe	
6	PAULY Reagens (diazot. Sulfanils.)	ockergelb 0.5	ockergelb 0.5	ockergelb 0.5	
7	Diazot. *p*-Nitroanilin	ockergelb 1	orange 0.5	ockergelb 1	
8	2,6-Dichlorchinonchlorimid (1% in Äthanol)	violett 1	blau 1	violett 1	Farben werden nach 5 Min. Erhitzen auf 105° braunrot
9	Echtblausalz B Merck	orange 0.01	violett 0.01	rot 0.01	

Chromatogramm 1: Das synthetische Tetrahydrocannabinol nach ADAMS[14] besteht aus mindestens 3 Isomeren, von denen, wie berichtet[2], durch Craig-Verteilung zwei kristallin erhalten wurden (Schmp. 128° und 62–63°). Ein drittes Isomere vom Schmp. 86–87° wurde in sehr geringen Mengen bei einer wiederholten Gegenstromverteilung isoliert.

Chromatogramm 2: Das Tetrahydrocannabinol nach TODD[15] besteht aus mindestens 2 Isomeren, die mit keinem der Syntheseprodukte nach ADAMS identisch sind.

Chromatogramme 3–5: Cannabidiol lässt sich säurekatalytisch mit verschiedenen Reagentien zu Tetrahydrocannabinolen unterschiedlicher Drehwerte cyclisieren. Die nach ADAMS mit äthanolischer HCl[16] und Pyridinhydrochlorid[16] dargestellten CBD-Isomerisierungsprodukte wurden chromatographisch je in 3 bzw. 2 Isomeren aufge-

trennt, das Umsetzungsprodukt mit *p*-Toluolsulfonsäure[17] war chromatographisch einheitlich, konnte jedoch auch nach Gegenstromverteilung nicht zur Kristallisation gebracht werden.

Chromatogramme 6 und 7: In einer früheren Mitteilung[2] berichteten wir über die Isolierung sehr geringer Mengen eines Tetrahydrocannabinol-Isomeren. Der Schmelzpunkt der nicht umkristallisierten Verbindung wurde mit 120–125° angegeben. In der Zwischenzeit gelang es, das Produkt durch Umkristallisieren zu reinigen und den

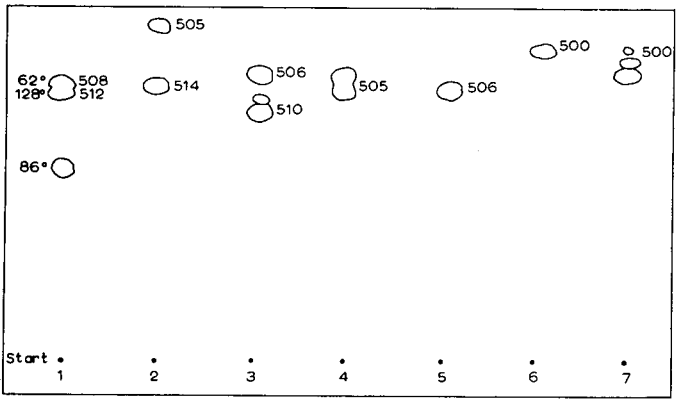

Fig. 2. Dünnschichtchromatogramme von Isomeren des Tetrahydrocannabinols. (1) synthetisch nach ADAMS; (2) synthetisch nach TODD; (3) CBD-Isomerisierungsprodukt mit äthanolischer HCl; (4) dgl. mit Pyridinhydrochlorid; (5) dgl. mit *p*-Toluolsulfonsäure; (6) genuin, Schmelzpunkt 146°; (7) genuin in Haschisch. Die Zahlen neben den Chromatogrammflecken bedeuten Wellenlängenmaxima, photometrisch bestimmt nach Besprühen der Chromatogramme mit Echtblausalz B und Elution der Farbstoffe mit Methanol.

Schmelzpunkt mit 146° genau zu bestimmen. Wie man aus dem Chromatogramm ersieht, ist diese Verbindung mit keinem der synthetischen und halbsynthetischen, dagegen mit einem der Tetrahydrocannabinole des Haschischextraktes (siehe unten) R_F- und farbidentisch.

DÜNNSCHICHTCHROMATOGRAPHISCHE UNTERSUCHUNG VON CANNABISEXTRAKTEN

Cannabis- und Haschischextrakte verschiedener Provenienz wurden dünnschichtchromatographisch in die Cannabinolverbindungen und eine grössere Anzahl weiterer phenolischer Komponenten getrennt (siehe Fig. 3).

Die in Fig. 3 zusammengestellten Chromatogramme wurden vergleichend ausgewertet und liessen folgende allgemeinen Zusammenhänge erkennen:

(1) Substanzen gleichen R_F-Wertes geben mit Echtblausalz B gleiche Anfärbung und sind daher vermutlich identisch.

(2) Die Substanzen V, VI und VII (in der linken Randspalte bezeichnet mit THC I, THC II und THC III), wurden in wechselnden Mengen auf allen Chromatogrammen wiedergefunden. Es sind, wie aus analytischen Daten eines durch Craig-Verteilung isolierten Gemisches von V, VI und VII hervorgeht*, Isomere des Tetra-

* Über eine Methode zur Trennung genuiner Tetrahydrocannabinole in präparativen Mengen berichten wir an anderer Stelle.

hydrocannabinols. Die Hauptmenge liegt bei THC I. Das in Haschischextrakten in nur geringen Mengen oder gar nicht nachweisbare THC III erwies sich als R_F- und farbidentisch mit dem aus deutschem Hanf isolierten Isomeren vom Schmelzpunkt 146° (siehe auch Fig. 2, Chromatogramme 6 und 7).

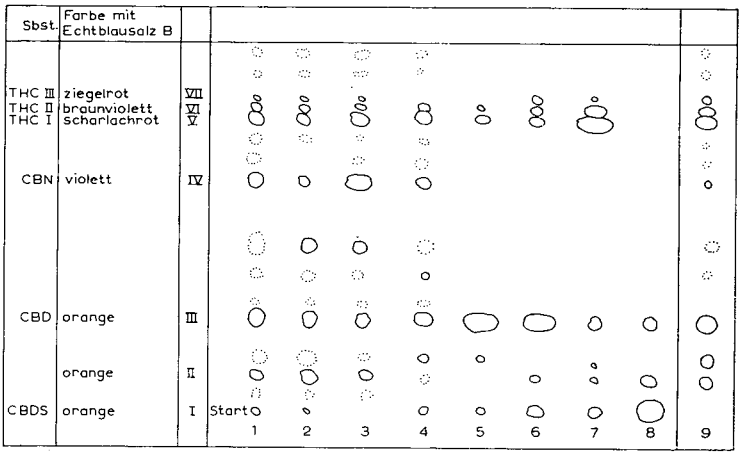

Fig. 3. Dünnschichtchromatogramme von Haschischextrakten und einem CBD-Pyrolyseprodukt. (1)–(4) Haschisch orientalischer Provenienz; (5) *Cannabis non indica*, Anbau Karlsruhe 1956; (6) dgl. 1957; (7) *Cannabis indica*, Karlsruhe 1957; (8) *Cannabis non indica*, Karlsruhe 1962; (9) CBD-Pyrolyseprodukt. Die gestrichelt eingezeichneten Flecke sind nur schwach sichtbar.

(3) In Haschischextrakten orientalischer Provenienz (Chromatogramme 1–4) ist kein CBDS, dagegen sind CBD, CBN und THC I, THC II und THC III in wechselnden Mengen nachweisbar. *Cananbis non indica* deutschen Anbaus (Chromatogramm 5) enthält viel CBD, wenig CBDS und THC, aber kein CBN. Diese Beobachtungen bestätigen die Literaturergebnisse[18], nach denen die Ausbildung cannabinolartiger Verbindungen nach Art und Menge überwiegend vom Reifezustand der Pflanze abhängt, wobei allmählich CBDS in CBD, THC und schliesslich CBN übergeht. Die Chromatogramme 6 und 7 demonstrieren dagegen eine Varietätsabhängigkeit der chemischen Zusammensetzung. *Cannabis indica* enthält viel mehr THC und weniger CBD als *Cannabis non indica*, das unter gleichen Wachstumsbedingungen kultiviert wurde.

(4) Die auf dem Chromatogramm zwischen CBDS und CBD sichtbare Substanz II zeigt die gleichen Farbreaktionen mit Echtblausalz B und dem Reagens nach BEAM wie CBD. In einem Pyrolyseprodukt des CBD (siehe unten) ist diese Substanz gleichfalls nachweisbar. Wir nehmen daher an, dass es sich um ein Isomerisierungsprodukt des Cannabidiols handelt.

(5) Sehr schonend extrahierter frischer Hanf enthält nur CBDS und CBD (Chromatogramm 8). Dies deutet darauf hin, dass nicht nur THC und CBN, sondern auch alle anderen chromatographisch nachgewiesenen Phenole Folgeprodukte der Diole sind. Diese Vermutung wird durch einen einfachen aber aufschlussreichen Versuch bestätigt. Pyrolytisch mehrere Stunden bei 250° unter der Einwirkung von wenig Luftsauerstoff und katalytischen Mengen Schwefelsäure behandeltes Cannabidiol lässt sich in 13 Flecke auftrennen (siehe Chromatogramm 9). Diese sind R_F- und farbidentisch mit den Komponenten der Haschischextrakte.

EXPERIMENTELLER TEIL

Es werden folgende Abkürzungen benutzt: CBDS = Cannabidiolsäure, CBD = Cannabidiol, THC = Tetrahydrocannabinol, CBN = Cannabinol. Alle zur Verwendung kommenden Lösungsmittel werden sorgfältig durch Destillation gereinigt.

Herstellung der Dünnschicht

Eine homogene Suspension von 30 g Kieselgel G, Merck, in 70 ml destilliertem Wasser wird nach der Methode von STAHL[19] mit dem Streichgerät der Fa. C. Desaga, Heidelberg, in einer 250 μ dicken Schicht auf 5 Glasplatten von 20 × 20 cm oder 20 Glasplatten von 5 × 20 cm aufgebracht, 10 Min. an der Luft liegengelassen und 1 Stunde bei 105° getrocknet. Die Platten werden in einem Exsikkator mit Blaugel aufbewahrt.

Imprägnierung der Dünnschicht

14.5 cm von der unteren Plattenkante entfernt wird die Adsorptionsschicht mit einem 3 mm breiten Spatel durch einen waagerechten Strich unterbrochen. Die Platte wird in einen 0.5 cm hoch mit einer Mischung von 60 Vol. % N,N-Dimethylformamid und 40 Vol. % CCl_4 gefüllten dampfdicht verschliessbaren Glastrog von 22 cm Länge, 22 cm Höhe und 5 cm Breite gestellt, an dessen Innenwänden zur Kammersättigung Filtrierpapierstreifen von 20 × 20 cm adhäsiv fixiert sind und in die Flüssigkeit eintauchen. Das Imprägnierungsmittel steigt gleichmässig über die Dünnschicht hoch und erreicht die Markierung in ca. 1.5 Stunden. Man lässt die Platte noch mindestens 15 Min. in dem Trog. Auf den Trenneffekt und die Laufgeschwindigkeit der Substanzen hat es keinen Einfluss, wenn die Platte noch längere Zeit (Stunden oder Tage) in der Flüssigkeit stehenbleibt. Die Chromatographie lässt sich so zweckmässig auch über Nacht vorbereiten.

Chromatographierte Substanzen

Cannabidiolsäure. Aus dem Diacetylester von SCHULTZ UND HAFFNER[20] gewonnen durch Verseifung mit $NaHCO_3$ nach SANTAVY und Mitarb.[18].

Cannabidiol. Schmp. 65–66°, $[\alpha]_D^{20} = -130°$ in Äthanol. Isoliert aus *Cannabis sativa non indica* durch Chromatographie und Craig-Verteilung[2].

Cannabinol. Schmp. 75–76°. Dargestellt aus Tetrahydrocannabinol nach ADAMS UND BAKER (siehe unten) durch Dehydrierung[21].

Tetrahydrocannabinol. (1) Synthetisch nach ADAMS UND BAKER[14]: Durch Kondensation von Olivetol mit 1-Methyl-cyclohexanon-(3)-carbonsäure-(4)-äthylester und anschliessende Umsetzung mit CH_3MgJ wird ein fast farbloses hochviskoses Öl gewonnen. Eine Gegenstromverteilung über 224 Stufen mit dem Lösungsmittelsystem *n*-Hexan–Methanol–Wasser (10:9:1) führt zur Isolierung eines Isomeren vom Schmp. 128° (farblose Nadeln), 62–63° (farblose Prismen) und 86–87° (farblose Nadeln). U.V.-Absorption des noch nicht beschriebenen Isomeren vom Schmp. 86–87°: λ_{max} 291 mμ (log ε = 3.48), 250 mμ (3.56), 258 mμ (4.25), 222 mμ (4.59) und 218 mμ (4.62); (gemessen in Methanol).

(2) Synthetisch nach TODD und Mitarb.[15]: Durch Kondensation von Olivetol mit Pulegon entsteht ein fast farbloses hochviskoses Öl, Drehwert $[\alpha]_D^{20} = +81°$.

(3) CBD-Isomerisierungsprodukte: Nach ADAMS und Mitarb. wird durch Umsetzung von kristallinem Cannabidiol mit äthanol. HCl[16] ein fast farbloses Öl vom

Drehwert $[\alpha]_D^{25} = -173°$, mit Pyridinhydrochlorid[16] ein schwach gelbes Öl vom Drehwert $[\alpha]_D^{23} = -246°$, mit *p*-Toluolsulfonsäurechlorid[17] ein farbloses Öl vom Drehwert $[\alpha]_D^{22} = -260°$ erhalten. Die Produkte sind Beam-negativ.

(4) Genuin: Isoliert aus *Cannabis sativa non indica* deutschen Anbaus durch Chromatographie und Craig-Verteilung[2]. Nach Umkristallisieren aus *n*-Heptan prismatische Säulen von Schmp. 146°. U.V.-Absorption: λ_{max} 212, 273 und 280 mμ (in Methanol).

Alle unter 1–4 beschriebenen Produkte zeigten die für Tetrahydrocannabinol zu erwartenden C,H-Verbrennungswerte, waren Beam-negativ und gaben sehr ähnliche I.R.-Spektren.

Extrakte

Für die Herstellung von Extrakten werden eingesetzt: gepresstes Haschischmaterial orientalischer Provenienz (Chromatogramme 1–4 in Fig. 3); luftgetrocknete und bei Raumtemperatur gelagerte weibliche Triebspitzen von *Cannabis sativa non indica*, Anbau Karlsruhe 1956 und 1962 (Chromatogramme 5 und 8), *Cb. non indica* und *Cb. indica*, Anbau Karlsruhe 1957 (Chromatogramme 6 und 7). Je 10 g Pflanzenmaterial werden in 50 ml Petroläther (Sdp. 40–60°) suspendiert und bei Raumtemperatur unter Stickstoff mit einem Ultraturrax zerkleinert und extrahiert. Es wird filtriert und der Rückstand 5 mal mit je 30 ml Petroläther wie oben beschrieben nachextrahiert. Die vereinigten Filtrate werden unter Stickstoff bei 20° in einem Rotationsverdampfer zur Trockne eingedampft.

CBD-Pyrolyseprodukt (Chrom. 9 in Fig. 3).

In einem offenen Schmelzpunktröhrchen werden 10 mg Cannabidiol 2 Stunden lang auf 250° erhitzt. Während dieser Zeit wird ein langsamer durch eine Waschflasche mit konz. H_2SO_4 geleiteter Stickstoffstrom aufgeblasen. Das Reaktionsprodukt ist fast farblos und in Petroläther löslich.

Auftragen der Substanzen und Chromatographie

Die für die Chromatographie vorbereitete Platte wird an der Luft bei Zimmertemperatur zum gleichmässigen Abdunsten von CCl_4 und überschüssigem N,N-Dimethylformamid so lange liegengelassen, bis sich die zunächst leicht transparente, graue Adsorptionsschicht nur noch schwach, aber noch deutlich sichtbar, von der nicht imprägnierten Schicht abhebt. Dazu werden je nach Raumtemperatur und Luftfeuchtigkeit $^3/_4$ bis $1^1/_4$ Stunden benötigt. Während dieser Zeit werden 2.5 cm oberhalb der unteren Schichtkante im Abstand von 1.5–2 cm je 2 μl 0.1 %ige Lösungen von CBDS, CBD, CBN und THC (Schmelzpunkt 62–63°) bzw. 1–5 %ige Lösungen der Cannabis- und Haschischextrakte in *n*-Hexan aufgetragen und mit Cyclohexan bis zur Markierung chromatographiert. Die Chromatographiedauer beträgt 20–30 Min. Um zu prüfen, ob die zu trennenden Komponenten weit genug gelaufen sind, wird die Adsorptionsschicht mit einer Glasplatte so abgedeckt, dass nur eine schmale Randzone mit einem Vergleichschromatogramm offenbleibt. Diese wird, wie unten beschrieben, mit einer Reagenslösung besprüht und gegebenenfalls von der Glasplatte abgestreift. Vor jeder erneuten Chromatographie lässt man die Platte zum Abdunsten des Fliessmittels 3 Min. an der Luft liegen. Die Chromatogramme der Cannabinolverbindungen nach ein- und mehrmaliger Chromatographie sind in Fig. 1, der Tetrahydrocannabinole in Fig. 2, der Haschisch- und Cannabisextrakte in Fig. 3 dargestellt.

Sichtbarmachen der Flecke und Dokumentation

Die Chromatogramme werden kurze Zeit an der Luft getrocknet und in horizontaler Lage mit einem der nachfolgenden Reagenslösungen so besprüht, dass die gesamte Adsorptionsschicht gleichmässig durchfeuchtet wird. Farbreaktionen und untere Nachweisgrenzen sind Tabelle I und Fig. 2 zu entnehmen.

1. Reagens nach Beam. 5% KOH in 99% Äthanol. CBDS und CBD, in Haschischextrakten und dem CBD-Pyrolyseprodukt, auch die Komponente II in Fig. 3 färben sich nach 5 Min. Erhitzen auf 105° blauviolett an, alle anderen phenolischen Komponenten bleiben farblos. Die Intensität der Anfärbung kann durch wiederholtes Besprühen und Erhitzen gesteigert werden.

2. Echtblausalz B. 15 mg Echtblausalz B Merck, Di-*o*-anisidintetrazoliumchlorid, werden in 20 ml *N*/10 NaOH kalt gelöst und wie oben aufgesprüht. CBDS, CBD und die Komponente II in Fig. 3 färben sich orange, Tetrahydrocannabinole und alle Cannabinol-fremden phenolischen Komponenten färben sich rot bis violett, CBN färbt sich dunkelviolett an.

Die Chromatogramme lassen sich nach Besprühen mit einer Kunststoffdispersion (z.B. Neatan Merck) von der Glasplatte abziehen und unter Klarsichtfolie im Dunkeln mindestens ein Jahr lang ohne sichtbare Veränderung der Farbnuance und -intensität aufbewahren.

DANK

Für die Überlassung von Cannabidiolsäurediacetat sind wir Herrn Prof. Dr. O. E. SCHULTZ vom Pharmazeutischen Institut der Universität Kiel zu Dank verpflichtet. Unser besonderer Dank gilt der Fa. Dr. W. Schwabe, Karlsruhe für den wiederholten Anbau von Cannabis und dem Bundeskriminalamt Wiesbaden, das uns grosszügig grössere Mengen Haschisch zur Verfügung stellte. Der Deutschen Forschungsgemeinschaft danken wir für eine Sachbeihilfe.

ZUSAMMENFASSUNG

Die Haschisch-Inhaltsstoffe Cannabidiolsäure, Cannabidiol, Cannabinol und mehrere Isomere des Tetrahydrocannabinols werden an Dimethylformamid-imprägnierten Kieselgel-Dünnschichten getrennt und mit Phenolreagentien spezifisch angefärbt. Die Chromatogramme von synthetischen und natürlichen Tetrahydrocannabinolen, Haschischextrakten und Pyrolyseprodukten des Cannabidiols werden vergleichend gegenübergestellt und diskutiert.

SUMMARY

The hashish constituents cannabidiolic acid, cannabidiol, cannabinol and several isomers of tetrahydrocannabinol were separated by thin-layer chromatography on silica gel impregnated with dimethylformamide, and specifically detected with phenol reagents. The chromatograms of synthetic and natural tetrahydrocannabinols, hashish extracts and the pyrolytic products of cannabidiol are compared and discussed.

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THE SEPARATION AND IDENTIFICATION OF PLANT PHOSPHOLIPIDS AND GLYCOLIPIDS BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY*

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INTRODUCTION

Recent development in thin-layer chromatography and its application to the analysis of lipids have proved to be very effective for the separation of animal phospholipids and cerebrosides[1,2]. In contrast, plant lipids contain other distinctive glycolipids of similar physical properties such as galactosyl glycerides, digalactosyl glycerides[3,4], polygalactosyl glycerides[5], phosphatidyl glycerol as well as the sulfolipid of BENSON[6]; their separation by chromatography is consequently a much more difficult operation. Nevertheless, clean separations of these plant lipids have been achieved by two-dimensional thin-layer chromatography. This communication sets forth the technique and its application to the fractionation of complex mixtures of polar plant lipids. Identification techniques and the value of thin-layer chromatography in autoradiography are also described.

EXPERIMENTAL

Lipid extracts

Total lipids were extracted from alfalfa leaves, potato leaves and tubers, and ^{14}C-containing *Chlorella* by homogenizing the tissue in hot 80 % ethanol for 2 min and extracting the residue with acetone, followed by a mixture of chloroform–methanol (2 : 1). The combined extracts were filtered, concentrated *in vacuo* and the lipid concentrate taken up in chloroform and washed thoroughly with water to remove non-lipid material. Stock solutions of lipid in chloroform were adjusted so that 2.5 μl solution was the equivalent of 1 mg fresh tissue.

Commercial soya lecithin, egg lecithin and animal kephalin were also used as reference material in chloroform–methanol solution at a concentration of 10 mg/ml.

Apparatus and solvents

Glass plates, 20 cm × 20 cm, were coated to a depth of 0.25 mm with Silica Gel G (Merck, Darmstadt) applied in a suspension of 25 g gel in 55 ml distilled water. The

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plates were activated by a final drying at 100° for 30 min. The chromatography jars were lined with filter paper to ensure saturation of the enclosed space with solvent vapors.

Three different solvent systems were used for the development of thin-layer chromatograms:

Solvent A: chloroform–methanol–water (65:25:4)[1].
Solvent B: diisobutyl ketone–acetic acid–water (80:50:10)[7].
Solvent C: hexane–diethyl ether–acetic acid (90:10:1)[8].

Spotting and development

For one-dimensional thin-layer chromatograms, 1–10 μl quantities of lipid extract were spotted in the conventional manner and allowed to develop in equilibrated jars until the solvent front had climbed a distance of 17 cm. The times required for development were: 2 h for solvent A, 3 h for solvent B and only 1 h for solvent C.

Two-dimensional thin-layer chromatograms were prepared by applying the lipid sample as a single spot at the right-lower corner of the plate and using solvents A and B as developing systems. At the end of the first run in solvent A, the plates were removed from the chromatography jars, dried for 30 min and then placed in the second solvent after clockwise rotation through 90°.

Detection of spots

After the developing solvents were removed, the lipids were detected on the chromatogram in the following manner.

A. General methods

(1) *Iodine vapor*[9]. The spots were revealed by iodine vapors and were outlined with a pencil. The iodine was then removed *in vacuo*, and the plates used again with more specific reagents.

(2) *Aqueous 20% perchloric acid*. The chromatoplates, after preheating at 100° for 5 min, were sprayed with an aqueous 20% perchloric acid solution. Most lipids appeared as brown spots, which could be intensified by further heating. Two spots, U_1 and U_2, tentatively designated as esterified sterol glycoside and sterol glycoside respectively gave atypical reddish spots.

B. More specific methods

(1) *Ninhydrin*. The chromatoplates were stained with 0.2% ninhydrin in 99% *n*-butanol and 1% pyridine for phosphatidyl ethanolamine and phosphatidyl serine. When the chromatoplates were heated at 100° prior to spraying, no further heating was necessary for the appearance of the spots.

(2) *Modified Dragendorff reagent*[10]. Choline-containing phospholipids appeared immediately on spraying with this reagent. Galactolipids also yield orange spots, but these appeared more slowly.

(3) *Perchloric acid–Schiff reagent*[11]. The chromatoplates were damply sprayed with an aqueous 0.5% sodium periodate solution. After 5 min, the chromatoplates,

while still wet, were exposed to SO_2 gas to remove excess periodate and then stained with an aqueous 0.5% *p*-rosaniline solution, freshly decolorized by bubbling SO_2 gas. After full development of the color (about 1 h), the background can be lightened by spraying with a 1% perchloric acid solution. All phospholipids and glycolipids appeared as well defined blue and purple spots on a yellowish background.

The sequence of sprays that we have followed was: the chromatoplates were first exposed to iodine vapors. The spots were outlined, iodine removed and the chromatoplates stained with periodate–Schiff reagents to detect glycolipids. Alternatively, after the iodine vapor, the chromatoplates were sprayed with ninhydrin to detect the amino-phospholipids, then with Dragendorff reagent to detect choline-containing phospholipids and finally with perchloric acid to char all the lipid spots.

C. Method for radioactive lipids

Radioactive lipids gave excellent autoradiograms when the chromatoplates were placed powder-side down on Kodak, single coated, X-ray film. No damage to the plate or loss of radioactive lipid was encountered. Because the spots on thin-layer plates are so compact, an exposure of only 2 days was required for clear pictures of spots containing about 2 mμC ^{14}C.

Identification by alkaline hydrolysis

A modified Dawson procedure[5,12] using toluene–pyridine (10:1) as solvents and 0.1 *N* methanolic potassium hydroxide followed by neutralization with Dowex 50 (H^+), was used to deacylate lipids that could not be fully identified on thin-layer plates by spray reagents. Preparative, one-dimensional, thin-layer chromatography in solvents A or B furnished material for the hydrolysis. Two-dimensional paper chromatography[12] was employed to separate the glycotic portions of the unknown lipids. Spots were identified by a comparison of R_F values[13–15].

RESULTS AND DISCUSSION

One-dimensional thin-layer chromatography of the plant lipid extracts gave only incomplete resolution of the components. This inferior performance was confirmed by hydrolysis and paper chromatography of allegedly pure spots. Resolution of all mixtures into single components was, however, achieved by two-dimensional chromatography using solvents A and B (Fig. 1). Phospholipids separated suitably in solvent A, but travelled much less rapidly in solvent B. The latter solvent was, however, more effective for the separation of glycolipids and sterol glycosides. This two-dimensional system effected therefore a clean separation of all lipid components.

It has not been possible to identify all components on the chromatograms with the various chemical sprays. All lipids were located either with iodine vapors, 20% perchloric acid or periodate–Schiff spray reagents, but these reagents lacked specificity for distinguishing phosphatidyl inositol from phosphatidyl glycerol or phosphatidic acid, and the monogalactosyl glyceride from the digalactosyl glyceride and the polygalactosyl glycerides. Only phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline were identified with certitude with the aid of ninhydrin or Dragendorff reagents. Because of these limitations it has been necessary to insure

unambiguous identification through hydrolysis and comparison of R_F values on paper chromatography of their deacylated products.

The above scheme has been used to separate potato tuber lipids into 17 components of which 12 have been identified. Their R_F values, relative intensities and re-

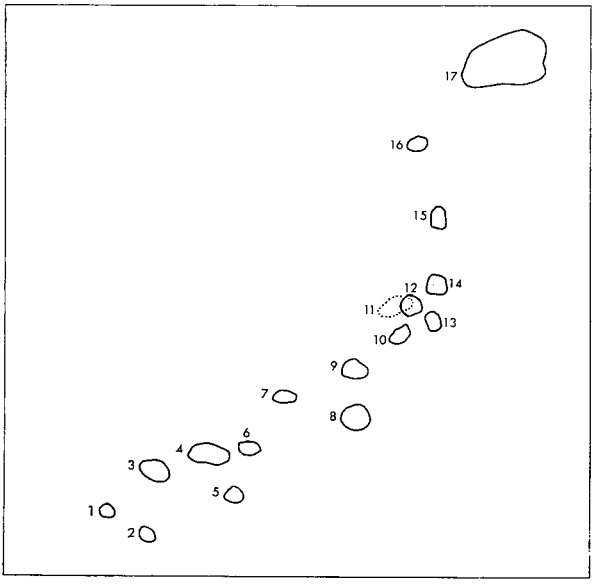

Fig. 1. Tracing of a two-dimensional thin-layer chromatogram of phospholipids and glycolipids from potato tuber extracts. The chromatogram was developed in chloroform–methanol–water (65:25:4) (solvent A) in the x-direction and then in diisobutyl ketone–acetic acid–water (80:50:10) (solvent B) in the y-direction. The spots were detected with iodine, followed by staining with 20% perchloric acid. The tracing was made from a contact photograph. Numbers refer to those in Table I.

sponse to various chemical sprays are indicated in Table I. Iodine was found to be the most sensitive chemical to detect all spots on chromatograms, but it failed to detect compound 12. Perchloric acid, although generally less sensitive than iodine, produced a reddish color, characteristic of sterols, with compounds 12 and 15. Hydrolysis of compound 15 in 0.1 N methanolic potassium hydroxide produced fatty acids and a derivative with the same R_F value as compound 12. This suggests that compound 15 is similar to compound 12, but has in addition a fatty acid portion. Further degradation of compound 15 by acid hydrolysis freed sterols and sugars. On the basis of these observations, compounds 12 and 15 have been tentatively identified as sterol glycoside and esterified sterol glycoside respectively. Details of their chemical characterization will be reported later.

When the above mixture of plant lipids was analysed by the procedure of KIDMORE AND ENTENMAN[2], only 12 of the 17 compounds were separated.

SUMMARY

The thin-layer chromatographic properties of plant phospholipids and glycolipids have been determined. Two-dimensional thin-layer chromatography, using chloro-

TABLE I

R_F VALUES AND RELATIVE INTENSITIES OF PLANT PHOSPHOLIPIDS AND GLYCOLIPIDS

No.	Components	$R_F \times 100$ in solvents[a]		Spray reagents[b]					Rel. intensity[c]	$R_F \times 100$ in PW[d]	
		A	B	A_1	A_2	B_1	B_2	B_3		Found	Reported[4]
1	Phosphatidyl serine	14	6	+	+	+			w		
2	Unidentified	21	2	+	+	—		+	w		
3	Phosphatidyl inositol	23	14	+	+	—		+	m	9	12
4	Phosphatidyl choline	33	18	+	+	—	+	+	s		
5	Unidentified	36	9	+	+			+	w		
6	Sulfolipid	42	22	+	+	—	—	+	m	18	18
7	Phosphatidyl glycerol	48	30	+	+	—		+	m	42	36
8	Digalactosyl glyceride	62	25	+	+	—	+	+	vs	48	46
9	Phosphatidyl ethanolamine	62	35	+	+	+	—	+	s		
10	Unidentified	71	41	+				+	m		
11	Unidentified	70	47					+	vw		
12	Unknown U_2[e]	73	48	—	r	—	—	+	s		
13	Unidentified	77	44		+	—		+	m		
14	Monogalactosyl glyceride	77	51	+	+	—	+	+	vs	62	62
15	Unknown U_1[f]	78	65	+	r	—	—	+	s		
16	Phosphatidic acid	74	79	+	+	—	—	+	m		
17	Neutral lipids	92	97	+	r	—	—	+	vs		

[a] R_F values of intact lipids.
[b] + = positive spot; — = negative spot; r = reddish spot.
[c] vw = very weak; w = weak; m = medium; s = strong; vs = very strong. This is applicable to all spray reagents.
[d] R_F values of compounds after deacylation. PW = phenol saturated with water.
[e] Tentatively identified as sterol glycoside.
[f] Tentatively identified as esterified sterol glycoside.

form–methanol–water (65:25:4) and diisobutyl ketone–acetic acid–water (80:50:10) solvent systems, yielded separation of all the major lipids, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl inositol, digalactosyl glyceride, monogalactosyl glyceride and the sulfolipid, as well as other minor lipids. Two partially characterized glycolipids, probably sterol glycosides, were found in the potato lipid extracts.

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ZUR PAPIERCHROMATOGRAPHISCHEN AUFTRENNUNG VON PFLANZENLIPIDEN

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Die Analyse von Pflanzenlipiden und die Untersuchung ihrer funktionellen Aufgaben ist schwierig durchzuführen, da vor einer Bestimmung der einzelnen Stoffe ihre Abtrennung aus den sehr komplex zusammengesetzten Lipidextrakten erforderlich ist. Hierzu standen in der Vergangenheit eine Reihe präparativ-chemischer Methoden zur Verfügung, deren Genauigkeit für analytische Zwecke jedoch nicht ausreichend war[1,2]. Einen Fortschritt bedeutete daher die Einführung der Chromatographie an Kieselgelsäulen, obwohl auch hierbei keine voll befriedigenden Auftrennungen erzielt werden konnten[3].

Die Ergebnisse, die durch Anwendung üblicher papierchromatographischer Methoden erhalten wurden, waren ebenfalls nicht sehr ermutigend. BENSON UND MARUO[4] konnten zwar Lipidextrakte aus Pflanzen in vier verschiedene Hauptfraktionen auftrennen; trotzdem wurde in dieser wie auch in späteren Arbeiten die zuerst von DAWSON[5] angewandte Deacylierungstechnik vorgezogen, bei der die nach schonender hydrolytischer Abspaltung der Fettsäurereste entstehenden wasserlöslichen Spaltprodukte chromatographisch identifiziert wurden. Aus dem Ergebnis dieser Bestimmungen wurde dann auf die Zusammensetzung der nativen Lipide geschlossen.

Mit dieser Methode konnten in verschiedenen Pflanzen zwei neue Phosphatide, drei Glykolipide und ein Sulfolipid nachgewiesen werden[4,6–8]. Ein Nachteil des Verfahrens besteht jedoch darin, dass zur Vermeidung von Reaktionen, die über die Abspaltung der Fettsäuren hinausgehen, die Hydrolysebedingungen ausserordentlich genau kontrolliert werden müssen und dass Diesterverbindungen die gleichen Spaltprodukte wie die entsprechenden Lysoverbindungen ergeben[9].

Die von MARINETTI und Mitarb.[10,11] entwickelte papierchromatographische Technik an mit Kieselgel imprägniertem Papier wurde bei der Untersuchung von Phosphatiden tierischer Herkunft in vielen Fällen erfolgreich angewandt[12]. Bei der Auftrennung der *Chlorella*-Lipide liessen sich auf diesem Wege zunächst keine befriedigenden Resultate erzielen (FERRARI, nach Zit. 14). KATES[13,14] konnte jedoch durch eine zweimalige Entwicklung mit dem gleichen Lösungsmittel den Trenneffekt so weit steigern, dass der Nachweis von mindestens 11 Lipidkomponenten im Blattextrakt von *Phaseolus multiflorus* gelang. Allerdings trat auch hier noch eine Überlagerung einzelner Flecke auf.

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Bei der Untersuchung der Lipide verschiedener Pflanzenarten liessen sich von uns an einem kürzlich im Handel erschienenen Kieselgelpapier (Schleicher & Schüll 289) sehr gute Auftrennungen dieser komplex zusammengesetzten Gemische erzielen. Obwohl die Identifizierung aller aufgefundenen Komponenten noch nicht abgeschlossen ist, soll an dieser Stelle doch schon kurz über unsere Erfahrungen bei der Auftrennung pflanzlicher Lipide berichtet werden.

METHODIK

1. Herstellung der Lipidlösungen

Die Lipidextraktion wurde in Anlehnung an das Verfahren von BLIGH UND DYER[15] in folgender Weise vorgenommen: 100 g frische Blätter werden in 100 ml Chloroform und 200 ml Methanol ca. 3 Min. im Starmix homogenisiert; danach werden weitere 100 ml Chloroform und etwa 1 Min. später 100 ml dest. Wasser hinzugefügt. Nach intensivem Mischen wird der Extrakt durch ein Filter abgesaugt. Der Filterrückstand mitsamt dem Filter wird nochmals kurz in je 100 ml Chloroform und Methanol homogenisiert; anschliessend wird das Homogenisat 15 Min. unter Rückfluss gekocht. Nach dem Abkühlen wird der Extrakt abgesaugt, mit 90 ml dest. Wasser versetzt und mit der ersten Lösung vereinigt. Nach mehrstündiger Aufbewahrung in der Kälte haben sich der tiefgrüne Lipidextrakt und die gelbbraune Waschphase, die die wasserlöslichen Verbindungen enthält, gut voneinander getrennt. Die organische Phase wird vorsichtig abgenommen und im Vakuum unterhalb von 35° zur Trockne gebracht. Zur Chromatographie wird eine 5 %ige Lösung der Lipide in Benzol–Amylalkohol (1:1) hergestellt.

Diese Rohlipidlösungen wurden auch zum Auffinden der günstigsten Lösungsmittelsysteme benutzt. Zur Identifizierung einzelner Flecke und zur Ermittlung von R_F-Werten kamen darüber hinaus gereinigte Testpräparate zur Anwendung.

2. Chromatographische Technik

Die Trennung wurde bei Raumtemperatur (18–22°) nach der aufsteigenden Methode in Glasstutzen mit eingeschliffenem Deckel durchgeführt. Die für das Kieselgelpapier (Schleicher & Schüll 289) brauchbaren Lösungsmittelsysteme sind in Tabelle I

TABELLE I

LÖSUNGSMITTELSYSTEME

(Angaben in Volumenteilen)

Lösungsmittel	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
Tetrahydrofuran	45	45	45	—	—	—	—	—	—	—
Wasser	4	6	6	12	1	3	1	1	6	6
Diisobutylketon	9	5	5	—	5	30	10	10	—	30
Methylisobutylketon	—	—	—	—	—	26	8	8	40	26
Methyläthylketon	—	—	—	—	—	20	10	8	20	20
Chloroform	—	10	—	—	40	110	90	100	180	110
Eisessig	—	6	—	—	—	—	—	10	45	45
Ameisensäure (98 %ig)	—	—	—	—	—	30	20	10	—	—
Äthylacetat	—	—	—	64	—	—	—	—	—	—
Isopropanol	—	—	—	24	—	—	—	—	—	—
Methanol	—	—	—	—	9	—	—	—	—	—

angegeben. Die verwendeten Lösungsmittel sollen frei von Peroxyden sein, da sonst die Trennungen und auch die Ninhydrinreaktion gestört werden; das Tetrahydrofuran und die Ketone wurden deshalb über Eisen-(II)-sulfat aufbewahrt und vor Gebrauch frisch destilliert.

Zur Sichtbarmachung der aufgetrennten Verbindungen wurden verschiedene Farbreaktionen benutzt. So verwendeten wir zum *allgemeinen Lipidnachweis* die Anfärbung mit Rhodamin B. Um die störende stärkere Untergrundfärbung zu vermeiden, die bei Einwirkung einer rein wässrigen Farbstofflösung auf Kieselgelpapier auftritt, benutzten wir schliesslich eine 0.01 %ige Rhodamin B-Lösung in 25 %igem Methanol unter Zusatz von 0.5 % Ammoniak. Die Chromatogramme wurden 5 Min. in das Farbbad gelegt, anschliessend mehrere Male in dest. Wasser gewaschen und schliesslich im feuchten Zustand im U.V.-Licht betrachtet; dabei lassen sich unterschiedliche Farbtönungen bei den einzelnen Lipiden beobachten (siehe Tabelle III). Neben der Rhodaminfärbung konnten die Lipide ausserdem durch Anfärbung mit Joddämpfen[16] oder Protoporphyrin[17] sichtbar gemacht werden.

Einzelne Lipidbestandteile liessen sich auf den Chromatogrammen zusätzlich mit den folgenden Farbreaktionen nachweisen.

(*a*) *Cholin* durch Besprühen mit Dragendorff-Reagens (Stammlösung: 1 g Kaliumwismutjodid in 100 ml Methanol und 50 ml Eisessig lösen. Vor Gebrauch im Verhältnis 1:2 mit Methanol verdünnen).

(*b*) *Phosphorsäure* durch Einlegen der Chromatogramme in ein nach HAHN UND LUCKHAUS[18] modifiziertes Zinzadze-Reagens (Herstellung: 6.85 g $Na_2MoO_4 \cdot 2\,H_2O$ und 400 mg Hydrazinsulfat in 100 ml Wasser lösen, 250 ml konz. Schwefelsäure p.a. und nach dem Abkühlen weitere 600 ml Wasser hinzufügen); der Reagensüberschuss kann anschliessend mit Methanol ausgewaschen werden.

(*c*) *NH_2-Gruppen* durch Besprühen mit Ninhydrin[19].

(*d*) *Ungesättigte Bindungen* durch Einwirkungen von OsO_4-Dämpfen[20].

(*e*) *Kohlenhydrate* durch Reaktion mit Perjodat–Fuchsinschwefliger Säure[21], durch Besprühen mit Anilinphthalat[22] oder Triphenyltetrazoliumchlorid[23].

Die angegebenen Färbungen wurden an Parallelchromatogrammen durchgeführt; es ist jedoch auch möglich, die Färbung mit Ninhydrin, Dragendorff, Rhodamin B und Zinzadze in der angegebenen Reihenfolge an einem Chromatogramm hintereinander auszuführen.

ERGEBNISSE

Die mit den in Tabelle I zusammengestellten Lösungsmittelsystemen und den zur Verfügung stehenden Testsubstanzen gefundenen R_F-Werte sind aus Tabelle II zu ersehen; es handelt sich dabei um Mittelwerte von mehreren Chromatogrammen mit jeweils einer Steighöhe von ca. 25 cm. In fast allen Fällen wurden die einzelnen Flecke gut voneinander getrennt; lediglich Phosphatidylserin zeigte in den säurefreien Lösungsmitteln die Tendenz, einen länger auseinandergezogenen Fleck zu bilden, der die angrenzenden Komponenten teilweise überdeckte. Dies Verhalten könnte einmal dadurch verursacht sein, dass auch die gereinigten Testsubstanzen noch Gemische darstellen, in denen die einzelnen Komponenten sich durch die verschiedenen Fettsäurereste unterscheiden. Andererseits ist auch die Möglichkeit gegeben, dass Phosphatidylserin und seine Salze eine unterschiedliche Wanderungsgeschwindigkeit besitzen, wie an Kieselgelsäulen bereits früher festgestellt worden ist[24].

TABELLE II

R_F-WERTE VON TESTSUBSTANZEN

Testsubstanz	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
Lyso-Phosphatidylcholin	0.06	0.09	0.13	0.14	0.13	0.31	0.26	0.14	0.10	0.09
Sphingomyelin	0.10	0.16	0.25	0.10	0.25	0.39	0.28	0.27	0.18	0.18
Phosphatidylcholin	0.17	0.25	0.32	0.21	0.45	0.48	0.44	0.40	0.42	0.36
Phosphatidylserin	0.32	0.40	0.49	0.17	0.22	0.41	0.38	0.29	0.35	0.33
Phosphatidyläthanolamin	0.42	0.49	0.53	0.63	0.61	0.52	0.49	0.46	0.64	0.66
Lyso-Phosphatidyl-äthanolamin	0.27	0.31	0.44	0.49	0.36	0.33	0.30	0.17	0.25	0.20
Phosphatidylinosit	0.24	0.33	0.51	0.09	0.20	0.15	0.27	0.08	0.09	0.09
Cerebrosid I	0.51	0.56	0.61	0.68	0.51	0.23	0.25	0.13		0.13
Cerebrosid II	0.79	0.83	0.81	0.88	0.75	0.51	0.50	0.48	0.74	0.50
Cerebrosid III	0.83	0.85	0.85	0.88	0.75	0.59	0.56	0.56	0.86	0.68

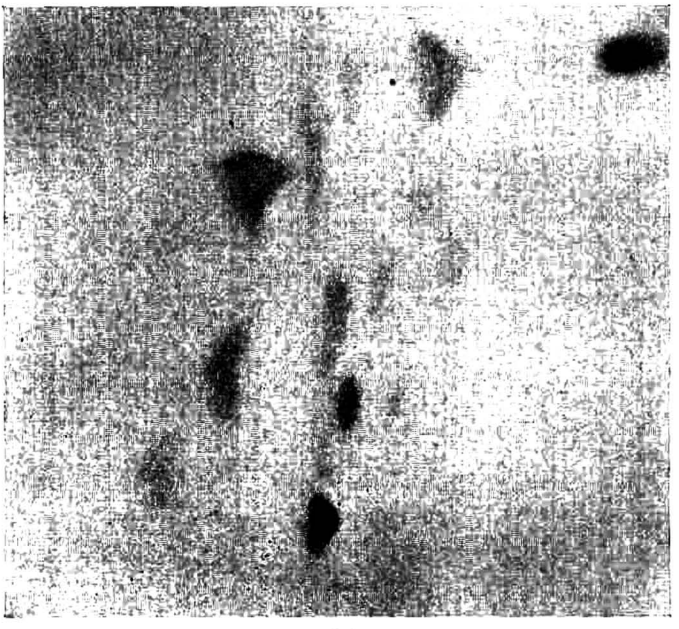
Auch nach Auftrennung von frisch gewonnenen Phosphatidextrakten aus Rinderhirn, Kaninchenherz und verschiedenen Pflanzen wurde Phosphatidylserin als langgezogener Fleck erhalten; es ist daher nicht anzunehmen, dass diese Überlappung durch eine Zersetzung unseres Testpräparates verursacht wurde.

Inzwischen haben THIELE UND WOBER[25] mitgeteilt, dass sie am gleichen Kieselgelpapier unter Verwendung der von uns schon früher mitgeteilten[26] Lösungsmittelsysteme III und VI ebenfalls eine gute Auftrennung von Phosphatiden erzielt haben; die von ihnen angegebenen R_F-Werte stimmen grösstenteils mit den von uns gefundenen überein. Einige Abweichungen lassen sich wahrscheinlich damit erklären, dass ihre Trennungen bei 2°, unsere jedoch bei 18–22° durchgeführt wurden.

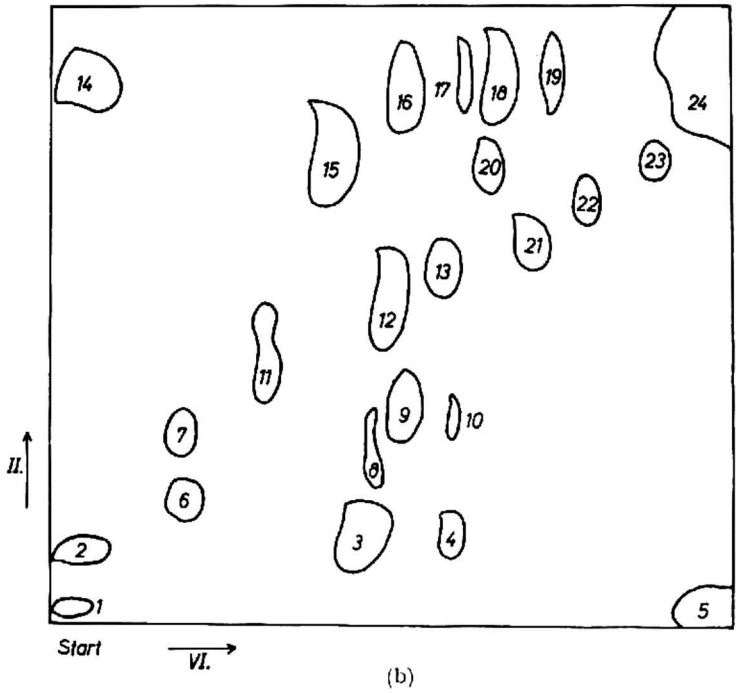
Bereits bei den ersten Versuchen erwies es sich als unmöglich, alle Komponenten eines Lipidextraktes aus Pflanzen im eindimensionalen Chromatogramm aufzutrennen. Daher wurde die zweidimensionale Technik angewendet, wobei sich zeigte, dass mit einer effektiven Lauffläche von 14 × 14 cm bereits sehr gute Auftrennungen möglich sind; die Laufzeit war dann sehr kurz und betrug insgesamt etwa 3 Stunden. Zusätzlich benutzten wir auch grössere Chromatogramme mit Laufflächen von 16 × 18 cm und 18 × 24 cm. Abgesehen davon, dass auf diese grössere Lipidmengen aufgetragen und dadurch Komponenten nachgewiesen werden konnten, die nur in geringer Konzentration vorlagen, fanden wir jedoch keinen Vorteil in der Verwendung grösserer Papierformate, da die schärfsten Trennungen auf den kleinen Laufflächen erzielt wurden.

Zur Auftrennung von Lipidrohextrakten haben sich die Lösungsmittelkombinationen II oder III (1. Lauf mit der Faserrichtung) und VI, VII oder VIII (2. Lauf) bewährt. Bei der Untersuchung der Lipide aus Zuckerrübenblättern wurden dabei 23 verschiedene Komponenten gefunden (siehe Tabelle III und Fig. 1), von denen auf Grund verschiedener Farbreaktionen 4 als Glykolipide und 10 als Phosphatide eingestuft wurden; 9 weitere Komponenten ergaben lediglich eine Anfärbung mit Rhodamin B, ohne dass jedoch auf Grund anderer Reaktionen eine Zuordnung möglich war.

Mit der angegebenen Methode lässt sich in relativ kurzer Zeit die Auftrennung eines Lipidgemisches erreichen; über ihre Anwendung zur Differenzierung der Lipide gesunder und viruskranker Pflanzen wird an anderer Stelle berichtet werden.



(a)



(b)

Fig. 1 (a). Auftrennung eines Lipidextraktes aus den Blättern vergilbungskranker Zuckerrüben (Lösungsmittelsysteme II und VI; Anfärbung mit OsO_4). (b). Lage der Lipide im zweidimensionalen Chromatogramm. (Lösungsmittelsysteme II und VI; Erläuterung siehe Tabelle III).

TABELLE III

FARBREAKTIONEN DER PFLANZENLIPIDE (= ERLÄUTERUNG ZU FIG. 1)

Fleck Nr.	*Anfärbung mit*						*Vorläufig identifiziert als*
	Rhodamin B	*Dragendorff*	*Ninhydrin*	*OsO_4*	*Zinzadze*	*Perjodat-Schiff*	
1	rot	±	+	—	—	—	—
2	orange	—	—	—	—	—	—
3	hell-orange	+ + +	—	+ + +	+ + +	—	Phosphatidylcholin
4	± orange	—	—	+	+	—	Phosphatid
5	rot	—	—	—	—	—	—
6	orange	—	—	+	+	+	Phosphatidylinosit
7	orange	—	—	+	+	+	Phosphatid
8	blau-rot	±	+	+	+	—	Phosphatidylserin
9	rosa-orange	±	+	+ + +	+ + +	—	Phosphatidyläthanolamin
10	orange	±	+	+	+	—	Phosphatid
11	orange	—	—	+ +	—	+	Glykolipid
12	orange	—	—	+ + +	+ +	+ + +	Phosphatid
13	± orange	—	—	+	—	—	—
14	rot-orange	—	—	±	—	—	—
15	gelb	±	—	+ + +	—	+ + + *	Glykolipid
16	rosa-rot	—	—	+ +	—	+	Glykolipid
17	rosa-rot	—	—	+	—	—	—
18	gelb	±	—	+ + +	—	+ + + *	Glykolipid
19	orange	—	—	±	—	—	—
20	± orange	—	—	+	+	+	Phosphatid
21	orange	—	—	+	+	+	Phosphatid
22	orange	—	—	+	—	—	—
23	orange	—	—	+	—	—	—
24	—	—	—	—	—	—	Farbstoffe, freie Fettsäuren, etc.

* Reagierten zusätzlich mit Anilinphthalat und Triphenyltetrazoliumchlorid.

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Wir sind den Herren Prof. Dr. L. HÖRHAMMER, München, und Prof. Dr. E. KLENK, Köln, für die freundliche Überlassung von gereinigten Testsubstanzen zu Dank verpflichtet.

ZUSAMMENFASSUNG

Durch zweidimensionale Chromatographie auf Kieselgelpapier gelingt es, Lipidrohextrakte in kurzer Zeit aufzutrennen. Bei der Untersuchung von Zuckerrübenblattextrakten wurden insgesamt 23 verschiedene Lipidkomponenten aufgefunden, von denen 10 als Phosphatide und 4 als Glykolipide charakterisiert werden konnten. 9 Komponenten liessen sich noch nicht näher klassifizieren.

SUMMARY

Crude lipid extracts may be separated rapidly by two-dimensional chromatography on a new type of silicic acid-impregnated paper. In the examination of lipid extracts of sugar beet leaves 23 different spots have been observed; 10 of these have been characterized as phospholipids and 4 as glycolipids, while the remaining 9 lipid spots could not yet be classified.

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A MODIFIED APPARATUS FOR CARRIER-FREE PREPARATIVE CONTINUOUS ELECTROPHORESIS

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INTRODUCTION

BARROLLIER, WATZKE AND GIBIAN[1] have succeeded in carrying out electrophoretic separations in a free-flowing buffer solution without sacrificing the advantages of an apparatus needing a carrier. The merits of this system have already been pointed out by HÖLZER, NOACK AND WIEK[2], HÖLZER AND WIEK[3], and SCHWARTZKOPFF AND HÖLZER[4], but non-observance of strict control of the working conditions in continuous-flow electrophoresis gives rise to considerable errors.

The direction of migration of a particle in the separating chamber is the result of the following factors: represented by vectors (a) the rate of flow of the buffer solution through the apparatus as the vertical, and (b) the electrophoretic migration at a defined field intensity and a defined pH-value as the horizontal component. If during the experiment a vector quantity is changed, the direction of migration of the particle also changes. Thus the prerequisite for good selectivity is the constancy with time of the vectors at each individual point within the chamber. If the vectors do not act with the same magnitude on the individual points within the chamber, the only result will be that the fractions do not follow a straight course, the separation sharpness remaining unaffected. However, under unfavourable conditions the separating capacity of an apparatus designed for a theoretical optimum capacity is not then attained.

In the apparatus described by BARROLLIER[1] and his co-workers a capillary flow of buffer solution passes between two water-cooled, horizontal glass plates. Strips of filter paper are positioned between the plates on both sides to act as spacers and at the same time serve to connect the troughs to the electrodes. The separating buffer is fed dropwise on to the lower, somewhat longer glass plate, is led through cellulose into the separating chamber and is delivered at the front to small strips of paper. The sample is added through holes bored in the upper glass plate. The electrode troughs are filled with buffer solution of approximately twice the concentration of the separating buffer solution and are rinsed continuously.

As the glass plates, which are 50 cm wide, are only spaced from each other by the filter paper at the edge, sagging of the top plate may occur after extensive use, thus causing distortions in the flow of the buffer solution. This phenomen of sagging was prevented by interpositioning small discs (for example of perspex or teflon);

the flow was not disturbed by this arrangement. In this way it was also possible to define the distance between the plates, which may be varied from 0.3 to 0.8 mm. At the same time the filter paper was replaced by fibre glass paper in order to prevent the absorption of proteins.

It was now found that the rate at which the buffer solution was imbibed by the small strips of paper or fibre glass paper at the front edge did not remain constant. This is explained by the fact that the small strips form a system of multiple siphons, their delivery depending on the quality of the front edge of the bottom glass plate and its wetting conditions.

Delivery of a constant quantity of buffer solution per unit time through a strip may, however, only be expected if the position of the particular strip with respect to the glass plate and the surface character of the latter remain the same. But this can only be assumed under theoretically ideal conditions. In practice, vibrations in the environment can cause the small strip to shift slightly, and pollution of the glass plate by small air bubbles cannot be avoided. The mechanism by which the liquid is conveyed depends on both the capillary system inside the strips and that between the latter and the glass surface. But as the velocity of a liquid within a capillary tube varies with the third power of the radius, a considerable disturbance is caused even by an infinitely small change. Owing to the inconstancy of flow rate of the buffer solution with time, the most essential condition for a continuously operating electrophoresis apparatus is not met.

It is also very difficult to reproduce test results exactly when new glass plates have to be used, as they are not usually of equal quality even when manufactured with the greatest care.

In order to avoid the above-mentioned disadvantages, BARROLLIER's apparatus has been modified in cooperation with the manufacturer* and the modifications are described and illustrated in Figs. 1–4 below.

APPARATUS

First of all, the two glass plates are connected to each other on all four sides by silicone rubber (Wacker-Chemie, München, Germany), which is very resistant to chemical attack. The top glass plate has at its rear end seven holes through which the buffer solution is led into the chamber (Fig. 1). At the forward end of the separating chamber the bottom glass plate is provided with 49 holes through which the buffer solution is fed to a collecting device. This device is made of Trovidur and constructed as follows: it consists of 49 cups which are connected via a system of flexible tubes to the holes in the bottom glass plate. All the cups have the same capacity. At predetermined intervals, set by means of an electric timing unit, small pins of the same standard size plunge into the cups and the buffer solution in the cups is thereby displaced and flows through a system of receptacles and flexible tubes to the collecting vessels. In order to prevent the buffer solution returning to the chamber, the bottom of each cup is provided with a small pin that functions as a non-return valve. The supply of the buffer solution to the chamber and thus to the cups is effected from a vessel located at the rear portion of the apparatus. Fresh buffer solution is constantly pumped from

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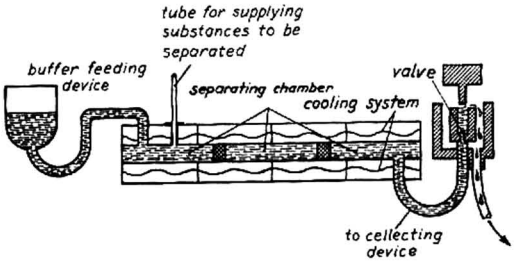

Fig. 1. Longitudinal section of the apparatus, diagrammatic.

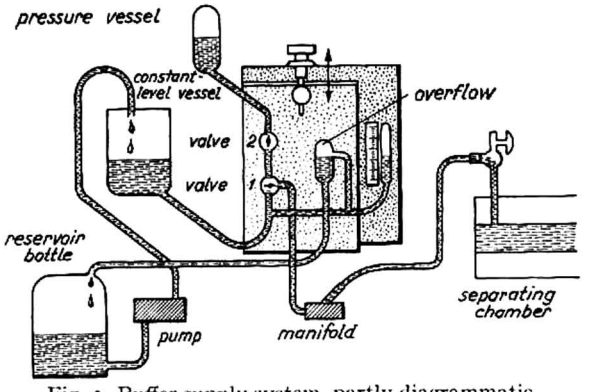

Fig. 2. Buffer supply system, partly diagrammatic.

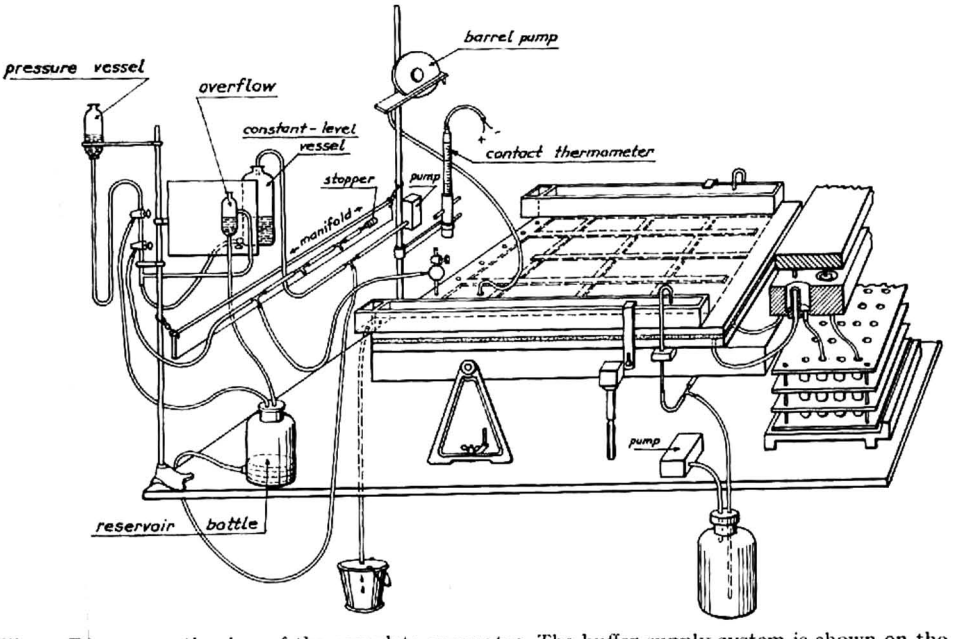

Fig. 3. Diagrammatic view of the complete apparatus. The buffer supply system is shown on the left of the illustration. The separation chamber with the two electrode troughs attached to its sides and the rinsing system for the electrode troughs are shown in the middle. The sample is fed into the chamber by the metering pump via a hose connection. The right-hand part of the illustration shows the buffer extraction system and the lines leading to the collecting vessels.

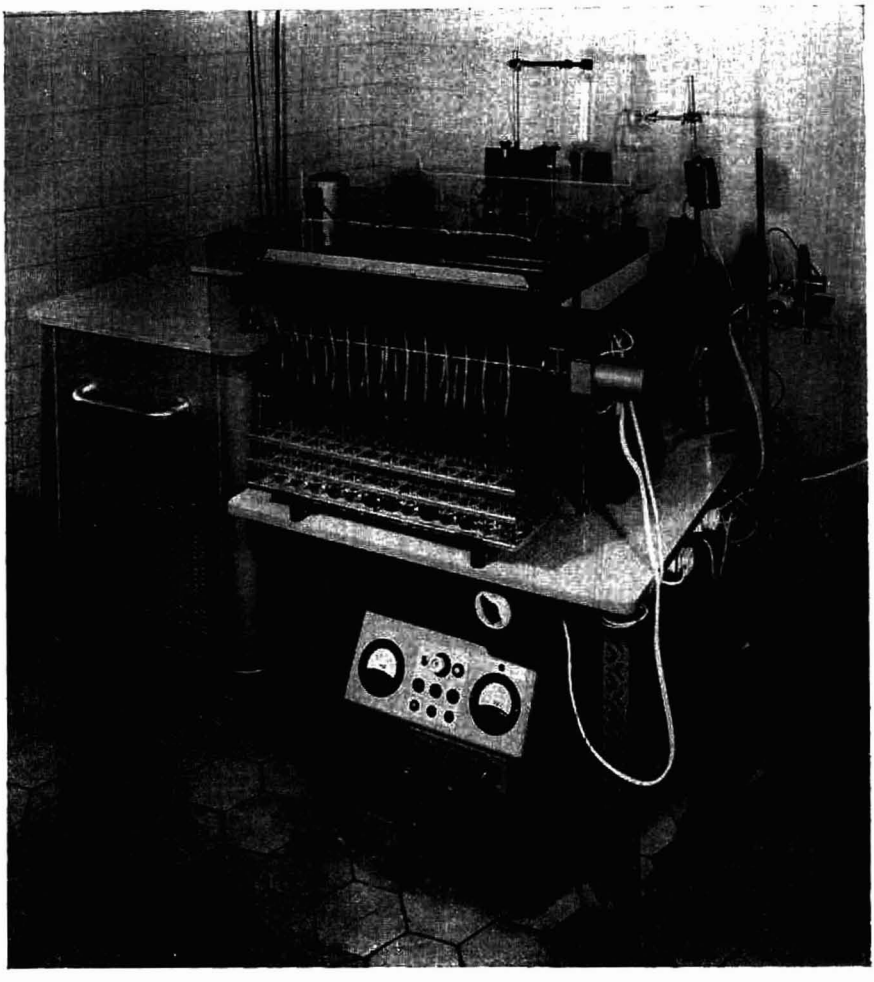

Fig. 4. A photograph showing the whole separating system. In the middle, the separation chamber, on the left the cooling aggregate, and under the table, the electric generator.

a reservoir bottle (Fig. 2), into this vessel which is provided with an overflow device. In this way the liquid level in the vessel is maintained (constant-level vessel, (see Fig. 2), but can be varied with the aid of the adjustable overflow device which in Fig. 2 is shown as the drip vessel. The constant-level vessel, the gap separating the plates and the delivery cup represent a system of communicating tubes; this ensures that the same level of liquid is also maintained in the cups. Buffer solution may be drawn from another vessel (pressure vessel) in which the liquid level is considerably higher for filling the chamber and rinsing it quickly after a test.

The troughs for the electrodes are cemented to the top plate. Holes in the top plate, which also extend through the bottoms of the troughs for the electrodes, connect the troughs with the separating chamber. Small tubes made of Trovidur are glued into these holes. To prevent the buffer solution in the troughs mixing with the buffer solution intended for the separation, the small tubes are firmly packed with

asbestos and glass wool. In this way hydrostatic effects of the buffer solutions in the troughs and the separating chamber are also avoided. Rinsing the troughs for the electrodes prevents the occurrence of polarization.

The connection of the constant-level vessel with the separating chamber is effected via a manifold having seven branches, which are seven narrow flexible tubes leading to the separating chamber. This constitutes a shunt that must have an effect on the flow of electric current. This effect was checked by measuring the resistance after filling the chamber and the system of flexible tubes with a buffer solution of veronal/veronal-sodium, ionic strength $\mu = 0.05$. In the system of tubes the resistance was 2 megohms, whereas the resistance in the chamber, which is of much greater cross-section, was as low as 8.4 kiloohms. Therefore the current flowing through the shunt can be practically neglected.

Measurements of the potential drop at the diaphragms between trough and chamber showed a drop of approximately 15% of the voltage applied to the terminals. The electric field in the chamber proved to be homogeneous.

Checks on the conductivity and the actual reaction of the buffer solution, both in the chamber and in the troughs for the electrodes, gave constant results under prolonged and varying test conditions

As in the previous arrangement a terminal voltage of 500 to 3000 volt can be applied. In the case of a chamber width of 50 cm this would correspond to 10 to 60 volt/cm. The flow rate of the buffer solution is determined by the interval at which the pins plunge into the delivery cups and by the liquid level of the latter. To control the liquid level, the constant-level vessel is connected via an ascending tube with a graduated scale so that the initial value may be determined and reproduced at any time. Thus the time for the buffer solution to pass through the system may be set for values from 20 to 360 min.

As in the unmodified apparatus, there are five holes in the top glass plate for feeding in the substance to be separated. Depending on the test conditions chosen, and on the substance to be separated, one of the holes is selected for introducing the substance into the system. In contrast to the older system, however, the holes must now be sealed to prevent the chamber from draining dry. This is achieved by fitting the holes with small pins of Trovidur which project approximately 15 mm above the glass plate. A flexible hose capable of being plugged is pulled over these pins. The substance is delivered through a metering pump of the rotary barrel type. The selectivity attainable and the constancy of the test results in this modified arrangement for carrier-free preparative continuous electrophoresis were shown by investigating the separation of human serum proteins.

EXPERIMENTAL AND RESULTS

Test conditions

Buffer: Veronal/veronal-sodium buffer solution, pH 8.6, ionic strength $\mu = 0.025$.

Terminal voltage: 1800 volts = 36 volt/cm, 100 mA.
Cooling: Inflow 0°, effluent + 3°.
Time for passage of buffer solution: 210 min.
Height of separating chamber: 0.5 mm.

Sample: 0.8 ml/h were fed into the chamber continuously. This corresponds to a

capacity of 19.25 ml serum = approx. 1.5 g protein in 24 h. The serum was undiluted and undialyzed.

Detection: The protein concentration in the collecting vessels was determined by the biuret reaction. The proportions in the fractions were found by evaluating Gauss distribution curves. The tests specified under A, C, and D were carried out in a Beckman spectrophotometer, wavelength 540 mμ, slit 0.02, whereas an Eppendorf photometer, wavelength 546 mμ, in connection with a macro trough was used for the tests listed under B.

Test A

In order to determine the order of accuracy of results under constant test conditions a normal serum (protein content 7.3 g%) was separated seven times.

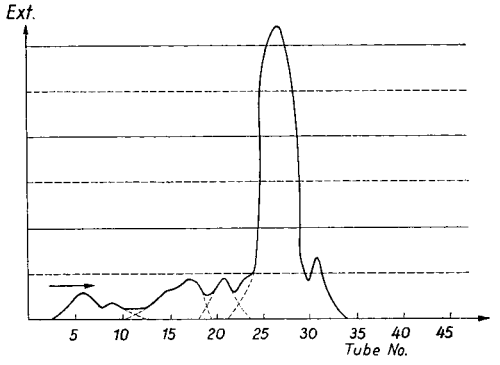

Fig. 5. Protein fractionation of a normal human serum.

Mean values (M) for the protein in the separated fractions of serum, together with the standard deviation (S.D.) and the standard error of the mean (S.E.), were calculated and are shown in Table I.

TABLE I

STATISTICAL ACCURACY OF REPEAT PROTEIN FRACTIONATIONS OF A NORMAL HUMAN SERUM

	Protein fraction			
	Albumin (%)	*α-Globulin* (%)	*β-Globulin* (%)	*γ-Globulin* (%)
M	68.47	8.61	13.14	9.77
S.D. ±	0.77	0.47	0.50	0.60
S.E.	0.29	0.18	0.19	0.23

Test B

In order to evaluate quantitative determinations of separated protein in fractions from pathological serum a range of normal values was determined by separating serum samples from 15 healthy persons. The mean (M) and the extreme values (E.V.) are given in Table II.

TABLE II

THE RANGE OF VALUES OBTAINED FOR PROTEIN FRACTIONS FROM NORMAL SERUM

	Albumin (%)	*α-Globulin* (%)	*β-Globulin* (%)	*γ-Globulin* (%)
M	63.5	10.7	11.1	14.8
E.V.	56.2–71.5	6.8–14.0	8.2–13.6	11.7–18.5

Tests C and D

Both of these separations were carried out with pathological sera which are illustrated in Figs. 6 and 7. The results are given in Table III.

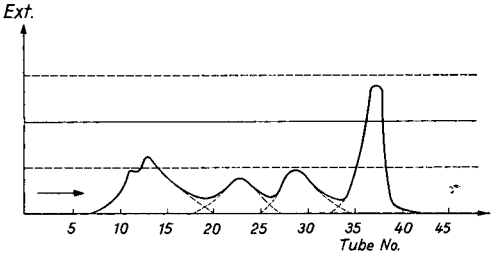

Fig. 6. Liver cirrhosis serum.

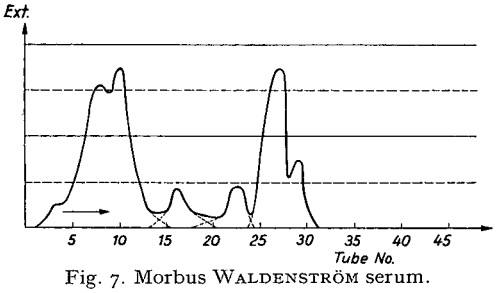

Fig. 7. Morbus WALDENSTRÖM serum.

TABLE III

PERCENTAGE PROTEIN IN FRACTIONS FROM PATHOLOGICAL SERUM SAMPLES

Sample	*Protein content* g%	*Albumin* (%)	*α-Globulin* (%)	*β-Globulin* (%)	*γ-Globulin* (%)
Liver cirrhosis serum	6.3	40.0	10.0	16.5	33.5
Morbus WALDENSTRÖM serum	9.4	29.5	6.5	6.5	57.5

DISCUSSION

The intention underlying the modification of the apparatus for carrier-free preparative continuous flow electrophoresis designed by BARROLLIER and his co-workers was to retain the separating principle but to eliminate the factors affecting the separating

process which, on account of their very limited control, gave rise to frequent trouble. To attain this end, it was necessary to redesign the entire buffer supply and extraction system.

This object seems to have been realized, judging by the separation results obtained with human serum proteins. There is satisfactory selectivity and only a very low margin of error present in the apparatus; this is comparable to the values given in the literature (RIVA[5]). The constancy of the separation results obtained is satisfactory.

In contrast to the previous design, the flow rate of the buffer solution can be mechanically adjusted and it is now possible, after modifying or cleaning of the apparatus or even use of a second apparatus, to reproduce the test conditions exactly and automatically. Automatic operation of the apparatus reduces the amount of work required to a minimum, and when starting a test the laborious filling process for the chamber, which often had to be repeated several times because of the formation of air bubbles, is no longer necessary. After the first filling, which is easily and rapidly effected by using the pressure vessel and tilting the whole chamber into a upright position, the apparatus is serviceable at all times and is capable of being put into operation with a switch.

A detailed analysis of the protein fractions present in the collecting vessels does not fall within the scope of this paper. Evaluation of the Gauss distribution curves and checks by means of the paper electrophoresis are not sufficient for qualitative analysis of the fractions and their purity yield. Further chemical, physical, and possibly serological methods are required for this purpose. Only the latter will show whether the peak before the albumin is equivalent to pre-albumin and whether the two peaks that always occur in the range of the gamma globulins are due to a true separation or only to a pseudo-fractionation.

SUMMARY

It has been shown that inconsistent results are obtained in carrier-free continuous electrophoresis according to BARROLLIER and his co-workers, in spite of all the advantages of the system. The apparatus was therefore modified. The electrophoretic separation of human serum proteins was chosen as an example to show that it is possible to achieve constant test conditions and satisfactory separation results with this modification.

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A RAPID METHOD FOR THE QUALITATIVE ANALYSIS OF MIXTURES OF INORGANIC IONS BY PAPER ELECTROMIGRATION

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INTRODUCTION

Several authors have studied the separation and subsequent identification of inorganic ions by paper electromigration. No attempt will be made to give a complete survey of these researches, as several reviews are available and new ones appear periodically (see ref. 1 and the reviews appearing every second year in *Analytical Chemistry, e.g.* ref. 2).

Generally speaking these researches concern a small number of ions which have been subjected to a preliminary separation by means of a group-reagent. Proposals for the separation by electromigration of mixtures of a large number of ions are rare, and have not been worked out into a scheme of analysis up till now. Recently GROSS[3] studied the electromigration of a large series of ions in formic acid and ammonium carbonate.

We have been looking for a method by which as many ions as possible could be identified by their different path lengths in the supporting electrolyte together with colours or fluorescence developed with suitable reagents.

In preliminary experiments with a group of about ten ions, the following supporting electrolytes were tried: citric acid, oxalic acid, malonic acid (all three in various concentrations), and also tartaric acid, gluconic acid, ammonium citrate and sodium potassium tartrate. Of these, oxalic acid and malonic acid were discarded on account of their tendency to form precipitates with various ions. Solutions of ammonium citrate and sodium potassium tartrate increase the conductance and have an appreciable buffering capacity; this last property is disadvantageous in several identification reactions. Of the remaining acids, citric acid, in 1 N solution, gave the best results.

In the following experiments we studied the behaviour of the ions: Ag^+, AsO_3^{3-}, AsO_4^{3-}, Au^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Ce^{3+}, Cl^-, Co^{2+}, Cr^{3+}, CrO_4^{2-}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ga^{3+}, Hg^{2+}, In^{3+}, K^+, La^{3+}, Li^+, Mg^{2+}, Mn^{2+}, MnO_4^-, MoO_4^{2-}, Na^+, Ni^{2+}, Pb^{2+}, Sb^{3+}, SeO_3^{2-}, SeO_4^{2-}, Sn^{2+}, Sn^{4+}, Sr^{2+}, TeO_3^{2-}, Th^{4+}, Ti^{4+}, UO_2^{2+}, VO^{2+}, WO_4^{2-}, Y^{3+}, Zn^{2+}, and Zr^{4+}.

The results obtained indicate that, in the medium chosen, a large number of the above-mentioned ions can be identified clearly after electromigration for one hour or less in some cases.

EXPERIMENTAL

Apparatus

A number of paper strips (Whatman No. 1 chromatographic paper, 80 × 2 cm) were sandwiched between two flat glass plates, the ends of each strip coinciding with a slit

bored in the upper glass plate. The cathode- and anode-slits were 75 cm apart. They were filled with an agar gel (14 % in 1 *N* citric acid) to ensure electrical contact between the carbon electrodes and the strips. The gel also served as an absorbent for the products of electrolysis. A cellophane strip between the gel and the paper strips acted as a barrier for the transport of liquid from the former to the latter (see Fig. 1).

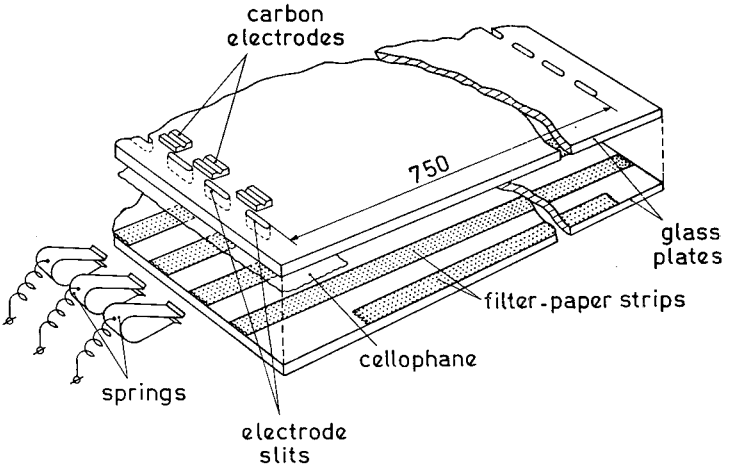

Fig. 1. Electromigration apparatus used in this work.

Evaporation was reduced by inserting two extra strips, soaked in the electrolyte solution, along the long sides of the glass plates and by loading the plates with iron blocks of several kg weight. Contact between the extra paper strips and the cellophane membranes should be avoided.

Control experiments with arabinose showed that electroendosmosis is nil, while the fact that the current in each strip remains fairly constant during the experiments (the applied potential being kept constant) indicates that evaporation is insignificant.

Any high-voltage d.c.-source, capable of maintaining a potential gradient of about 30 V/cm and a current of 2–3 mA in each strip, may be used.

Reagents and other solutions

Solutions of the ions (of known concentration) in 1 *N* citric acid were used. The solutions of the developing reagents, together with the treatment after spraying, are summarized in Table I. The reactions with gases mentioned there, can be performed by hanging the paper strips in a glass cylinder in which an atmosphere of the particular gas is maintained.

General procedure

(*a*) *Pretreatment of the paper strips.* A pile of 6–10 paper strips was soaked with 1 *N* citric acid, and after draining off the surplus liquid the pile of strips was placed between two thick layers of dry filter paper and a brass cylinder was rolled over them twice; the amount of liquid retained by the strips was approximately equal to their own weight. The strips were placed on the lower glass plate, parallel to each other and about 1 cm apart.

TABLE I

REAGENT SOLUTIONS

Reagent	*Solution*	*Treatment after spraying with the reagent*
Dithiooxamide	Satd. soln. in 96% ethanol	In NH_3 atmosphere
Oxine	1% soln. in 80% ethanol	In NH_3 atmosphere, then examine in U.V. light (maintain NH_3 atm.)
Dithizone	0.025% soln. in 80% acetone	In NH_3 atmosphere
Alizarin	Satd. soln. in 96% ethanol	In NH_3 atmosphere; then spray with boric acid soln.
Boric acid	Satd. soln. in water	
HNO_3–glycerol[10]	1% conc. HNO_3 and 5% glycerol in 96% ethanol	Dry, then spray with an ammoniacal soln. of Ag^+, place in U.V. light during 20 min
o-Tolidine*	0.2% soln. in 10% acetic acid	
Rhodizonic acid	Freshly prepared 0.2% soln. of the Na salt in water	
K ferrocyanide	2% soln. in water	
Morin	1 part of a 1% soln. in 96% ethanol is mixed with 4 parts of conc. HCl	Observe in U.V. light
Dithiol	0.2 g is dissolved in 100 ml of a 1% aqueous soln. of Na_2CO_3 by occasional shaking during some hours. The soln. is stabilized by adding so much thioglycolic acid that an opalescence appears	Spray with 2 *N* hydrochloric acid; then in NH_3 atmosphere
Chloranilic acid**	0.1% soln. in ether	Observe in U.V. light
K iodide	5% soln. in water	
Titan yellow	Satd. soln. in water	Dip in 0.5 *N* NaOH and spray again with the reagent.
Diphenylcarbazide*	1% soln. in 96% ethanol, add 1 drop of 1 *N* H_2SO_4	

* Before applying the reagent oxidize by the following procedure: spray with 4 *N* NaOH, bring the strip in bromine vapour, destroy the remaining bromine in NH_3 atmosphere. Some speed is required.

** For this reagent a dip technique was used[11]: the strip was dipped in the ethereal solution of the reagent, then washed with fresh ether.

(*b*) *Application of the sample solution.* A piece of white sewing yarn (*e.g.* Brook's patent glacé thread No. 60, 0.15 mm thick) of sufficient length was soaked for at least 5 min in a few drops of the sample solution, wiped lightly with a piece of filter paper and placed across the paper strips at a distance of approximately 30 cm from the anode. Care was taken to draw the thread taut. Strips and thread were covered with the upper glass plate, which pressed them together. After 10 min the thread was removed, its position on the strips was marked with a short pencil line and the upper glass plate, fitted beforehand with cellophane membranes and agar gel, was again put into position.

TABLE

RELATIVE MOBILITIES WITH RESPECT TO THE Co^{2+}-ION, COLOUR

Reagent and medium:		*Dithio-oxamide* NH_3		*Oxine*				*Dithizone* NH_3		*Alizarin*				*Glycerol* $-HNO_3-$ $AgNO_3$ *U.V.*		*Oxidation then o-tolidine*	
				NH_3		NH_3 *U.V.*				NH_3		*Boric acid*					
Ion	*Relative mobility*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*
Tl^{+}	179 ± 1	b	15	b	5	d	5	v	10	—		—		—		bl	15
Ba^{2+}	120 ± 3	—		—		yf	16	—		—		—		—		—	
Sr^{2+}	119 ± 2	—		—		yf	8	—		—		—		—		—	
Ca^{2+}	119 ± 2	—		—		yf	4	—		—		—		—		—	
Mg^{2+}	118 ± 1	—		y	1.8	yf	0.9	—		—		—		—		—	
Mn^{2+}	113 ± 3	—		y	7	d	2.5	—		v	1.1	v	10	b	0.9	bl	6
Fe^{2+}	107 ± 1	—		gr	0.8	d	1	—		bl	0.3	v	1	gr†	0.4	—	
Cr^{3+}	100 . .	—		—		—		—		—		—		—		bl	5
Co^{2+}	100	by	0.8	by	3	d	1	v	0.2	v	0.3	v	5	—		—	
Cd^{2+}	96 ± 2	—		y	5	yf	2	o	0.8	—		—		—		—	
Zn^{2+}	91 ± 2	—		y	3	yf	1	rv	0.5	v	3	v	5	—		—	
Be^{2+}	90 ± 2	—		y	10	yf	1	—		rv	1	rv	1	—		—	
Ni^{2+}	85 ± 5	v	0.9	y	10	d	2.5	v	0.8	rv	10	rv	10	—		—	
Pb^{2+}	57 ± 2	d	4	y	3	d	0.5	rv	0.7	bl	2	v	2	—		bl	4
Cu^{2+}	45 ± 3	g	1	y	3	d	3	gr	0.5	v	1	v	0.5	—		—	
La^{3+}	45 ± 2	—		y	3	yf	2.3	—		bl	3	v	3	—		—	
Ce^{3+}	39 ± 1	—		d*	2.5	d	2	—		bl	1.1	bl	1	—		bl	5
Y^{3+}	32 ± 2	—		y	2	yf	0.8	—		v	1	v	1	—		—	
Al^{3+}	29 ± 2	—		y	2	yf	0.5	—		rv	3	rv	3	—		—	
In^{3+}	26 ± 1	—		y	6	yf	3	—		rv	1.6	rv	1.8	—		—	
UO_2^{2+}	17 ± 2	—		by	2	d	2	—		rv	1.4	rv	1.4	—		—	
Sn^{2+}	6 ± 1	—		y	30	yf	10	o	1.5	rv	1.4	o	1.7	b†	1	—	
Ag^{+}	6 ± 1	b	0.5	y	2	yf	1	v	1	—		—		d	0.8	—	
Ga^{3+}	2 ± 1	—		y	3	yf	3	—		rv	1	rv	1	—		—	
VO^{2+}	2 ± 1	—		y	2.5	d	2	—		o	1	r	1	d	1	bl	3
Hg^{2+}	0	—		y	7	d	5	o	0.8	—		v	6	—		—	
AsO_3^{3-}	0	—		—		—		—		—		—		by	2	—	
Th^{4+}	—9 ± 2	—		y	10	d	2	—		v	6	rv	2	—		—	
Bi^{3+}	—9 ± 3	b	1.4	y	1.4	yf††	1.4	o	0.5	v	0.6	v	0.6	—		—	
Fe^{3+}	—18 ± 3	—		gr	1	d	1	—		v	3	v	7	b	1.1	—	
AsO_4^{3-}	—18 ± 4	—		—		—		—		—		—		b	2	—	
Sb^{3+}	—26 ± 2	b	15	y	10	yf††	10	—		v	10	rv	10	d	7	—	
CrO_4^{2-}	—28 ± 1	—		y	10	d	10	—		rv	10	rv	4	—		g†	0.9
Au^{3+}	—42 ± 2	b	15	gr	20	d	20	g	20	rv	20	v	10	b	2	g†	3
Sn^{4+}	—44 ± 5	—		y	2	yf	2	—		o	2	o	2	—		—	
Ti^{4+}	—45 ± 3	—		y	10	yf	5	—		v	10	rv	5	—		—	
Zr^{4+}	—52 ± 2	—		y	8	yf	5.5	—		rv	5	rv	6	—		—	
MoO_4^{2-}	—57 ± 2	—		y	10	d	10	—		—		—		—		—	

* Appearing after some time; † appears at once; ** disappears within a short time, except when the concentration of the ion is high; †† in higher concentrations: d.

II

REACTIONS (C) OF THE IONS AND THEIR SENSITIVITY (S) (mg/ml)

Rhodizonic acid		*K ferrocyanide*		*Hydrogen sulphide*				*Morin U.V.*		*Dithiol*				*Chloranilic acid*		*Special reagents*
				Citric acid		NH_3				*2N HCl*		NH_3				
C	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	*C*	*S*	
—		—		—		—		—		—		—		gr	9	KI: y 15
r**	3	—		—		—		—		—		—		—		
r**	10	—		—		—		—		—		—		by	35	
—		—		—		—		—		—		—		y	16	
—		—		—		—		—		—		—		y	20	Titan yellow: o 15
—**		—		—		—		—		—		—		y	10	
—**		bl	1.7	d	1	d	1	—		gr	3	br	1	gr	3	
—		—		—		—		—		—		—		—		Diphenylcarbazide: r 5
—		—		—		d	0.8	—		gr	1	gr	1	—		
—**		—		y	7	y	7	—		—		—		v	3	
—**		—		—		—		—		—		—		—		
—		—		—		—		—		—		—		v	10	
—		—		—		gr	3	—		gr	3.5	gr	3.5	—		
rv	0.7	—		gr	2	gr	2	—		y	3	—		b	4	
b	2	b	3	b	6	b	6	—		gr	5	gr	5	v	10	
o**	3	—		—		—		—		—		—		y	9	
—		—		—		—		—		—		—		y	11	
—		—		—		—		—		—		—		—		
—		—		—		—		—		—		—		rv	3	
o**	4	—		—		—		yf	10	—		—		y	15	
o**	2.5	b	2.5	—		—		—		—		—		o	2.5	
r**	5	—		—		—		—		r	10	—		v	30	
—		—		d	0.8	d	0.8	—		y	3	y	3	—		
—		—		—		—		yf	1	—		—		v	3	
r**	5	—		—		—		—		—		—		gr	3	
—**		g*	5	b	10	b	10	—		—		—		—		
—		—		y	2.5	y	2.5	—		—		—		—		$AgNO_3$: y 10
—		—		—		—		yf	15	—		—		—		
rv**	0.6	—		b	0.6	b	0.6	—		o	1.7	o	1.7	—		KI: y 0.4
—**		bl	0.9	gr	2	gr	2	—		gr	1	gr	1	gr	3	
—		—		—		—		—		—		—		—		$AgNO_3$: y 10
—		—		y	10	y	10	yf	15	y	10	—		—		
—		—		—		—		—		—		—		—		Diphenylcarbazide r 5
—		g	7	b	7	b	7	—		b	10	b	10	gr	7	
—		—		—		—		—		r	2	—		—		
—		—		—		—		yf	10	—		—		—		
—		—		—		—		yf	6	—		—		—		
—		—		—		—		—		g	3	g	3	v	10	

Abbreviations used: bl = blue; by = brown-yellow; g = green; o = orange; rv = red-violet; yf = yellow fluorescence; b = brown; d = dark, black; gr = grey; r = red; y = yellow; v = violet.

Finally the electrodes were clamped on and the plates were loaded with the iron blocks.

(*c*) *Electromigration and detection.* A voltage of approximately 30 V/cm was applied across the strips, for one hour, after which the position of the various ions was determined.

(*d*) *Determination of the relative mobilities.* The relative mobilities, defined by:

$$\text{relative mobility of ion X} = \frac{\text{distance travelled by ion X} \times 100}{\text{distance travelled by the } Co^{2+} \text{ ion on the same strip}},$$

were evaluated from experiments with solutions of each of the ions together with the Co^{2+} ion.

(*e*) *Sensitivity determinations.* Decreasing concentrations of each of the ions studied were examined by the technique described above to determine the limits of detection.

RESULTS

The relative mobilities of several of the ions mentioned in the first section of this paper, together with standard deviations, are given in Table II. Their colour reactions with the developing reagents and the sensitivities of these reactions, are also given. 1 *N* citric acid cannot be used as a medium for those ions listed in Table III, for the reasons stated.

TABLE III

IONS THAT ARE DIFFICULT TO DEAL WITH IN THE CITRIC ACID MEDIUM

Species	*Reason*	*Other supporting electrolytes permitting a separation*
K^+, Na^+, Li^+	Detection with chloranilic acid, following the procedure of BARRETO AND BARRETO[11] gave negative results	0.1 *M* $(NH_4)_2CO_3$[3,5,12]; 0.75 *M* formic acid[3]
Cl^-	Is transferred to the agar gel around the anode under the conditions described	2% $(NH_4)_2CO_3$[4]
MnO_4^-	Not stable in the medium	1 *M* $(NH_4)_2CO_3$
SeO_3^{2-}, SeO_4^{2-}, TeO_3^{2-}	Too small differences in mobility	SeO_3^{2-} and TeO_3^{2-} in 2% $(NH_4)_2CO_3$ [4]; all three in 0.4 *N* Na_2SO_4 or 0.1 *N* H_2SO_4[13]
WO_4^{2-}	Is not easily detected in the 1 *N* citric acid medium	0.1 *M* lactic acid[14]; 1 *M* $(NH_4)_2CO_3$

APPLICATION OF THE METHOD IN QUALITATIVE ANALYSIS

A solution of the sample in 1 *N* citric acid is prepared. In the case of Bi^{3+} and Sb^{3+} where it is impossible to obtain such a solution due to hydrolysis, hydrochloric acid is added to the sample until a clear solution is obtained. Small amounts of HCl do not affect the results, but excess should be avoided.

The presence or absence of the Co^{2+} ion is established in a preliminary experiment. Electromigration for 15 min is sufficient to identify the ion as a yellow-brown zone after spraying the strip with dithiooxamide and neutralizing in an atmosphere of NH_3. If the result is negative or dubious, a $Co(NO_3)_2$ solution in 1 *N*

citric acid is added to the sample mixture, such that the concentration of Co^{2+} is approximately 5 g/l. Analysis of this solution is carried out as described above.

The position of the Co^{2+} ion (which travels about 20 cm), is determined by spraying one strip with dithioöxamide solution; the position of some other ions is also revealed by this reagent. Another strip is sprayed with oxine solution and examined. In general, many ions can be identified from the relative mobilities with respect to Co^{2+}; this is occasionally aided by characteristic colours or fluorescence of the oxinates. Confirmation is obtained by applying the other reagents mentioned in Table II to the remaining strips, preferably in the order given. If then the Co^{+2} ion is not found again, an additional spray with dithiooxamide, *e.g.* on the back of the paper, can be helpful.

DISCUSSION

One advantage of the procedure is that there is no perceptible electro-endosmosis; another one is that diffusion of products of electrolysis to the working zone is inhibited by the agar gel and the cellophane.

Because of the capillary action of the half-wet paper, we used the thread-technique described above (by which the initial zone width is only 2–3 mm), instead of the common methods with pipets, etc., which give unduly wide zones. In control experiments, spectrophotometric and titrimetric measurements showed that the quantity of solute transferred to the paper amounts to the portion present in 2.5–4 cm thread length, though only 2 cm is in actual contact with the paper. A disadvantage is that differences in diffusion velocities give rise to alterations in the ratios of the concentrations of the ions during their transport to the paper, *e.g.*, in the original solution the ratio of Fe^{3+}:Ag^{+} and Fe^{3+}:PO_4^{3-} was 1:1 but on the paper the former was 1:1.5 and the latter was 1:1.1. Finally it was found that the amount of solute transferred is hardly influenced either by variation of the humidity of thread and paper, the time of contact or the pressure applied to the thread.

In spite of the precautions taken, the humidity of the paper cannot be fully controlled and the ions will not cover strictly equal distances in the various paper strips. Therefore the use of the Co^{2+} ion as a reference standard for each strip appeared to be inevitable. The Co^{2+} ion has been chosen as a standard because it moves rather quickly and is easily detected. However, this introduces the necessity of a preliminary experiment to determine the presence of Co^{2+} in the mixture.

For reproducible results, the electromigration time should be kept roughly constant.

Most of the anions show only small differences in mobility and give rise to broad zones. This combined with the fact that anions are not easily detected, makes their analysis rather difficult. Though these objections are not fully eliminated it is better to avoid the possible disturbing action of the citric acid and use $(NH_4)_2CO_3$ as supporting electrolyte, a method described by LEDERER[4]. We recommend this electrolyte also for alkali metals[3,5]. Other groups that cannot be separated or distinguished by the method described are Al^{3+}–Y^{3+}, lanthanides, and Sr^{2+}–Ba^{2+}.

Though no relative mobilities are reported by EVANS AND STRAIN[6] and by MUKERJEE[7], the sequence in their experiments with restricted groups of ions is in general the same as the one reported here for the same supporting electrolyte. LEDERER[8] found a somewhat different sequence in 1 % citric acid.

As may be concluded from Table II the sensitivity of the method is not very high for some ions, *e g.* the alkaline earth metals. This can be explained by the fact that the sample solution is diluted during the diffusion from the thread and the subsequent electromigration. Also there will be competition between the complex formed by the ions with the citric acid and the developing reagents. However, many ions can be detected in concentrations ranging from 1:100 to 1:1000 (w/v), and an experienced analytical chemist may be able to detect even lower concentrations.

The oxidation reaction before application of *o*-tolidine should be performed quickly but carefully; too heavy spraying with $4N$ NaOH and too prolonged treatment with bromine weaken the paper. This reaction for Cr^{3+} is not always reliable as the zones of Cr^{3+} and Co^{2+} coincide, and the presence of a reagent, used for the detection of the latter, interferes with the identification of the former. For this reason no definite value for the relative mobility of Cr^{3+} can be given. It is advisable to oxidise the Cr^{3+} to CrO_4^{2-} before the electromigration. The citric acid does not perceptibly reduce this ion during the experiment, even at a hydrochloric acid concentration of $2N$, except in the presence of Mn^{2+} or Ce^{3+}.

The excess of acid necessary to keep some ions (Bi^{3+}, Sb^{3+}, Sn^{4+} and Ti^{4+}) in solution does not affect the relative mobilities of the cations. The mobilities of the anions are slightly lower because of the initially high ionic concentration and thus lower field strength in the anodically moving zones (see also EDWARD[9]). However, Au^{3+}, moving principally as the chloroaurate (III) ion (as concluded from the appearance of chloride in the zone after precipitation of the metal), is 10% faster in the presence of $2N$ hydrochloric acid. The effect of hydrochloric acid on Fe^{3+} is more serious though; in $2N$ acid the relative mobility was nearly twice the original value. The sensibility to small local differences in hydrochloric acid concentration may be the cause of the corrugated appearance and trailing of the Fe^{3+} zone, and if Fe^{3+} and Au^{3+} are present together with hydrochloric acid interference with the detection of some of the faster moving ions could occur.

In conclusion, the composition of a sample, containing a mixture of ions can be determined by the above technique within a short time. The method has been incorporated in the students curriculum in this laboratory.

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SUMMARY

A method for qualitative analysis of mixtures of inorganic ions by paper electromigration in $1N$ citric acid at 30 V/cm is described. A thread technique for the application of the sample is described, which gives narrow initial zones. Thirty-eight ions are characterized by their relative mobilities with respect to the Co^{2+} ion and their colour reactions with spray reagents; the sensitivities have also been determined. Analysis of mixtures by this technique is satisfactory and rapid.

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THE PAPER CHROMATOGRAPHIC IDENTIFICATION OF COMPOUNDS USING TWO REFERENCE COMPOUNDS*

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INTRODUCTION

R_F values are subject to significant variation if the experimental conditions are changed in a non-reproducible manner from one experiment to another. Well known reasons for such variations are changes in the dimensions of the apparatus, the grade of paper and its slight heterogeneity, the direction (ascending, descending, or horizontal) and the length of the solvent flow, the duration of development, the equilibration time, the temperature, the composition of the solvent travelling along the paper, the foreign ions and impurities in the solvent or on the paper, the volume of solvent used, the concentrations of the substances being chromatographed, the nature of the mixture being chromatographed and previous mode of treatment (type of desalting, etc.), the distance between the starting point and the source of solvent, the losses of solvent from the tank, the irregular travelling of the solvent along the paper, the incorporation of any other liquid or vapours for special purposes, and many other variables.

It is therefore generally accepted that the R_F values are not sufficient for the identification of a substance. In order to establish with certainty the identity of a substance, it has to be chromatographed simultaneously with an authentic sample of the suspected compound; but this entails the necessity of having a large collection of chemical substances, which is obviously a great disadvantage especially in cases of investigations on substances not commercially available, unstable compounds, etc.

In their classical communication on paper chromatography CONSDEN, GORDON AND MARTIN[1] pointed out that there are variations in the R_F values of a substance even when duplicate runs are made simultaneously in the same chamber. However, since then an enormous number of publications have dealt with the nature and the extent of the variations of R_F values caused by each of the above-mentioned conditions as well as their unforeseen changes during the development. As a result of these studies information has been obtained about the factors which may change the R_F values, and the way of controlling them in order to obtain reproducible results. Although an R_F variability of less than $\pm$ 0.02 has been achieved[2,3] in a few cases, such precision is not usually obtained in practice, so that the main problem *of identifying a substance without having to chromatograph simultaneously an authentic sample* remains unsolved, since all of the aforesaid publications approach this problem in a qualitative way.

An attempt to solve this problem quantitatively is described in the present report.

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The introduction of relative values, R_K, given by the ratio

$$R_K = \frac{\text{Distance of the studied substance X from the start}}{\text{Distance of the reference substance K from the start}}$$

can obviously be considered as a first step. R_K values are generally considered to be more reliable criteria for the identification of a substance than its R_F values; *i.e.* not the individual R_F values themselves, but *the pattern* of a chromatogram is more important for characterizing the components of a mixture.

EXPERIMENTAL

Procedure

A number of compounds belonging to different classes of organic substances were submitted to a series of paper chromatographic separations as follows:

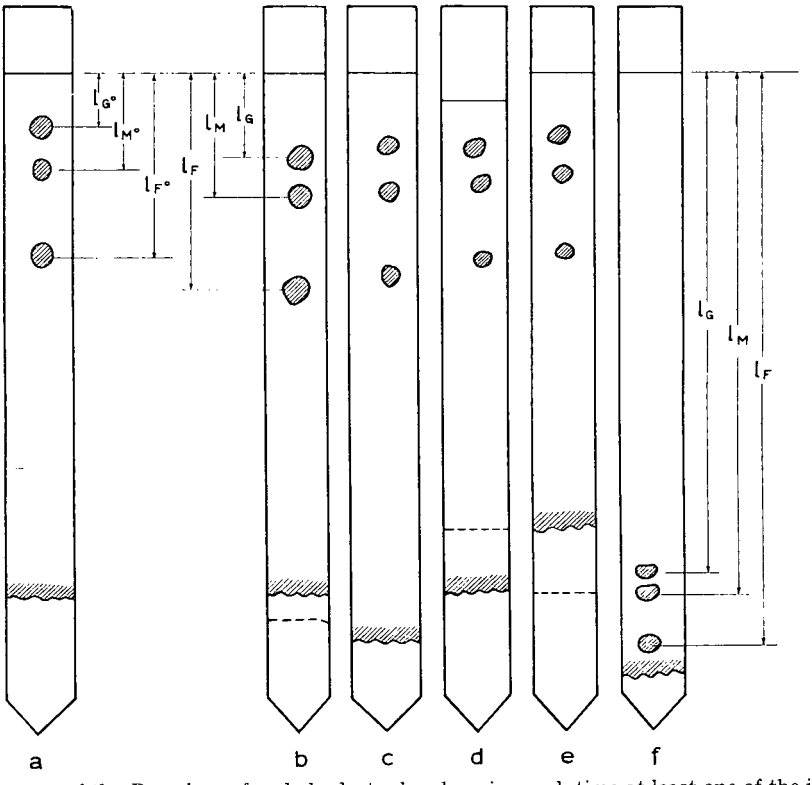

Fig. 1. Changes of the R_F values of carbohydrates by changing each time at least one of the initial experimental conditions. The initial experimental conditions, strip (a), were as follows: solvent, ethyl acetate–acetic acid–water 70:15:15 (v/v/v); method, descending (all glass apparatus, Shandon, 30 × 17 × 55 cm); time of development, 12 h; pre-equilibration time, 4 h; volume of solvent in the tank, 60 ml; carbohydrates studied, galactose (G), mannose (M) and fucose (F); temperature, 20°; paper, Whatman No 1. The conditions changed each time were the following: strip (b), the water content of the solvent (slightly increased). Strip (c), the time of development (16 h). Strip (d), the distance between starting point and solvent source. Strip (e), pre-equilibration of the tank (none). Strip (f), the tank was replaced by another constructed of wood; losses of solvent through cracks of this tank could not be prevented. Development time, 24 h.

A paper chromatogram was first developed under the conditions described by another worker. Next, several chromatograms of the same substance were carried out, but at least one of the initial conditions was changed each time. A comparison of the R_F values thus obtained illustrated the manner in which variations of the R_F values are caused.

The experimental conditions, together with any changes, are given in the legends to the figures and tables.

Results

The results thus obtained were very instructive, although many of them have been described before.

The variations in R_F value for carbohydrates given in Fig. 1 are very similar to those obtained with all hydrophilic substances studied in this laboratory.

The type of variation in R_F values of polar lipids illustrated in Fig. 2 occurs mainly in paper chromatographic runs of all lipophilic substances (glycerides, free fatty acids,

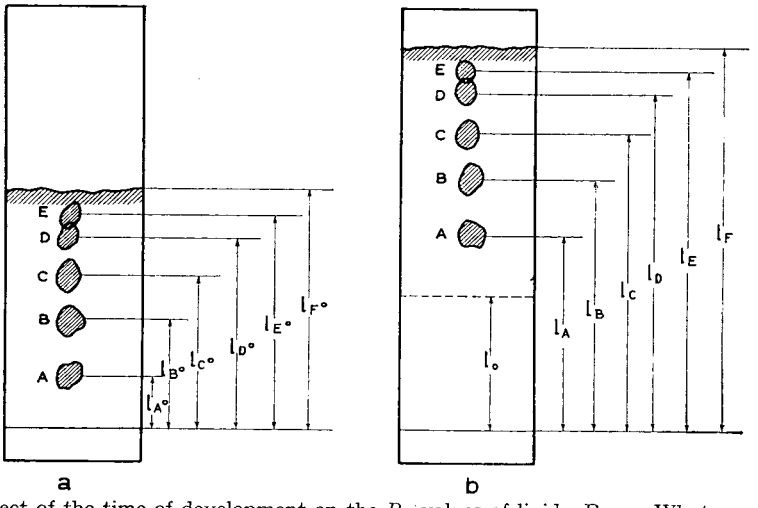

Fig. 2. Effect of the time of development on the R_F values of lipids. Paper, Whatman No. 1 impregnated with silicic acid; solvent, 85 vol. of chloroform–acetone 4:1 (v/v) mixed with 15 vol. of glacial acetic acid–water 9:1 (v/v); method, ascending (all glass apparatus, Shandon); time of development, 3 and 6 h respectively for strips (a) and (b). Lipids studied, sphingomyelin (A), phosphatidylcholine (B), phosphatidylserine (C), phosphatidylethanolamine (D) and cerebroside (E), 30 μg each spot. Volume of solvent in the tank, 200 ml; pre-equilibration time, 1 h; temperature, 20°

sterols, etc.). However, similar results were also observed during a paper chromatographic investigation of hydrophilic deacylation products of phosphatides, and also in cases in which ideal conditions were not fulfilled (*e.g.* strip (f) in Fig. 1).

CLASSIFICATION OF THE R_F VARIATIONS

The variations caused by the aforesaid experimental conditions to the mobilities of compounds submitted to chromatography can be classified into the following three groups:

1. Variations which do not change the R_F values themselves, but change the distances of the spots from the starting point, *i.e.* influence the separability of the substances submitted to chromatography (see strips (a) and (c) in Fig. 1). Variations of this type are not contradictory to the postulate of the constancy of R_F values, therefore:

$$R_F^\circ = R_F \tag{1}$$

The symbols R_F° and R_F are used throughout the present report for tabular and measured R_F values, respectively. The term *tabular R_F values* is here used to define any set of R_F values either given in the literature, or uniquely determined in the laboratory, *i.e. tabular R_F values are sets of values taken as models, and which one tries to reproduce.* Any other R_F values of the same group of compounds are here defined as *measured R_F values.*

2. Variations which change the R_F values without changing the ratios of the distances of the spots from the starting point, *e.g.*, $l_G^\circ : l_M^\circ : l_F^\circ = l_G : l_M : l_F$ [see strips (a), (b), (d), and (e) in Fig. 1]. Variations of this type can be expressed as follows:

$$R_F^\circ = a \cdot R_F \tag{2}$$

where a is a coefficient. The postulate of the constancy of R_F values continues to be true, if allowance is made for a transfer of the solvent front (dotted lines in strips (b), (d), and (e) of Fig. 1).

It should be noted that, long ago, PARTRIDGE AND WESTALL[4] pointed out that such a linear variation was caused by temperature alterations. According to these authors, in order to obtain greater constancy between the R_F values observed, the R_F's determined at a temperature t° have to be corrected to the standard R_F's at 20° by multiplying by the factor

$$a = \frac{R_F \text{ of glucose at } 20^\circ}{R_F \text{ of glucose at } t^\circ}$$

3. Variations which change both the individual R_F values and the ratios of the distances of the spots from the starting line, but still do not change the pattern of the chromatogram if allowance is made for a transfer of the starting line (dotted line in strip (b) of Fig. 2). In such cases the separability of the spots is obviously not affected, *i.e.* the distances between the spots remain constant ($l_B^\circ - l_A^\circ = l_B - l_A, l_D^\circ - l_C^\circ = l_D - l_C$, etc.; Fig. 2). Variations of this type can be expressed as follows:

$$(R_F^\circ)_X = \frac{l_X^\circ}{l_F^\circ} = \frac{l_X - l_0}{l_F - l_0} = \frac{l_F}{l_F - l_0} \cdot (R_F)_X - \frac{l_0}{l_F - l_0} \tag{3}$$

where $(R_F^\circ)_X$ and $(R_F)_X$ are the tabular and measured R_F values of a substance X respectively.

Although an examination of each of the above-mentioned variables is neither possible, nor practically important in ordinary paper chromatographic work, it has been shown that the following general equation:

$$R_F^\circ = a \cdot R_F + b \tag{4}$$

can be applied experimentally.

This equation has been based on the following postulates:

(i) Any kind of R_F variations can be classified in one of the previously mentioned three groups, *i.e.* there are no factors which can influence differently the individual R_F values of the compounds co-chromatographed or, at least, such factors if they exist, cannot cause significant changes in the R_F's.

(ii) All R_F variations are additive properties, *i.e.* the coefficients a and b of eqn. (4) represent in each case the sum of many individual coefficients a_1, a_2, a_3, ..., or b_1, b_2, b_3, ..., corresponding to different experimental variables which are not individually of any practical importance.

It is obvious that coefficients a and b can be determined experimentally by measuring in each case the R_F values of two authentic samples:

$$a = \frac{(R_F^\circ)_A - (R_F^\circ)_B}{(R_F)_A - (R_F)_B} \quad \text{and} \quad b = (R_F^\circ)_A - a \cdot (R_F)_A \tag{5}$$

where $(R_F^\circ)_A$, $(R_F^\circ)_B$ are the tabular R_F values, and $(R_F)_A$, $(R_F)_B$ the measured ones of the two reference compounds A and B.

It should be noted that although eqns. (3) and (4) look very similar, they are actually different, namely, the sum of the two parameters of eqn. (3) is obviously equal to one, while this is not necessarily true in the case of the general eqn. (4).

APPLICATIONS

One-dimensional run

An acidic hydrolyzate of a polysaccharide was submitted to paper chromatographic separation using the isopropanol–water 80:20 (v/v) solvent system. The tabular R_G values of various monosaccharides in this system (Table I) were taken from SMITH[5].

TABLE I

R_G VALUES OF MONOSACCHARIDES ON WHATMAN NO. 4 PAPER, TAKEN FROM SMITH[5]

(Solvent: isopropanol–water, 80:20)

Compound	R_G	*Compound*	R_G
Rhamnose	1.52	Sorbose	1.02
Ribose	1.37	Glucose	1.00
Fucose	1.35	Galactose	0.80
Xylose	1.30	Glucosamine	0.65
Arabinose	1.12	Muramic acid	0.62
Mannitol	1.10	Galactosamine	0.50
Dulcitol	1.05	Inositol	0.35
Sorbitol	1.05	Glucuronic acid	0.20
Mannose	1.05	Galacturonic acid	0.15
Fructose	1.05		

The chromatogram is illustrated in Fig. 3, where it is evident that this hydrolyzate contains five components, one of which (spot C) is galactose. The measured distances of the spots from the starting line are: $l_A = 4.1$ cm, $l_B = 8.7$ cm, $l_C = 11.0$ cm,

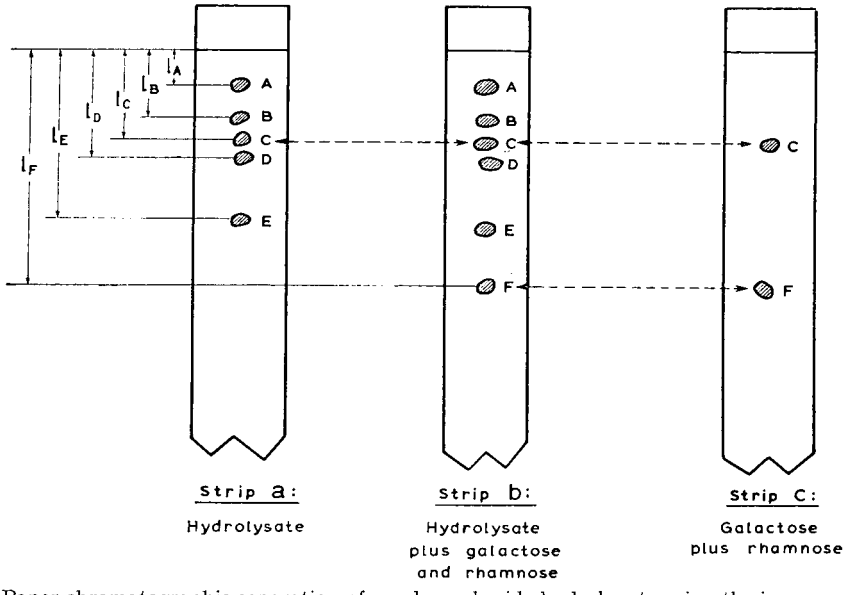

Fig. 3. Paper chromatographic separation of a polysaccharide hydrolyzate using the isopropanol–water 80:20 (v/v) solvent system. Method, descending, 24 h. Paper, Whatman No. 1.

$l_D = 14.1$ cm, $l_E = 18.7$ cm, $l_F = 22.1$ cm. Using eqn. (5) for the two reference compounds, galactose and rhamnose,

$$a = \frac{(R_G^\circ)_F - (R_G^\circ)_C}{l_F - l_C} = \frac{1.52 - 0.80}{22.1 - 11.0} = 0.065 \qquad b = 0.80 - 0.065 \times 11.0 = 0.085$$

Then, according to eqn. (4), the following R_G°'s are calculated:

Spot A: $0.065 \times 4.1 + 0.085 = 0.35$ (Inositol)
Spot B: $0.065 \times 8.7 + 0.085 = 0.65$ (Glucosamine)
Spot D: $0.065 \times 14.1 + 0.085 = 1.00$ (Glucose)
Spot E: $0.065 \times 18.7 + 0.085 = 1.30$ (Xylose)

These calculations have been made using R_G°'s instead of R_F°'s, and l_X's instead of $(R_F)_X$'s in eqns. (4) and (5). Coefficients a and b calculated in this way are different from the ones obtained if the aforesaid replacement had not taken place, but obviously lead to the same R_G° values.

It is evident that the evaluation of paper chromatographic results by this method leads to reliable findings in nearly all cases, whereas the older method of using a single reference compound gives confusing results. For instance, in the case examined above, using galactose (spot C) as the reference compound, the following results are obtained:

$(R_g)_A = 4.1:11.0 = 0.37$	$(R_G^\circ)_A = 0.37 \times 0.80 = 0.30$ (?)
$(R_g)_B = 8.7:11.0 = 0.79$	$(R_G^\circ)_B = 0.79 \times 0.80 = 0.63$ (Muramic acid)
$(R_g)_D = 14.1:11.0 = 1.28$	$(R_G^\circ)_D = 1.28 \times 0.80 = 1.02$ (Sorbose)
$(R_g)_E = 18.7:11.0 = 1.70$	$(R_G^\circ)_E = 1.70 \times 0.80 = 1.36$ (Fucose or Ribose)

Here, R_g's are the relative R values measured with respect to galactose. Misleading results are also obtained using rhamnose (spot F) as the reference compound; *i.e.* calculating as described above, the following data are obtained:

$(R_G°)_A = 0.28$ (?)
$(R_G°)_B = 0.60$ (Muramic acid ?)
$(R_G°)_D = 0.97$ (Glucose ?)
$(R_G°)_E = 1.28$ (Xylose ?)

Two-dimensional run

A mixture of 10 amino acids was chromatographed by the two-dimensional technique using *n*-butanol–acetic acid–water 120:30:50 (v/v/v) for the first direction and phenol–water–ammonia 160:40:1 (v/v/v) for the second one. The tabular $R_F°$ values of the chromatographed amino acids (Table II) were taken from SMITH[5]. The chromatogram is illustrated in Fig. 4, and the measured R_F values are given in Table II.

TABLE II

TABULAR AND MEASURED R_F VALUES OF TEN AMINO ACIDS IN TWO SOLVENT SYSTEMS

	Amino acid	*n-Butanol–acetic acid–water 120:30:50 (v/v/v)*		*Phenol–water–ammonia 160:40:1 (v/v/v)*	
		$R_F°$	R_F	$R_F°$	R_F
A.	Aspartic acid	0.23	0.210	0.17	0.150
B.	Cystine	0.05	0.075	—	0.380
C.	Glutamic acid	0.28	0.250	0.26	0.260
D.	Serine	0.22	0.200	0.35	0.340
E.	Threonine	0.26	0.235	0.48	0.485
F.	Alanine	0.30	0.265	0.58	0.600
G.	Histidine	0.11	0.118	0.72	0.735
H.	Valine	0.51	0.415	0.78	0.805
I.	Phenylalanine	0.60	0.485	0.84	0.875
J.	Leucine	0.72	0.570	0.85	0.880

Now suppose that two of these amino acids, aspartic acid (spot A) and leucine (spot J) were used as the two reference compounds, all the others being unknown. According to eqn. (5) for the first solvent system:

$$a = \frac{0.72 - 0.23}{0.57 - 0.21} = 1.36 \quad \text{and} \quad b = 0.72 - 1.36 \times 0.57 = -0.055$$

Then using eqn. (4), the following $R_F°$ values are calculated:

$(R_F°)_B = 1.36 \times 0.075 - 0.055 = 0.047$ (Cystine)
$(R_F°)_C = 1.36 \times 0.250 - 0.055 = 0.285$ (Glutamic acid)
$(R_F°)_D = 1.36 \times 0.200 - 0.055 = 0.217$ (Serine)
$(R_F°)_E = 1.36 \times 0.235 - 0.055 = 0.265$ (Threonine)
$(R_F°)_F = 1.36 \times 0.265 - 0.055 = 0.305$ (Alanine)
$(R_F°)_G = 1.36 \times 0.118 - 0.055 = 0.115$ (Histidine)
$(R_F°)_H = 1.36 \times 0.415 - 0.055 = 0.509$ (Valine)
$(R_F°)_I = 1.36 \times 0.485 - 0.055 = 0.605$ (Phenylalanine)

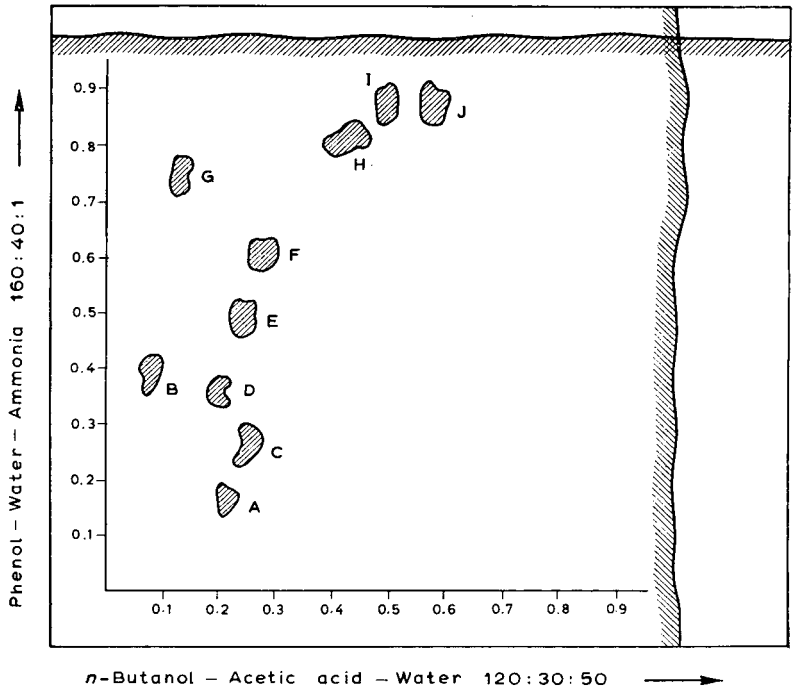

Fig. 4. Two-dimensional chromatogram of ten amino acids.

Similarly, for the second solvent system:

$$a = \frac{0.85 - 0.17}{0.88 - 0.15} = 0.93 \quad \text{and} \quad b = 0.85 - 0.93 \times 0.88 = 0.032$$

$(R_F°)_B = 0.93 \times 0.380 + 0.032 = 0.386$ (Cystine)
$(R_F°)_C = 0.93 \times 0.260 + 0.032 = 0.274$ (Glutamic acid)
$(R_F°)_D = 0.93 \times 0.340 + 0.032 = 0.348$ (Serine)
$(R_F°)_E = 0.93 \times 0.485 + 0.032 = 0.485$ (Threonine)
$(R_F°)_F = 0.93 \times 0.600 + 0.032 = 0.590$ (Alanine)
$(R_F°)_G = 0.93 \times 0.735 + 0.032 = 0.716$ (Histidine)
$(R_F°)_H = 0.93 \times 0.805 + 0.032 = 0.782$ (Valine)
$(R_F°)_I = 0.93 \times 0.875 + 0.032 = 0.847$ (Phenylalanine)

Multiple development

In multiple development the mobility of a substance can theoretically be calculated as follows:

$R_1 = R_F$
$R_2 = R_1 + R_F \cdot (1 - R_1) = 2\,R_F - R^2_F$
$R_3 = R_2 + R_F \cdot (1 - R_2) = 3\,R_F - 3\,R^2_F + R^3_F$
.....
$R_n = 1 - (1 - R_F)^n$

where R_1, R_2,... R_n are the measured R_F values after one, two,..., n, developments respectively. The last of the above equations can be expressed as follows:

$$R_F = 1 - \sqrt[n]{1 - R_n} \tag{6}$$

But owing to the fact that in multiple development the previously described R_F variations are much more marked compared with ones in single development, no attempt until now has been made to calculate R_F values using eqn. (6), *i.e.* to identify unknown compounds by means of this equation.

However, a theoretical examination of this problem from the viewpoint developed in this paper led to the following very interesting results, which have been experimentally verified.

If eqn. (4) (solved with respect to R_F) is used in calculations of the mobility of a substance after a multiple development one obtains:

$R_1 = a' \cdot R_F^\circ + b'$ (where $a' = 1/a$ and $b' = -b/a$)
$R_2 = R_1 + a' \cdot R_F^\circ \cdot (1 - R_1) + b' \cdot (1 - R_1) = 1 - (1 - R_1)^2$
$R_3 = 1 - (1 - R_1)^3$
.....
$R_n = 1 - (1 - R_1)^n$

By transforming the last equation,

$$R_F^\circ = c - d \cdot \sqrt[n]{1 - R_n} \tag{7}$$

where c and d are coefficients derived from a and b,

$$c = \frac{1 - b'}{a'} = a + b \quad \text{and} \quad d = \frac{1}{a'} = a$$

It is evident that eqn. (7) can be used to calculate the tabular R_F° values of unknown substances, *i.e.* to identify them by their R_n values measured after n developments in a certain solvent system. Two reference compounds are again required for the calculation of coefficients c and d of eqn. (7):

$$d = \frac{(R_F^\circ)_A - (R_F^\circ)_B}{\sqrt[n]{1 - (R_n)_B} - \sqrt[n]{1 - (R_n)_A}} \quad \text{and} \quad c = (R_F^\circ)_A + d \cdot \sqrt[n]{1 - (R_n)_A} \tag{8}$$

It should be noted that by similar, but more complex, calculations it can be shown that eqn. (7) leads to correct results even in cases where the coefficients a' and b' are not exactly identical in the repeated developments of the same chromatogram. In such a case if $a_i = a' + y_i$ and $b_i = b' + z_i$ (where y_i and z_i are small as compared to a' and b'), factors such as $y_i^2 R_F$, $a' \cdot y_i \cdot R_F^2$, etc. can obviously be neglected. In this manner an equation similar to eqn. (7) results, in which the coefficients c and d are not equal to $(a + b)$ and a respectively, but still have the same values for all compounds co-chromatographed.

Experimental verification. A mixture of 10 amino acids was chromatographed three times by the ascending technique in the *n*-butanol–acetic acid–water 120:30:50

(v/v/v) solvent system, for which the tabular $R_F°$ values are given in Table II. The R_3 values experimentally determined are:

A. Aspartic acid	0.505	F. Alanine	0.605
B. Cystine	0.210	G. Histidine	0.320
C. Glutamic acid	0.580	H. Valine	0.800
D. Serine	0.490	I. Phenylalanine	0.860
E. Threonine	0.555	J. Leucine	0.915

Now suppose, as previously, that aspartic acid and leucine were the two reference compounds, all other spots being unknown. Using eqn. (8):

$$d = \frac{0.720 - 0.230}{\sqrt[3]{1 - 0.505} - \sqrt[3]{1 - 0.915}} = 1.40 \qquad c = 0.72 + 1.40 \times \sqrt[3]{1 - 0.915} = 1.335$$

Then, by eqn. (7) the tabular $R_F°$ values of the spots B to I are calculated as follows:

$$(R_F°)_B = 1.335 - 1.40 \times \sqrt[3]{1 - 0.210} = 0.045 \text{ (Cystine)}$$
$$(R_F°)_C = 1.335 - 1.40 \times \sqrt[3]{1 - 0.580} = 0.285 \text{ (Glutamic acid)}$$
$$(R_F°)_D = 1.335 - 1.40 \times \sqrt[3]{1 - 0.490} = 0.215 \text{ (Serine)}$$
$$(R_F°)_E = 1.335 - 1.40 \times \sqrt[3]{1 - 0.555} = 0.265 \text{ (Threonine)}$$
$$(R_F°)_F = 1.335 - 1.40 \times \sqrt[3]{1 - 0.605} = 0.305 \text{ (Alanine)}$$
$$(R_F°)_G = 1.335 - 1.40 \times \sqrt[3]{1 - 0.320} = 0.105 \text{ (Histidine)}$$
$$(R_F°)_H = 1.335 - 1.40 \times \sqrt[3]{1 - 0.800} = 0.515 \text{ (Valine)}$$
$$(R_F°)_I = 1.335 - 1.40 \times \sqrt[3]{1 - 0.860} = 0.605 \text{ (Phenylalanine)}$$

DISCUSSION

The present method of calculating tabular $R_F°$ values using two reference compounds seems to be applicable to paper chromatographic separations of any kind of organic substance. This is true even in cases where the results observed ought to be discarded as unreliable, if examined on the basis of the older method of one reference compound.

The only point which needs experimental verification is that the coefficients a and b of eqn. (4) are the same for all compounds co-chromatographed. It can be shown that in every chromatogram these coefficients are indeed the same for all compounds belonging to the same class of organic substances, but this is not true for compounds belonging to different organic classes. Thus, for all carbohydrates (pentoses, hexoses, etc.) the coefficients calculated for any given chromatogram were found to be equal. The coefficients a and b for amino acids were found to be different from the ones for co-chromatographed carbohydrates, although these differences were not of great significance for all the solvent systems. This can possibly be explained by the fact that although amino acids are similar to carbohydrates, as far as polarity is concerned, their separation on paper is also affected by their ampholytic nature; this influence being in some cases strong, and in other cases weak.

In some cases this difference in the coefficients a and b for co-chromatographed compounds can obviously lead to invaluable deductions about the nature of unknown substances. For instance, during a study on an acidic hydrolyzate of a

fraction of milk lipids, the results illustrated in Fig. 5 were obtained. These indicate that the two constituents of this hydrolyzate (see spots A and B in Fig. 5) are probably not of a purely carbohydrate nature, since their chromatographic properties are rather similar to the ones of lipophilic substances, *i.e.* large negative *b* values, as in the case of Fig. 2.

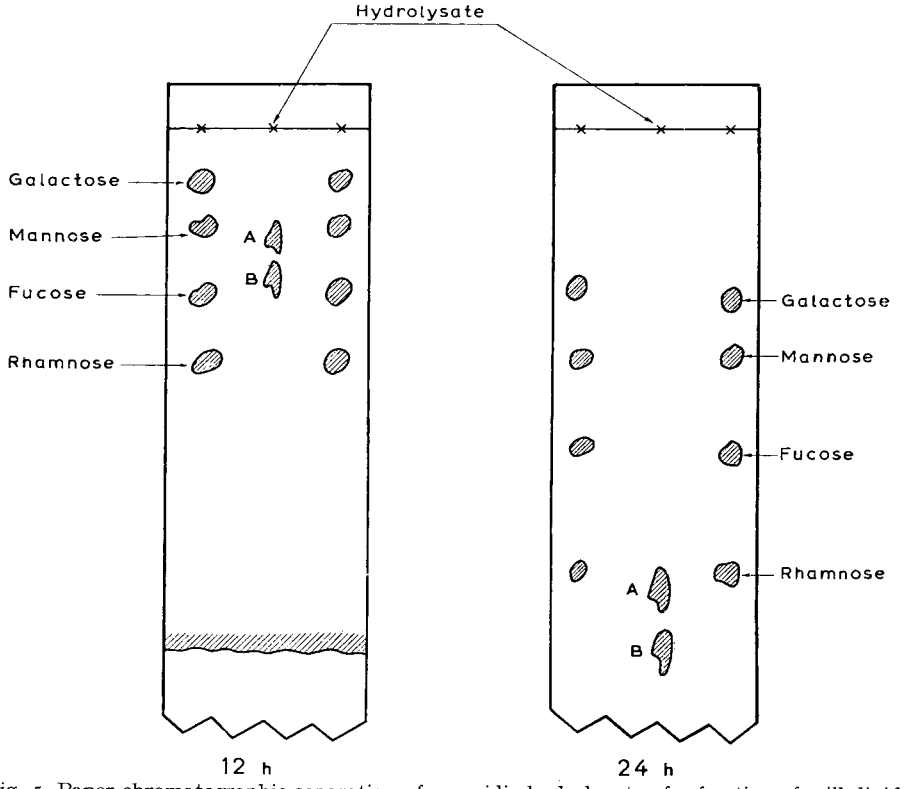

Fig. 5. Paper chromatographic separation of an acidic hydrolyzate of a fraction of milk lipids in ethyl acetate–pyridine–water 2:1:2 (v/v/v). All spots were located by both the silver nitrate and the aniline phthalate reagents.

SUMMARY

A general method using two reference compounds for the paper chromatographic identification of unknown substances is described.

The method devised is based on the fact that any kind of important variations to the paper chromatographic mobility of various substances co-chromatographed caused by different experimental conditions, can be classified into three groups of linear variations.

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PAPIERCHROMATOGRAPHISCHER NACHWEIS VON AMINOSÄUREN MIT DEHYDROASCORBINSÄURE

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In Zusammenhang mit präparativen und analytischen Arbeiten über die Dehydroascorbinsäure fanden wir in der Literatur einige Hinweise über ihre Reaktivität auf Amine und Aminosäuren. ABDERHALDEN[1] erkannte bei verschiedenen Aminosäuren nach Zusatz von Ascorbinsäurelösung schwache Farbveränderungen, die sich bei Zugabe älterer Lösungen verstärkten. Vor allem Histidin zeigte nach kurzer Zeit eine rötlich violette Färbung; aber auch Alanin oder Phenylalanin wiesen nach bestimmten Zeiten charakteristische Veränderungen auf. PECHERER[2] beobachtete ebenfalls bei einer Reihe von Aminosäuren nach Zusatz der von ihm dargestellten Dehydroascorbinsäure eine Farbveränderung nach Rot, die besonders beim Erhitzen auftrat.

Wir haben diese bisher kaum beachtete Reaktion aufgegriffen und versucht, sie auf das Papier zu übertragen. Die notwendige Dehydroascorbinsäure als Reinsubstanz wurde nach einem eigenen Verfahren aus Ascorbinsäure durch Oxydation mit Selendioxyd hergestellt[3]. Wir verteilten zunächst 20 Aminosäuren auf dem Papier und besprühten mit Dehydroascorbinsäurelösungen verschiedener Zusammensetzung und zum Vergleich auch mit Ninhydrinreagens. Mit Dehydroascorbinsäure erhält man nach Erwärmen auf 100° rosarote Flecke unterschiedlicher Farbtiefe, die gut begrenzt erscheinen. Nicht erkennbar waren Oxyprolin und Prolin, während Phenylalanin, Tyrosin und Tryptophan nur schwache Reaktionen gaben (Tabelle I).

Um die Empfindlichkeitsgrenze des Aminosäurenachweises mit Dehydroascorbinsäure kennenzulernen, wurden Verdünnungsreihen von 0.2 bis 50 μg Aminosäure papierchromatographisch verteilt und mit Dehydroascorbinsäure sichtbar gemacht (Tabelle II). Dabei zeigte sich, dass Oxyprolin und Prolin auch nachweisbar sind, sich aber nur sehr schwach anfärben, die Grenze liegt bei 25 μg. Phenylalanin, Tyrosin und Tryptophan können bis etwa 10 μg und alle übrigen Aminosäuren bis herab zu etwa 2 μg identifiziert werden.

Die typische Rosafärbung des Aminosäurefleckes nach dem Erwärmen setzt das Vorhandensein einer bestimmten Dehydroascorbinsäurekonzentration voraus. Papierchromatographisch verteilte Aminosäuren wurden mit Dehydroascorbinsäurelösungen verschiedener Konzentration behandelt (Tabelle III). Eine Rosafärbung tritt erst bei einer Konzentration von 0.05 % Dehydroascorbinsäure auf, während die 1 %ige Lösung sehr farbtiefe Flecke gibt. Nach unseren Erfahrungen dürfte die 0.5 %ige Dehydroascorbinsäurelösung zur Erkennung von Aminosäuren auf dem Papier ausreichend sein. Die Erhitzungszeiten (5 Min.) müssen ebenso wie die Temperaturen (100°) möglichst konstant gehalten werden, da längeres Erwärmen oder Steigerung der Temperatur die Flecke graubraun verfärben.

TABELLE I

FARBREAKTIONEN EINIGER AMINOSÄUREN MIT DEHYDROASCORBINSÄURE IM VERGLEICH MIT NINHYDRIN

Nr.	*Aminosäure*	*Sprühreagens*			
		A	*B*	*C*	*Ninhydrin*
1	Alanin	rosa rot	rosa-rot	rosa-rot	violett
2	Asparagin	rosa-rot	rosa-rot	rosa-rot	violett
3	Asparaginsäure	rosa-rot	rosa-rot	rosa-rot	violett
4	Cystin	bräunlich-rot	bräunlich-rot	bräunlich-rot	violett
5	Glutaminsäure	rosa-rot	rosa-rot	rosa-rot	violett
6	Glykokoll	rosa-rot	rosa-rot	rosa-rot	violett
7	Histidin	violett-rot	violett-rot	violett-rot	violett
8	*d,l*-Homocystein	rosa-rot	rosa-rot	rosa-rot	violett
9	Lysin	rosa-rot	rosa-rot	rosa-rot	violett
10	Leucin	rosa-rot	rosa-rot	rosa-rot	violett
11	Isoleucin	rosa-rot	rosa-rot	rosa-rot	violett
12	*d,l*-Methionin	rosa-rot	rosa-rot	rosa-rot	violett
13	Oxyprolin	—	—	—	schwach gelb
14	Phenylalanin	schwach rosa	schwach rosa	schwach rosa	violett
15	Prolin	—	—	—	schwach gelb
16	Serin	rosa	rosa	rosa	violett
17	Threonin	rosa-rot	rosa-rot	rosa-rot	violett
18	Tyrosin	schwach rosa	schwach rosa	schwach rosa	—
19	Tryptophan	schwach rosa	schwach rosa	schwach rosa	schwach violett
20	Valin	rosa-rot	rosa-rot	rosa-rot	violett

TABELLE II

EMPFINDLICHKEITSGRENZEN DES AMINOSÄURENACHWEISES MITTELS DEHYDROASCORBINSÄURE

Nr.	*Aminosäure*	*Erkennbar ab µg*	*Farbton*
1	Alanin	2	rosa
2	Asparagin	3	rosa
3	Asparaginsäure	2	rosa
4	Cystin	2	bräunlich-rot
5	Glutaminsäure	1	rosa-rot
6	Glykokoll	2	rosa-rot
7	Histidin	2	violett-rot
8	*d,l*-Homocystein	3	rosa
9	Lysin	3	rosa
10	Leucin	2	rosa
11	Isoleucin	3	rosa
12	*d,l*-Methionin	2	rosa
13	Oxyprolin	25	schwach violett-blau
14	Phenylalanin	10	schwach rosa
15	Prolin	25	schwach gelb
16	Serin	2	rosa
17	Threonin	3	rosa
18	Tyrosin	10	schwach rosa
19	Tryptophan	10	schwach rosa
20	Valin	2	rosa

TABELLE III

FARBBILDUNG IN ABHÄNGIGKEIT VON DER DEHYDROASCORBINSÄUREKONZENTRATION

Dehydroascorbinsäurelösung (%)	*Farbton*	*Dehydroascorbinsäurelösung (%)*	*Farbton*
0.001	schwach bräunlich-gelb	0.05	schwach rosa
0.002	schwach bräunlich-gelb	0.1	rosa
0.005	schwach bräunlich-gelb	0.2	rosa
0.01	schwach bräunlich-gelb	0.5	rosa
0.02	schwach bräunlich-gelb	1.0	rot

Unter Einhaltung der beschriebenen Versuchsbedingungen ist die Dehydroascorbinsäure jedenfalls recht gut zur Identifizierung von Aminosäuren auf dem Papier geeignet. Wie am Beispiel eines Originalchromatogramms zu ersehen ist, kann man Glykokoll nach papierchromatographischer Verteilung im Bereich von 1 bis 50 μg deutlich sichtbar machen (Fig. 1).

Einer quantitativen Auswertung der Chromatogramme stehen zunächst Hemmnisse im Wege. Die rosa Farbverbindungen lassen sich zwar mit Wasser aus dem Papier herauslösen, sind aber sehr instabil und liefern demzufolge keine befriedigend reproduzierbaren Werte. Auch das zur Stabilisierung der Ninhydrinflecke verwendete Kupferreagens nach KAWERAU UND WIELAND[4] führte zu keinem sichtbaren Erfolg.

Die Farbreaktion dürfte ähnlich verlaufen, wie die Umsetzung zwischen Ninhydrin und Aminosäuren; Alanin spaltete neben Ammoniak noch Kohlendioxyd und Acetaldehyd ab[2]. Untersuchungen über den möglichen Reaktionsablauf zwischen Dehydroascorbinsäure und Aminosäuren sind im Gange.

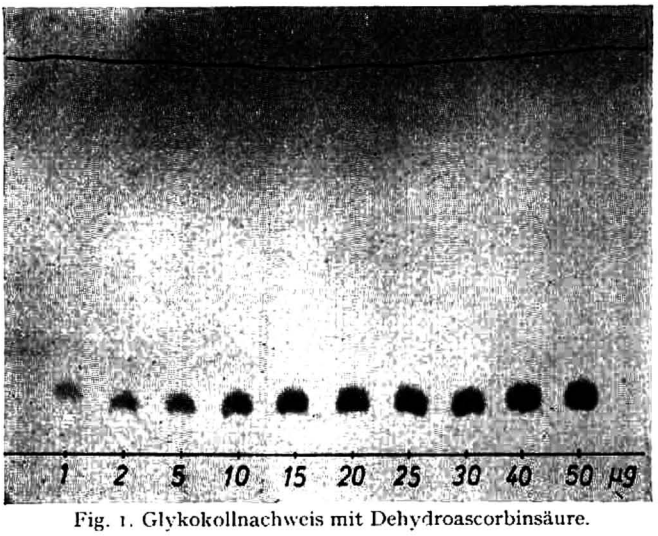

Fig. 1. Glykokollnachweis mit Dehydroascorbinsäure.

VERSUCHSTEIL

1. Nachweis der Aminosauren mit Dehydroascorbinsäure

(*a*) *Sprühreagentien.* Reagenslösung A: 0.1 g Dehydroascorbinsäure wurden in 5 ml etwa 60° warmem Wasser gelöst und mit Butanol zu 100 ml aufgefüllt.

Reagenslösung B: 0.5 g Dehydroascorbinsäure wurden wie bei A gelöst.

Reagenslösung C: 0.5 g Dehydroascorbinsäure wurden in 5 ml etwa 60° warmem Wasser gelöst, mit 10 ml Essigsäure gemischt und schliesslich mit Butanol zu 100 ml aufgefüllt.

Ninhydrinreagens nach CRAMER[5]: 0.2 g Ninhydrin wurden in 95 ml Butanol gelöst und mit 5 ml 2 *N* Essigsäure vermischt.

(*b*) *Arbeitsweise.* Jeweils 10 mg der Aminosäuren wurden in 5 ml Wasser gelöst; Cystin und Tyrosin wurden in einer Mischung aus 3 ml Wasser und 2 ml Äthanol und 1 Tropfen 25 %iger Salzsäure gelöst.

Auf 4 verschiedenen Papierbogen 23.5 × 28 cm (Schleicher & Schüll 2043 bM) wurden jeweils 10 μl (= 20 μg) der Aminosäurelösungen aufgetragen und an der Luft getrocknet. Nach 12stündiger Sättigungszeit wurde mit dem Gemisch Butanol–Essigsäure–Wasser (4:1:1) (V/V/V) aufsteigend nach der Zylindermethode chromatographiert. Die getrockneten Chromatogramme, d.h. die 4 Proben von jeder Aminosäure, wurden mit den Reagenslösungen A bis C bzw. mit dem Ninhydrinreagens besprüht. Die mit Reagenslösung A bis C behandelten Chromatogramme wurden in einem auf 100° vorgeheizten Trockenschrank 5 Min. getrocknet, die mit Ninhydrinreagens behandelten 5 Min. bei 70° getrocknet (Tabelle I).

2. Empfindlichkeit des Aminosäurenachweises mit Dehydroascorbinsäure

Verdünnungsreihen von 0.2, 0.5, 1, 2, 3, 5, 10, 25 und 50 μg der geprüften Aminosäuren (Tab. I) wurden unter gleichen Versuchsbedingungen (1b) chromatographiert, anschliessend mit Reagenslösung A (1a) besprüht und 5 Min. bei 100° getrocknet (Tab. II).

3. Ermittlung der Dehydroascorbinsäurekonzentration zum Nachweis der Aminosäuren

Auf verschiedenen Papierbogen 23.5 × 28 cm (Schleicher & Schüll 2043bM) wurden jeweils 25 μg der Aminosäuren (Alanin, Asparagin, Asparaginsäure, Glutaminsäure, Glykokoll, Lysin, Leucin, *d,l*-Methionin, Serin und Valin) nebeneinander aufgetragen und nach 12stündiger Sättigungszeit mit dem Gemisch Butanol–Essigsäure–Wasser (4:1:1) (V/V/V) aufsteigend nach der Zylindermethode chromatographiert. Nach dem Trocknen wurde mit Dehydroascorbinsäurelösungen verschiedener Konzentrationen besprüht und 5 Min. bei 100° getrocknet (Tabelle III).

ZUSAMMENFASSUNG

Es wird über eine Farbreaktion zum papierchromatographischen Nachweis von Aminosäuren mit Dehydroascorbinsäure berichtet. Aminosäuremengen bis herab zu 2 μg sind als rosarote Flecke erkennbar.

SUMMARY

A colour reaction is described for the detection of amino acids on paper chromatograms by means of dehydroascorbic acid. Amounts of amino acids down to 2 μg appear as pink-red spots.

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AMÉLIORATION D'UN SYSTÈME SOLVANT À HAUT POUVOIR SÉPARATEUR PERMETTANT L'ANALYSE CHROMATOGRAPHIQUE SUR PAPIER À DEUX DIMENSIONS DES MÉLANGES D'AMINO-ACIDES DE GRANDE MOBILITÉ (ISOLEUCINE, LEUCINE, MÉTHIONINE, PHÉNYLALANINE, TRYPTOPHANE, TYROSINE, VALINE)

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(Reçu le 10 avril, 1963)

Un grand nombre de systèmes solvants ont été proposés pour la chromatographie des aminoacides sur papier. Cependant il n'est pas possible d'obtenir en une seule chromatographie, même à deux dimensions, la séparation *complète* des 18 aminoacides présents dans les hydrolysats de protéines. De plus, en chromatographie à deux dimensions, les aminoacides se répartissent, en général, sur la feuille de papier en deux groupes: celui des aminoacides de faible mobilité et celui des aminoacides de grande mobilité*. En vue de l'emploi de la chromatographie sur papier à l'analyse rapide de la composition en aminoacides des peptides dérivant des protéines, il est nécessaire d'avoir à sa disposition un procédé permettant l'analyse complète de ces deux groupes d'aminoacides. Nous n'envisagerons ici que le problème de la séparation complète des aminoacides de grande mobilité.

MATÉRIEL ET CONDITIONS OPÉRATOIRES

Toutes les chromatographies sont réalisées dans des cuves (105 l) pour 4 feuilles (57 × 47 cm) de papier Whatman No. 4 (surface utile: 43 × 33 cm), par chromatographie descendante avec écoulement continu du solvant (des dents, 2 × 2 cm, sont découpées au bas de la feuille de papier); dans ces conditions la surface sur laquelle peuvent se répartir les taches d'aminoacides est de 43 × 33 cm. Les chromatogrammes sont tous équilibrés avec l'atmosphère de la cuve pendant 4 h avant de démarrer le développement; l'atmosphère de la cuve est créé par le "fond de cuve" constitué par 500 ml de solvant mobile. Ce "fond de cuve" est renouvelé après dix chromatographies.

Les conditions opératoires particulières sont indiquées dans chaque cas, dans les légendes des Figs. 1, 2 et 3.

Les solvants mobiles sont préparés avec des produits purs Prolabo (Paris), excepté l'alcool amylique tertiaire (produit Flucka).

* Pour une revue récente voir réf. 1.

RÉSULTATS ET DISCUSSIONS

Actuellement, le système solvant présentant le plus haut pouvoir séparateur pour les aminoacides de grande mobilité est celui, imaginé par BOISSONNAS[2], utilisant respectivement en 1-ère et 2-ème dimension les solvants:

(I) *tert.*-butanol–méthyléthylcétone–eau (4:4:2)

(II) *tert.*-butanol–méthanol–eau (4:5:1).,

Avec ce système solvant, les taches des aminoacides de grande mobilité se répartissent uniformément sur la plus grande partie de la feuille de papier chromatographique* tandis que les autres aminoacides (ceux formant le groupe des aminoacides de faible mobilité) restent près de l'origine. Toutes les taches des aminoacides de grande mobilité sont bien séparées spécialement lorsque le développement du chromatogramme (feuille 57 × 47 cm) est réalisé avec écoulement continu des solvants dans les deux dimensions et en s'aidant d'un marqueur coloré (phénol sulfone phtaléine) de mobilité chromatographique légèrement plus grande que celle de l'aminoacide le plus rapide (ici le groupe leucine + isoleucine)[1,3,4] (voir Fig. 1).

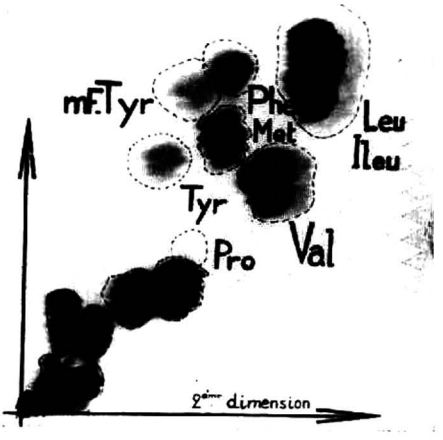

Fig. 1. Séparation chromatographique à deux dimensions des aminoacides de grande mobilité d'un hydrolysat de fraction protéinique principale *d'Escherichia* (100 µg) avec le système solvant (non modifié) DE BOISSONNAS[2]. Feuille (57 × 47 cm) de papier Whatman No. 4 (surface utile: 43 × 33 cm); écoulement continu des solvants dans les deux dimensions suivi grâce au marqueur coloré: phénolsulfone phtaléine: développement en 1-ère dimension: *tert.*-butanol–méthyléthylcétone–eau (4:4:2): en 2-ème dimension: *tert.*-butanol–méthanol eau (4:5:1). Ileu = isoleucine; Leu = leucine; Met = méthionine; "MeSO ex Met" = fraction de la tache de méthionine oxydée à l'air pendant le séchage (22°) du solvant ayant servi au développement en 1-ère dimension; Phe = phénylalanine; Pro = proline; Tyr = tyrosine; *m*-F-Tyr = *m*-fluorotyrosine; Val = valine.

Cependant, si ce système solvant permet de séparer parfaitement les aminoacides méthionine, phénylalanine, tryptophane, tyrosine, valine et le groupe leucine + isoleucine, ces deux derniers aminoacides sont toujours mêlés. Différents systèmes solvants:

* Pour des améliorations, par les modalités d'emploi, de ce système solvant, voir les réf. 1 (p. 679), 3 et 4.

méthyléthylcétone–pyridine–eau (70:15:15)[5];
alcool isoamylique–pyridine–eau (35:35:30)[6];
alcool *tert.*-amylique–eau (atmosphère de diéthylamine)[7];
méthyléthylcétone–acétone–eau (3:1:0.6)[8];

peuvent permettre de séparer plus ou moins complètement la leucine de l'isoleucine mais alors les distances entre les diverses taches d'aminoacides de grande mobilité sont moins importantes. Ainsi avons-nous tenté d'apporter diverses modifications au système solvant de BOISSONNAS afin de lui permettre de séparer la leucine de l'isoleucine tout en lui conservant son haut pouvoir séparateur vis à vis des taches des autres amino-acides de grande mobilité (tryptophane, tyrosine, phénylalanine, méthionine, valine)

La Fig. 2(A et B) montre le résultat obtenu lorsque l'on développe le chromato-

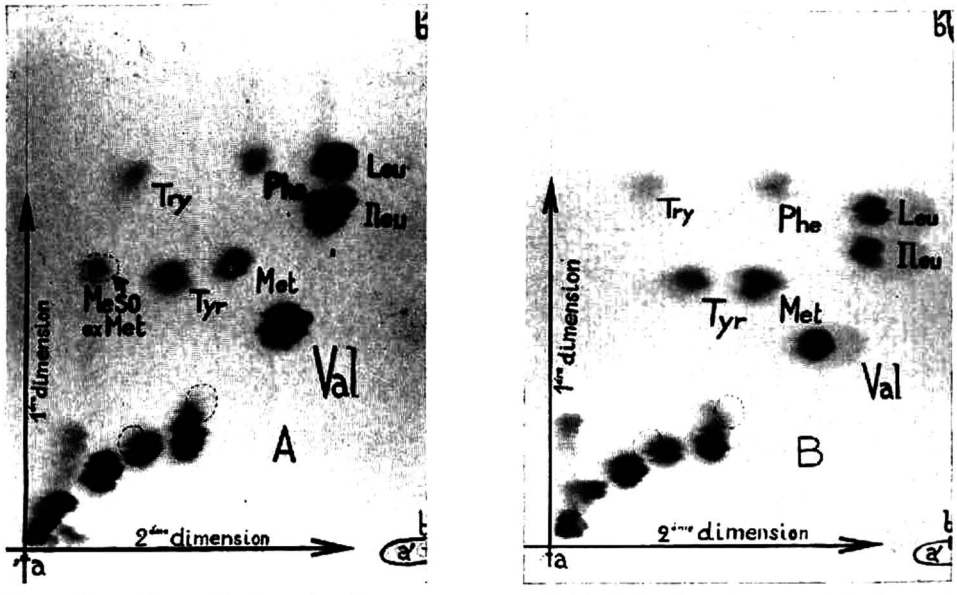

Fig. 2. Nouvelle combinaison de solvants permettant d'obtenir la séparation complète des sept aminoacides de grande mobilité: isoleucine, leucine, méthionine, phénylalanine, tryptophane, tyrosine, valine. Feuille (57 × 47 cm) de papier Whatman No. 4 (surface utile: 43 × 33 cm); écoulement continu des solvants dans les deux dimensions suivi grâce aux marqueurs colorés: en 1-ère dimension, méthyl orange III (b = départ; b′ = arrivée); en 2-ème dimension, phénol-sulfone phtaléine (a = départ; a′ = arrivée); développement en 1-ère dimension: alcool *tert.*-amylique–méthyléthylcétone–eau (6:2:2) pour le chromatogramme A, (4:4:2) pour le chromatogramme B; en 2-ème dimension: *tert.*-butanol–méthanol–eau (4:5:1); quantité d'aminoacide mise en oeuvre: 10 μg par tache.

gramme en 1-ère dimension avec le nouveau solvant (écoulement continu):

(Ia) alcool *tert.*-amylique–méthyléthylcétone–eau (4:4:2) ou (Ib) alcool *tert.*-amylique–méthyléthylcétone–eau (6:2:2) et en 2-ème dimension avec le solvant habituel de BOISSONNAS

(II) *tert.*-butanol–méthanol–eau (4:5:1)*.

* On peut, dans le solvant (II), remplacer le méthanol par de l'éthanol; le pouvoir séparateur du système solvant est conservé mais l'écoulement du solvant est ralenti; le seul avantage du solvant (III) (*tert.*-butanol–éthanol absolu–eau (4:5:1)) réside dans la réduction de sa toxicité par rapport à celle du solvant (II).

Comme on peut le voir dans le chromatogramme de la Fig. 3*, le pouvoir séparateur du nouveau couple de phases solvantes mobiles est encore plus élevé que celui du système solvant original. Les 7 aminoacides de grande mobilité—iscleucine, leucine, méthionine, phénylalanine, tryptophane, tyrosine, valine – sont complètement séparés sur un seul et même chromatogramme. L'emploi de marqueurs colorés appropriés (en 1-ère dimension: méthyl orange III; en 2-ème dimension: phénolsulfone phtaléine) de mobilité légèrement plus élevée que celle de l'isoleucine (aminoacide du groupe

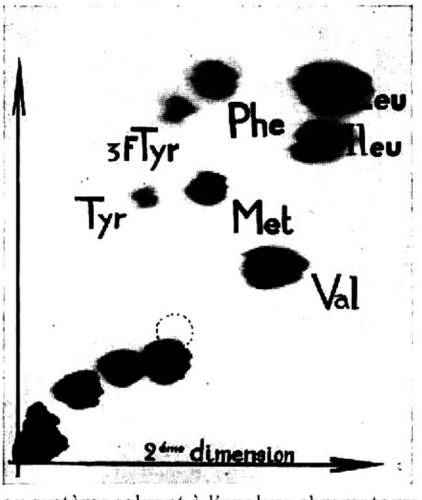

Fig. 3. Application du nouveau système solvant à l'analyse chromatographique des aminoacides de grande mobilité contenu dans un hydrolysat (50 μg) de la fraction protéinique principale d'*E.coli*[9]. Conditions opératoires identiques à celles de la Fig. 2; noter la séparation complète de la leucine et de l'isoleucine et l'amélioration de la séparation de la phénylalanine de la 3-fluorotyrosine (voir Fig. 1).

dont la mobilité est la plus grande) permet d'améliorer la reproductibilité des séparations obtenues d'un lot à l'autre d'une même qualité de papier. Toutes les conditions opératoires utilisées pour réaliser le développement de ces chromatogrammes sont indiquées dans les légendes des Figs. 2 et 3.

La composition du solvant (Ia, ou Ib) utilisé pour le développement du chromatogramme en 1-ère dimension a été imaginée en tenant compte des observations de WORK[7] qui a montré que la leucine et l'isoleucine peuvent être séparées par l'alcool *tert.*-amylique saturé d'eau (en atmosphère de diéthylamine); mais nous avons préféré le solvant (Ia) au (Ib) puisqu'on sait[10] que le système précédent n'a pas un très bon pouvoir séparateur pour les autres aminoacides de très grande mobilité et que la diéthylamine[11] gêne la révélation des taches d'aminoacides par la ninhydrine.

RÉSUMÉ

Ainsi en modifiant seulement légèrement le système solvant de BOISSONNAS a-t-on pu mettre au point un système solvant à haut pouvoir séparateur pour l'ensemble des 7

* L'hydrolysat analysé dans ce chromatogramme correspond aux protéines de la "fraction protéinique principale" (voir réf. 3) isolées d' *E.coli* ayant incorporé de la 3-fluorotyrosine au cours de sa croissance en présence de cet analogue de la tyrosine[9].

aminoacides de grande mobilité : isoleucine, leucine, méthionine, phénylalanine, tryptophane, tyrosine, valine.

SUMMARY

By modifying the solvent system of BOISSONNAS only slightly, a system was obtained that had a high separating power for the seven amino acids of high mobility, viz. isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine.

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THE BEHAVIOR OF HORMONALLY-ACTIVE PROTEINS AND PEPTIDES OF THE ANTERIOR PITUITARY ON CROSS-LINKED DEXTRAN POLYMER GELS*

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INTRODUCTION

The development of the techniques of gel filtration by PORATH AND FLODIN[1] offered a simple and rapid method for the fractionation of water-soluble substances. These workers demonstrated that with carbohydrates the separation was dependent on the molecular size of the substances. PORATH[2,3] demonstrated that these techniques were applicable to the separation of proteins, peptides, and amino acids. LINDNER, ELMQVIST AND PORATH[4] utilized the technique to fractionate an extract of posterior lobes of hog pituitaries into a hormonally-active fraction and a fraction of hormonally-inert material. They subsequently utilized the technique to further purify the active fraction and obtained an approximately twenty-fold increase in specific activity. PORATH AND SCHALLY[5] have utilized gel filtration to effect separations of a number of posterior pituitary hormones. They were able to separate α-melanocyte stimulating hormone (MSH) from lysine vasopressin and α-from β-MSH.

WILHELMI, FISHMAN AND RUSSELL[6], WILHELMI[7,8], and ELLIS[9,10] have described conditions for the preparation of extracts of the pituitary gland which contained a number of the gland's hormones. Originally the single extracts were separated into the various hormonal activities by salt or ethanol fractionation and by chromatography. WILHELMI[8] and ELLIS[10] have suggested schemes for separation during the initial extraction of the pituitary glands based on varying conditions of pH and ionic strength.

This communication reports the results of a study of the behavior of a number of the individual anterior pituitary hormones when subjected to gel filtration. This investigation was carried out under the pH and ionic strength conditions encountered in initial extracts of the pituitary. This study is an evaluation of the individual hormones, prior to the utilization of gel filtration in the fractionation of crude pituitary extracts.

METHODS

Gel filtration experiments were carried out using the cross-linked polysaccharide Sephadex G-50*** (medium particle size). The bed material was washed with distilled

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*** Pharmacia, Uppsala, Sweden.

water. Fine particles were removed by repeated washing and decantation. The Sephadex was suspended in the particular eluent to be used in the experiment and poured into columns of 1 cm^2 cross-sectional area to a height of 10 cm.

The following eluents were used in these experiments: (1) 0.15 *M* sodium chloride; (2) 0.02 *M* acetic acid; (3) 0.10 *M* hydrochloric acid; (4) pH 4.0, 0.02 *M* acetate buffer with 0.10 *M* ammonium sulphate; (5) pH 5.5, 0.02 *M* acetate buffer; (6) pH 5.5, 0.02 *M* acetate buffer with 0.30 *M* potassium chloride.

The following anterior pituitary hormone preparations* were used: (1) porcine (oxycellulose purified) adrenocorticotrophic hormone (ACTH) No. 216174-3; (2) bovine thyrotrophic hormone (TSH) No. R-216-174-1; (3) equine lactogenic hormone (LH) No. 216-171-15; (4) bovine somatotrophic hormone (STH) No. 216-176-4; (5) porcine follicle stimulating hormone (FSH) No. 216-175-6. Solutions containing 1 mg/ml were prepared and 100 μl aliquots used in the experiments.

All experiments were run at room temperature. Volumetric fractions of 1 ml were collected. These fractions were immediately analyzed using the LOWRY modification of the Folin reaction[11]. All readings were made at 740 mμ using a Coleman Universal Spectrophotometer. Void volume determinations were performed using 100 μl aliquots of a 5 % hemoglobin solution. The internal volume was determined using the following relation:

$$V_i = (V_t - V_0) \frac{W_r d}{1 + W_r}$$

where V_i is the internal volume; V_t is the total bed volume; V_0 is the void volume; W_r is the water regain (equal to 5.0 g/g); and d is the wet density (equal to 1.06 g/ml).

It was then possible to evaluate the degree of diffusion for the different hormone preparations using the following relation:

$$K_D = \frac{V_{el} - V_0}{V_i}$$

where K_D is the distribution coefficient for a solute between the internal and external solvent; V_{el} is the volume necessary to elute the solute from the polymer gel; V_i is the internal volume or the volume of solvent contained within the polymer gel; and V_0 is the void volume or volume of solvent external to the polymer gel.

RESULTS

Table I summarizes the K_D values obtained in this study for these hormones. As the K_D approaches 0 this indicates increasing exclusion of solute from the internal solvent phase. As the K_D approaches 1, this indicates complete diffusion of the solute within the internal solvent phase. The techniques employed permitted replicate determinations of the K_D to be made with an average deviation of $9.8 \cdot 10^{-3}$ K_D units.

Fig. 1 indicates the result of plotting the K_D value against the reciprocal of the Briggsian logarithm of the molecular weight. The linear relation which exists between these two sets of data is described by the equation:

$$K_D = \frac{k}{\log \text{M.W.}} - C$$

* Gift of DR. J. D. FISHER, Armour Pharmaceutical Co., Kankakee, Ill.

TABLE I

K_D VALUES OF ANTERIOR PITUITARY HORMONES

Hormone	*Molecular weight*	*K_D with different eluents*					
		1	*2*	*3*	*4*	*5*	*6*
ACTH	3,500	0.95	1.00	—	0.87	—	0.30
TSH	10,000	—	0.87	—	—	0.27	—
LH	40,000	0.49	—	0.34	0.32	0.20	—
STH	45,000	—	—	0.25	—	—	0.07
FSH	70,000	0.40	0.68	0.17	0.00	0.17	0.04

where K_D is the distribution coefficient or ratio between the concentrations of the solute in the void volume and internal volume; K is a constant evaluated from the slope; C is a constant equal to the value of the Y-intercept. The values obtained for K for the various eluent systems were determined to be: 7.25 with 0.15 M sodium chloride; 4.24 with 0.02 M acetic acid; 14.48 with 0.10 M hydrochloric acid; 11.90 with

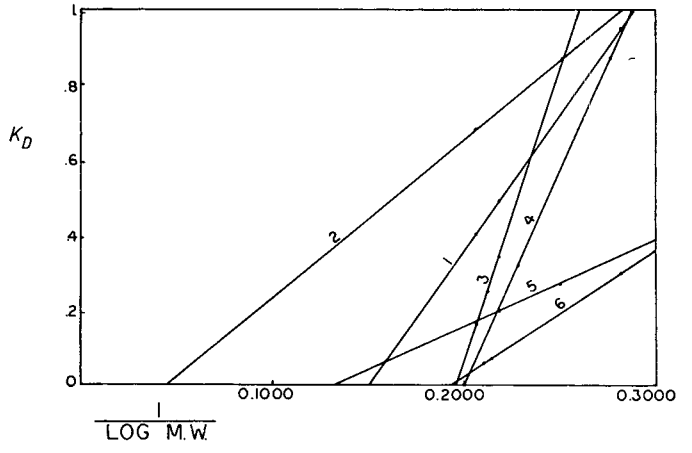

Fig. 1. Plot of K_D *versus* 1/log M.W. Eluent: (1) 0.15 M sodium chloride; (2) 0.02 M acetic acid; (3) 0.10 M hydrochloric acid; (4) pH 4.0, 0.02 M acetate buffer with 0.10 M ammonium sulphate; (5) pH 5.5, 0.02 M acetate buffer; (6) pH 5.5, 0.02 M acetate buffer with 0.30 M potassium chloride.

pH 4.0, 0.02 M acetate buffer with 0.10 M ammonium sulphate; 2.23 with pH 5.5, 0.02 M acetate buffer; 3.44 with pH 5.5, 0.02 M acetate buffer with 0.30 M potassium chloride.

The above results indicate that the individual anterior pituitary hormones studied (ACTH, TSH, LH, STH, and FSH-) behave on Sephadex G-50, under conditions of pH ranging from 1.0 to 6.8 and of salt concentration from 0.02 to 0.32 M, in a manner related to their molecular weights. The results further indicate that the distribution coefficients for these hormones on Sephadex G-50 can be predicted by the equation:

$$K_D = \frac{k}{\log \text{M.W.}} - C$$

DISCUSSION

Application of gel filtration, using materials such as Sephadex, seems suited to the fractionation of extracts of anterior pituitary. It is simpler and more economical with respect to time and to the valuable hormone products than salt or ethanol fractionation or multiple step chromatography. The possibility of interference with the observed, well-defined behavior in the presence of multiple hormonal protein components exists. This is presently being evaluated. The results obtained in these purely model systems do suggest that gel filtration on cross-linked dextran polymers can profitably be applied to the problem of fractionation and purification of extracts of anterior pituitary tissue for its hormonal activities.

SUMMARY

The possible application to the fractionation and purification of the various hormonal activities of the anterior pituitary has been examined. This investigation has dealt solely with the behavior of a number of the anterior pituitary hormones in model systems which parallel the conditions of pH and salt concentration found in extracts of anterior pituitary tissue. The behavior of these hormones, over the pH range 1.0–6.8 and salt concentration range of 0.02–0.32, is linearly related to the reciprocal of the log of their molecular weight. The distribution coefficient for a given hormone in a given system can be predicted by the equation:

$$K_D = \frac{k}{\log \text{M.W.}} - C$$

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PAPER CHROMATOGRAPHY OF MONOMER SUGARS USING QUATERNARY SALTS FOR IDENTIFICATION

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INTRODUCTION

A number of procedures utilizing aniline oxalate[1], cysteine–carbazole[2], 2,4-dinitrophenylhydrazine[3], resorcinol[4], naphthoresorcinol[5], orcinol[6], vanillin[7], *p*-anisidine[1], urea hydrochloride[1], *o*-phenylenediamine[8], diphenylamine–aniline[9], Benedict's solution[10], ammoniacal silver nitrate[11] have been used for the detection of sugars on paper chromatograms. The procedures yield color differences and are sensitive to ketoses and aldoses. However, uronic acids, 5-hexulosonic acids and sugar amines fail to be selectively differentiated by the above methods.

CHARGAFF *et al.*[12] used a *m*-phenylenediamine solution and found that complexes with unsubstituted sugars possessed a marked fluorescence in the ultra-violet. ROREM[13] has been successful in detecting and differentiating ketoses and aldoses with the aid of ultra-violet fluorescence of sulfosalicylic acid. ROREM[14] was able to detect phosphorylated sugars as well as amino acids by a fluorescence technique using quinine sulfate. BERA *et al.*[15] have also reported the use of cetyl-trimethyl-ammonium bromide (Cetavlon) as a spray reagent for the identification of sugars.

A major phase of our work is related to the identification of degradation products from acid mucopolysaccharides such as heparin, heparinoids, chondroitin sulfates and hyaluronic acid. A need developed during the course of our investigation for a chromagen which would permit identification of different monomer sugar classes, and subsequently different polymer fragments. This paper reports the use of organic reagents containing nitrogen which are substituted with alkyl or aryl groups capable of producing quaternary ammonium ions as possible identifying reagents. In this study, the ultra-violet fluorescence or, quenching, of the quaternary ammonium ion complex was used to detect and differentiate monomer sugars.

EXPERIMENTAL

Sample materials

The sugars or sugar derivatives used in this investigation were either obtained commercially or prepared in this laboratory. All of the samples were dissolved in water and were made up to a concentration of 1% (w/v). Multiples of one microliter aliquots were applied to the paper with a micro pipette in order to assess the sensitivity of the chromagen under investigation.

* Deceased.

Developing solvents

The following solvent systems were used:

(A) Pyridine–ethyl acetate–acetic acid–water (5:5:1:3)[16]
(B) Butanol–ethanol–water (4:1.1:1.9)
(C) *tert.*-Amyl alcohol–propanol–ethanol–water (4:1.3:0.5:2)
(D) Butanol–acetic acid–water (5:1.5:3.5)[17]
(E) Butanol–acetic acid–water (5:1.4:2.9)[17]
(F) Butanol–acetic acid–water (5:1.2:2.5)[17]
(G) *tert.*-Amyl alcohol–formic acid–water (4:1:1.5)
(H) Butanol–acetic acid–water (4.4:1.6:4.0)[17]

The above solvent systems are miscible at room temperature. In all cases descending chromatography was used.

Paper

Whatman No. 1 paper was found to be suitable for the procedure. Prior washing of the paper with the solvent in question was found to be beneficial, but not essential in qualitative work. Papers washed with water had little advantage over unwashed papers.

Detection

The following quaternary ammonium salts were used: (1) quinacrine hydrochloride, (2) Pyronin Y, (3) proflavine hydrochloride, (4) sparine hydrochloride, (5) methyl-dodecylbenzyl-trimethyl-ammonium chloride (Hyamine 1622), (6) cetyl-trimethyl-ammonium bromide (Cetavlon), (7) cetylpyridinium chloride, (8) cetyl-dimethyl-benzyl-ammonium chloride. Each of the reagents was prepared in stock solutions containing 500 mg in 200 ml of 80% ethanol. The stock solution was diluted 1:10 with 80% ethanol for spray application.

A long wave lamp with ultra-violet radiation in the 3600Å range was used for the identification of the quaternary complexes of the sugar samples.

These lights are constructed of a special high-transmitting self-filtering glass and hence require no secondary filter. At this wavelength, the sprayed chromatographic paper shows a faint fluorescence, and the substances under analysis appear as areas of quenching with slight coloration.

Procedure

After the chromatogram has been developed, the respective solvent is removed by first air drying at room temperature, followed by drying at 100° for 3–5 min. The paper is sprayed with the desired quaternary reagent. The sprayed paper is allowed to dry at room temperature under forced air conditions. The sprayed paper is then heated for 3–5 min at 100°.

Under ultra-violet light one can locate and identify the areas of quenching.

RESULTS AND DISCUSSION

The initial phase of this investigation (*cf.* Table I) relates the sensitivity of the respective quaternary compounds to the monomer sugars. From previous work[17] the

TABLE I

QUATERNARY EVALUATION

Solvent system (H): butanol–acetic acid–water (4.4:1.6:4.0)

Quaternary salt		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
sensitivity class		*2*	*3*	*1*	*3*	*3*	*3*	*3*	*2*
Glucose	R_F	0.47		0.46			0.50		0.45
	Color	green	none	green	none	none	white	none	white
Mannose	R_F	0.49		0.49			0.53		
	Color	lt. green	none	green	none	none	white	none	none
2,5-Anhydro-mannose	R_F	0.80		0.85			0.80		
	Color	violet	none	blue	none	none	white	none	none
Glucurone	R_F	0.49		0.53	0.53	0.55	0.55	0.54	0.54
	Color	yellow	none	orange	blue	white	white	white	white
Glucuronic acid	R_F	0.43		0.41	0.45	0.48	0.45	0.44	0.43
	Color	lt. green	none	orange	blue	white	white	white	white
Galacturonic acid	R_F	0.41		0.36	0.38	0.42		0.40	0.40
	Color	lt. green	none	orange	violet	white	none	white	white
Brucine salt of D-lyxo-5-hexulosonic acid	R_F	0.78	0.83	0.82	0.83	0.76	0.79	0.79	0.76
	Color	violet	violet	blue	violet	violet	violet	white	violet
Brucine salt of D-xylo-5-hexulosonic acid	R_F	0.79	0.82	0.82	0.83	0.77	0.79	0.80	0.77
	Color	violet	violet	blue	violet	violet	violet	white	violet
Galactosamine	R_F	0.32	0.32	0.33	0.31	0.37		0.36	0.35
	Color	lt. green	violet	violet	blue	white	none	white	white
Glucosamine	R_F	0.33	0.35	0.34	0.35	0.37		0.39	0.44
	Color	violet	violet	violet	white	white	none	white	white

butanol–acetic acid–water solvent system (H) was selected for the initial phase. The following classification of sensitivity was chosen and was used to select the most appropriate quaternary compound.

Class 1: 10–50 μg of sugar produces visually detectable level of color,

Class 2: 50–100 μg of sugar produces visually detectable level of color,

Class 3: greater than 100 μg of sugar produces visually detectable level of color.

Sugars are reported in terms of both R_F values as well as the color of the complex under ultra-violet light.

It has been demonstrated from this work that proflavin hydrochloride is the most sensitive of the reagents examined. With solvent system (H) and proflavin hydrochloride (3), the hexoses appear as green spots; the amines appear as violet spots; whereas the uronic acids and lactones appear as orange spots. The brucine salts of the 5-hexulosonic acids, however, appear as blue spots.

Table II shows the influence of solvents upon the color of the quaternary complex when viewed under ultra-violet light using proflavin hydrochloride as the identifying reagent. It should be noted that galacturonic acid, galactosamine, glucosamine and 2,5-anhydromannose respectively exhibit different colors with different solvents. It is therefore possible to resolve a mixture containing a uronic acid, a 5-hexulosonic acid and a sugar amine and a hexose. As an example, (*cf.* Table II) this could be accomplished with glucosamine, galacturonic acid, brucine salt of D-lyxo-5-hexulosonic

TABLE II

SOLVENT EVALUATION

Chromagen (3): proflavin hydrochloride (18 h development time)

Solvent		*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Glucose	R_F	0.33	0.21	0.19	0.31	0.23	0.24	0.19	0.46
	Color	green	green	green	green	green	green	green	green
Mannose	R_F	0.34	0.28	0.29	0.39	0.30	0.30	0.24	0.49
	Color	green	green	green	green	green	green	green	green
2,5-Anhydro-mannose	R_F	0.70	0.16	0.20	0.08	0.43	0.24	0.37	0.85
	Color	orange	violet	violet	violet	violet	violet	orange	violet
Glucurone	R_F	0.77	0.38	0.42	0.45	0.40	0.40	0.35	0.53
	Color	orange	orange	orange	orange	orange	orange	orange	orange
Glucuronic acid	R_F	0.32	0.24	0.25	0.30	0.22	0.21	0.18	0.41
	Color	orange	orange	orange	orange	orange	orange	orange	orange
Galacturonic acid	R_F	0.16	0.22	0.21	0.24	0.18	0.12	0.11	0.36
	Color	orange	orange	orange	orange	violet	violet	violet	orange
Brucine salt of D-lyxo-5-hexulosonic acid	R_F	0.80	0.48	0.31	0.75	0.65	0.67	0.62	0.82
	Color	blue	blue	blue	blue	blue	blue	blue	blue
Brucine salt of D-xylo-5-hexulosonic acid	R_F	0.80	0.45	0.29	0.74	0.63	0.66	0.61	0.82
	Color	blue	blue	blue	blue	blue	blue	blue	blue
Galactosamine	R_F	0.26	0.06	0.18	0.22	0.08	0.11	0.09	0.33
	Color	green	green	violet	violet	violet	violet	violet	violet
Glucosamine	R_F	0.18	0.16	0.12	0.22	0.18	0.14	0.16	0.34
	Color	green	green	green	green	green	violet	green	violet

acid and glucosamine by first using solvent system (A) and chromagen (3), followed by a duplicate set of chromatograms using solvent system (E) and chromagen (3). An alternative would be to use solvent system (A) followed by solvent system (D) and again using chromagen (3) for both.

SUMMARY

A selective procedure has been developed for the identification of hexoses, sugar acids and lactones, sugar amines and 5-hexulosonic acid salts with the aid of a quaternary ammonium compound. When proflavin hydrochloride is complexed with the respective sugars, viewed under ultra-violet light, the monomers are identified by various R_F values and colors. The procedure is sensitive to 10 micrograms of sugar.

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IDENTIFICATION OF ACID MUCOPOLYSACCHARIDES BY PAPER CHROMATOGRAPHY*

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Acid mucopolysaccharides, a family of carbohydrate macromolecules found in connective tissue "ground substance", have been isolated and studied by several investigators[1-3]. Isolation techniques applicable to large masses of tissue yield sufficient purified acid mucopolysaccharide to permit accurate identification by chemical analysis. However, even tentative identification of these substances in plasma, urine, or tissue culture media may be difficult due to low concentrations and limited total sample. Methods are available for the quantitative measurement of acid mucopolysaccharides in such limited samples[4-6]. Although progress in qualitative identification of specific acid mucopolysaccharides in small samples is less satisfactory, chromatographic methods appear promising as a means of rapid, tentative identification of material available in amounts inadequate for exhaustive chemical analysis.

Solvent systems employing phosphate buffer in propanol or ethanol have been used to demonstrate metachromatic material in urine and plasma possessing chromatographic mobility resembling chondroitin sulfate[4,7]. While these systems tend to distinguish heparin from chondroitin sulfate (cartilage origin), hyaluronate always remained at the origin, making its identification doubtful by these methods. A solvent system using dilute ammonium formate in isopropanol has been devised which will completely resolve chondroitin sulfate and heparin[8]. In addition, a multiple solvent system employing silicated glass paper appeared to separate most of the known mammalian acid mucopolysaccharides[9]. This technique offers the advantage of speed of execution but seems useful only with highly purified mucopolysaccharide samples.

In view of the limited methodology available for chromatographic identification of acid mucopolysaccharides, we have developed a system which distinguishes the known mammalian mucopolysaccharides and offers several advantages: (1) it is rapid and reproducible, (2) it distinguishes hyaluronate by actual movement of the compound, and (3) significant protein contamination of acid mucopolysaccharides does not interfere. On the basis of the methods described below it appears feasible to identify hyaluronic acid, heparin, heparin monosulfate, and chondroitin sulfates A, B and C with some certainty. Identification of keratosulfate is less certain by these methods

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MATERIALS AND METHODS

Acid mucopolysaccharide reference compounds

Hyaluronic acid and chondroitin sulfate C were prepared from human umbilical cord by peptic digestion and alcohol fractionation[10]. Chondroitin sulfates A and B (human skin) and chondroitin sulfate C (shark cartilage) were prepared by papain digestion and alcohol fractionation; the final products were isolated as the sodium salts from methanolic solutions of the respective mucopolysaccharide-cetylpyridinium complexes[2]. Chondroitin was prepared by desulfation of chondroitin sulfate A[11]. Heparin samples included products from the Organon, Upjohn, and Roussel Companies. Examples of chondroitin sulfates A, B, and C, heparitin monosulfate, and keratosulfate were available as gifts from Dr. J. A. CIFONELLI, University of Chicago, and Dr. KARL MEYER, Columbia University. Hyaluronic acid prepared from human umbilical cord by electrodeposition was provided by Dr. SAUL ROSEMAN, The University of Michigan[12].

Oligosaccharides of hyaluronic acid and chondroitin sulfate A were prepared by prolonged hydrolysis with testicular hyaluronidase followed by dialysis of the digest. The aqueous dialysis bath was passed through a column of Dowex 50, H^+ resin and then concentrated by rotary evaporation and lyophilization.

Procedure

Ascending chromatography was carried out in 11 in. rectangular chromatography jars. Samples were spotted with micropipettes, using 20 to 40 μg of acid mucopolysaccharide in 5 to 10 λ of diluent. Development times ranging from 1–12 h were explored. Two to three hours was usually adequate to elicit the maximal resolution for a particular system.

For chromatograms which were to be developed by the "Type I" solvent systems an extra step was interposed before development in the solvent. After the sample spot was dry, the paper was immersed for 3 min in 0.1 % cetylpyridinium chloride in 0.004 M phosphate buffer, pH 7.0, and then allowed to dry over night at room temperature. Chromatograms to be developed in "Type II" solvent systems may be developed as soon as the sample spots are dry. Schleicher and Schuell 589 blue ribbon paper proved satisfactory for all solvent systems.

Solvent systems

Type I. In this group of solvents we have utilized and extended the information originally developed by SCOTT[2] regarding the solubility of various mucopolysaccharide–cetylpyridinium (MPS–CPC) complexes in salt solutions of varying ionic strength. After treating the chromatogram with 0.1% cetylpyridinium chloride, as noted above, the acid mucopolysaccharides are "fixed" and will remain at the origin if the chromatogram is developed with distilled water. However, if a salt solution of sufficient ionic strength to dissolve the MPS–CPC complex is used to develop the chromatogram, the mucopolysaccharide will move with the solvent front. "Type I" solvents, then, identify the solubility characteristics of a particular MPS–CPC complex *and the critical salt concentration is the one which will allow migration of the acid mucopolysaccharide to the solvent front.* Lithium chloride, magnesium chloride, aluminum chloride and ferric chloride solutions were examined in some detail as solvents.

After development of the chromatogram in one of these solvents, it is air dried and then placed in the 0.1 % CPC solution for 3 min, followed by 95% ethanol, 3 min; absolute ethanol, 3 min; acetone, 2 min and then stained.

Type II. As noted earlier, several investigators have used solvent systems composed of a dilute buffer and an organic solvent for the partial identification of the sodium or potassium salts of mucopolysaccharides. After carefully examining the ammonium formate–isopropanol system described by SPOLTER AND MARX[8], we ultimately settled on a minor modification which has reduced development time as its chief virtue. For routine purposes we used 0.04 *M*, pH 3.5 ammonium formate and methanol in a 45:55 ratio.

Staining chromatograms

Chromatograms are stained for 3 min in an Azure A solution made up as follows: 200 mg Azure A in a mixture of 25 ml water, 200 ml acetone and 750 ml methanol.

After staining, the chromatogram may be differentiated in 3:1 methanol–acetone until the background is adequately cleared, and then air dried.

RESULTS

Type I solvents

Magnesium chloride. A solvent system employing magnesium chloride allowed division of the several reference compounds into three groups. Figs. 1, 2, and 3 illustrate the solubilization and consequent mobility of different MPS–CPC complexes at three separate concentrations of magnesium chloride. Hyaluronate stains distinctly less metachromatically than the sulfated compounds, and the streaking is apparently

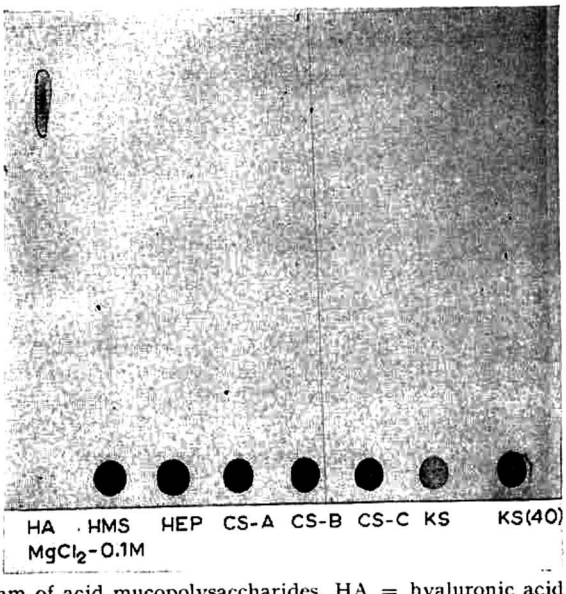

Fig. 1. Chromatogram of acid mucopolysaccharides. HA = hyaluronic acid; HMS = heparitin monosulfate; HEP = heparin; CS-A = chondroitin sulfate A; CS-B = chondroitin sulfate B (dermatan sulfate); CS-C = chondroitin sulfate C; and KS = keratosulfate. All applications were 5 μl volumes containing 20 μg of mucopolysaccharide except KS(40) which represents 40 μl, or 160 μg of mucopolysaccharide.

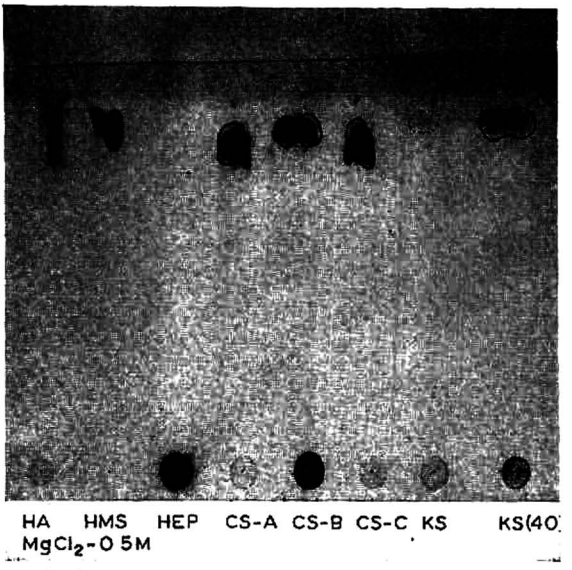

Fig. 2. Chromatogram of acid mucopolysaccharides. For abbreviations and conditions, see Fig. 1.

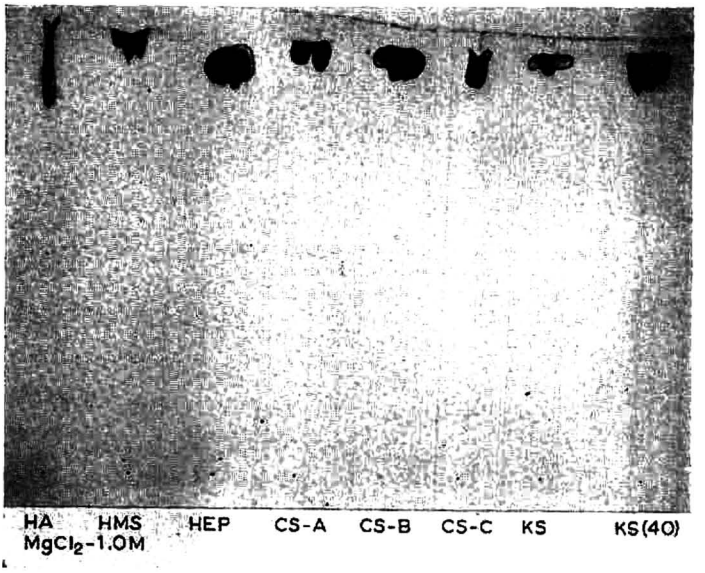

Fig. 3. Chromatogram of acid mucopolysaccharides. For abbreviations and conditions, see Fig. 1.

related to the very high molecular weight of viscous, relatively undegraded hyaluronate prepared by electrodeposition. Partially degraded hyaluronate (produced either by testicular hyaluronidase or by more drastic isolation methods) tends to move as a discrete spot near the front. Heparitin monosulfate and the three chondroitin sulfates stain with a similar deep blue metachromasia, while heparin has a reddish hue. Keratosulfate in equal concentration gave a pale metachromatic spot.

The concentrations of magnesium chloride indicated in Figs. 1, 2, and 3 were chosen primarily because they provide clear cut distinction of hyaluronate and heparin from each other and from the remaining compounds. Actually, magnesium chloride concentrations ranging from 0.05 M to 0.25 M will solubilize the hyaluronate–CPC complex without moving the sulfated compounds. In the range from 0.3 M to 0.5 M $MgCl_2$ the sulfated compounds other than heparin began to move with the solvent front. The possibility that the chondroitin sulfates, keratosulfate, and heparitin monosulfate might be separated by fine gradations in the ionic strength of the salt solution was examined. Magnesium chloride concentrations of 0.300 M, 0.330 M 0.350 M, 0.362 M, 0.375 M, 0.387 M, and 0.400 M tended to move heparitin monosulfate, chondroitin sulfate A, chondroitin sulfate C and chondroitin sulfate B in that order as the salt concentration was increased. However, the overlap of mobilities was so great as to preclude practical application in identification of an unknown.

Aluminum chloride and ferric chloride. Chondroitin sulfates A, B, and C, keratosulfate and heparitin monosulfate are not resolved by the magnesium chloride system, and in an effort to resolve this quintet many other solvents were examined. Aluminum chloride, 0.40 M, proved useful since it moved four of these five to the solvent front, leaving chondroitin sulfate B conspicuously at the origin (see Fig. 4). Ferric chloride, 0.5 M, will solubilize and move hyaluronate, keratosulfate and chondroitin sulfate A (Fig. 5). Heparitin monosulfate tends to move as a streak in this solvent. Of primary importance is the failure of chondroitin sulfates B and C to move.

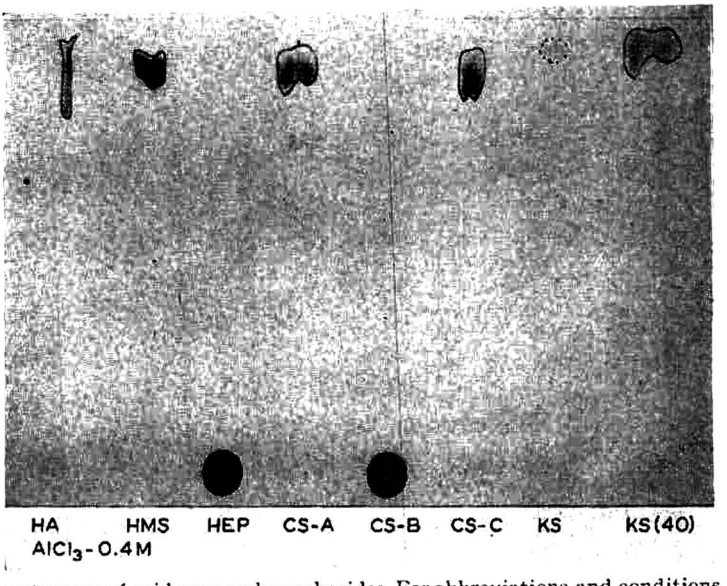

Fig. 4. Chromatogram of acid mucopolysaccharides. For abbreviations and conditions, see Fig. 1.

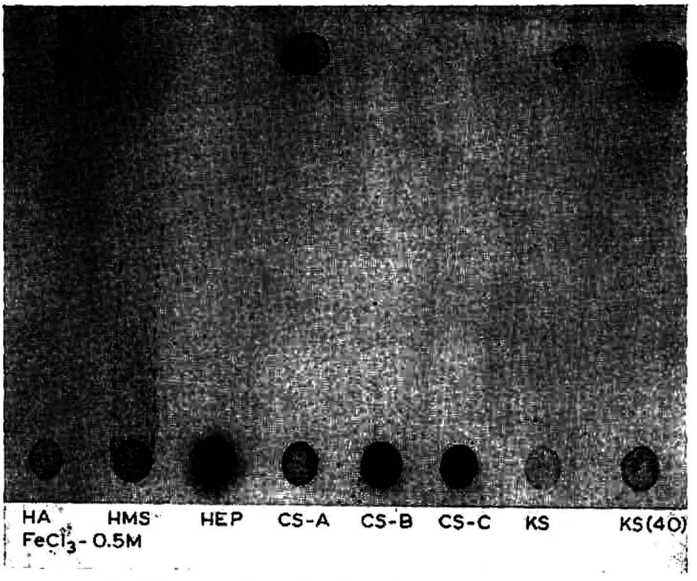

Fig. 5. Chromatogram of acid mucopolysaccharides. For abbreviations and conditions, see Fig. 1.

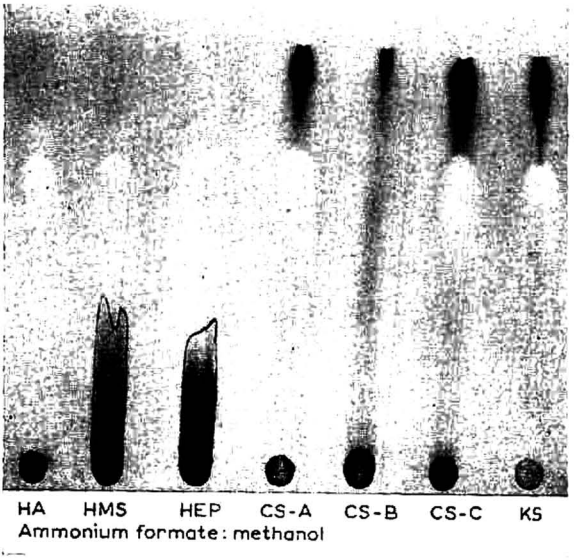

Fig. 6. Chromatogram of acid mucopolysaccharides. For abbreviations and conditions, see Fig. 1.

Type II solvent systems

The type II solvent system employed a dilute buffer in combination with one of the short chain alcohols. As Fig. 6 demonstrates, the Type II system also divides the mucopolysaccharide family into three distinct classes. At one extreme is hyaluronate which is essentially origin bound, and at the other extreme are the three chondroitin sulfates and keratosulfate which move with the solvent front. In between these extremes is the distinctive picture presented by heparin and heparitin monosulfate. These latter two substances exhibit a characteristic streak extending from the origin to a point about half way to the solvent front. Since heparin can be distinguished in the Type I system (1.0 M $MgCl_2$), it follows that an unknown which gave this streak effect in the Type II system and moved with 0.5 M $MgCl_2$ would be identified as heparitin monosulfate.

Table I summarizes the chromatographic behaviour of the various acid mucopolysaccharides. Distinctive patterns of behaviour occurred for five mucopolysaccharides, while chondroitin sulfate A and keratosulfate showed many similar characteristics.

TABLE I

COMPARATIVE BEHAVIOUR OF ACID MUCOPOLYSACCHARIDES IN DIFFERENT SOLVENT SYSTEMS

	0.1 M $MgCl_2$	0.5 M $MgCl_2$	1.0 M $MgCl_2$	0.4 M $AlCl_3$	0.5 M $FeCl_3$	Type II
Hyaluronic acid	F*	F	F	F	F	O**
Keratosulfate	O	F	F	F	F	F
Chondroitin sulfate A	O	F	F	F	F	F
Chondroitin sulfate B	O	F	F	O	O	F
Chondroitin sulfate C	O	F	F	F	O	F
Heparitin monosulfate	O	F	F	F	S***	S
Heparin	O	O	F	O	O	S

* "F" indicates substance moves to front.
** "O" indicates substance is origin bound.
*** "S" indicates substance streaks from origin.

It is important, however, to note that while keratosulfate in 20 μg amounts yielded spots of intensity equalling the chondroitin sulfates in the Type II systems, (Fig. 6), that this amount of keratosulfate is virtually not recorded in Type I systems. As seen in Figs. 2–5, eight times as much keratosulfate is required to produce spots of intensity comparable to other mucopolysaccharides. The precise reason for the apparent loss of keratosulfate from the Type I systems on reaching the critical salt concentration for the MPS–CPC complex is unclear. Fortunately, the absence of uronic acid in keratosulfate makes distinction from chondroitin sulfate A possible by simple chemical analysis, even with a limited sample. From a practical standpoint, it is probably reasonable to characterize a substance as chondroitin sulfate A, if 20 μg spots (based on uronic acid) behave according to the table. A mixture of chrondroitin sulfate A and keratosulfate obviously could not be separated by these systems.

The effect of several variables on the chromatographic systems

Several different salts of chondroitin sulfate A were prepared and tested in the Type II solvent system. Calcium, sodium, and ammonium salts behaved identically in this

system. We have varied the pH of the ammonium formate–methanol system between 3.5 and 7.1 without significant change in the chromatographic behaviour of the reference acid mucopolysaccharides. Pretreating the paper with a variety of substances, including sodium tetraborate and sodium silicate did not increase the discriminatory capability of the Type II system. Increasing the ionic strength of the buffer in the Type II system resulted in decreased mobility. Dialyzable oligosaccharides from chondroitin sulfate A migrated to the front in the ammonium formate–methanol system like the parent polymer. The oligosaccharide from hyaluronic acid also migrated with the front in this system, unlike the parent polymer which is bound to the origin. Neither oligosaccharide could be studied by the Type I system since the low molecular weight compounds, or their initial complex with CPC, are apparently dissolved in the original CPC bath and thus were lost from the paper.

The effect of protein contamination on the efficacy of these systems was tested for all the mucopolysaccharides. Each substance was dissolved in serum and then re-isolated by the euglobulin-heat precipitation method[6]. This final sample was dialyzed free of salt, lyophilized and solubilized in a small volume of water. These mucopolysaccharide samples contained up to 50% serum protein. The chromatographic behaviour of the serum protein-contaminated samples was identical to that shown by the protein-free acid mucopolysaccharides.

Monovalent salts, as lithium chloride, may be substituted for the three magnesium chloride solvents. When this is done, 0.2 *M*, 1.0 *M*, and 2.0 *M* lithium chloride solutions will yield the same data as the magnesium chloride solutions used here.

DISCUSSION

Resolution of mixtures of mucopolysaccharides may be undertaken to a limited extent by the methods outlined. Both heparin and hyaluronate may be identified in any mixture. Heparitin monosulfate may be distinguished when mixed with hyaluronate or heparin, but mixtures with the other sulfated compounds are not resolved with certainty. Further, mixtures of all the chondroitin sulfates and keratosulfate can not be completely resolved. In a mixture of chondroitin sulfates, one can identify chondroitin sulfate A, but can not distinguish between chondroitin sulfate B alone and the combination of chondroitin sulfate B and chondroitin sulfate C as the other components of the mixture.

Chondroitin sulfate C isolated in this laboratory from shark fin cartilage did not behave chromatographically like chondroitin sulfate C of human origin. In general, it required higher salt concentrations to solubilize the MPS–CPC complex. This is consistent with the higher degree of sulfation found in shark cartilage chondroitin sulfate C[13].

It should be recognized that in the chromatographic systems described in this report, that chondroitin (a desulfated chondroitin sulfate) is indistinguishable from hyaluronic acid. This material is only known to occur in the cornea, consequently confusion of chondroitin and hyaluronic acid is unlikely in most materials.

Mucopolysaccharide isolated from normal human urine was shown to consist primarily of chondroitin sulfate A by application of these chromatographic methods. Examination of mucopolysaccharides isolated from pooled blood donor plasma revealed two components, one of which chromatographed like chondroitin sulfate A.

Chromatography of mucopolysaccharides isolated from culture media which supported the growth of human connective tissue cells shows hyaluronic acid to be the predominant substance formed[14].

Chromatographic identification of acid mucopolysaccharides is necessarily tentative, and should, where possible, be used as an adjunct to chemical analysis. The possible use of the aluminum and ferric chloride systems in the preparative separation of acid mucopolysaccharides is under investigation.

SUMMARY

A multiple solvent paper chromatography system is described which tentatively identifies most of the known mammalian acid mucopolysaccharides, when these are examined singly. Certain mucopolysaccharides may be identified with reasonable certainty in complex mixtures of such substances. Protein contamination of significant degree does not appear to interfere with these methods. These chromatographic systems appear to be most useful for the rapid, tentative identification of acid mucopolysaccharides where only limited samples are available.

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A RAPID METHOD FOR THE IDENTIFICATION OF SMALL QUANTITIES OF LIPID-SOLUBLE VITAMINS AND QUINONES IN BIOLOGICAL MATERIAL*

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Increased knowledge of the function of fat-soluble vitamins and quinones in the living organism has made their rapid separation and identification of considerable interest. Various methods for their separation by paper chromatography are reported in the literature[1,3]. These methods, however, are not directly applicable to the original extracts of plant or animal tissues, since vitamins and quinones are usually present in very small concentrations and are masked by large amounts of accompanying lipids. These interfering substances cause poor separation of the vitamins and quinones. A preliminary purification of the extract, usually by saponification and column chromatography, is necessary before paper chromatographic methods can be applied. The preliminary treatments, however, very often lead to a partial destruction of the quinones and vitamins and thus result in considerable losses. Transformation products that were not originally detectable in the extracts may then become prominent. Furthermore, most of the fat-soluble quinones and vitamins are extremely sensitive to light and oxygen, and may be partially destroyed during the usual chromatography on paper (10 to 20 h). For the study of the function and biosynthesis of these compounds it was desirable to develop a rapid paper chromatographic method for their separation and identification, which could demonstrate the presence of these substances in biological systems before the application of the further purification processes.

By testing various papers and solvents we have been able to develop a simple and rapid method for the separation of vitamins and naturally occurring quinones. In comparison with existing chromatographic methods the present technique has several advantages, resulting from the rapidity of the chromatogram development (1 to 2 h) and the high adsorption capacity of the paper used (Schleicher and Schüll, No. 288). The quantity of the whole cell extract which can be applied to the paper is about 10 times greater than can be used with silicone- or paraffin-impregnated papers. This makes it possible to locate compounds present in extracts in such extremely low concentrations that they could not previously be detected. The substantially higher absorption capacity is also important for the separation of the components for further analytical purposes.

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EXPERIMENTAL

In the text and the figures the following abbreviations are used for the separated compounds:

A	= Vitamin A	PQ_9	= Plastoquinone A (9 isoprene units in side chain)
C	= β-Carotene	PQB	= Plastoquinone B (side chain unknown)
Chl	= Chlorophyll $a + b$	Q_{10}	= Coenzyme Q_{10}
D_2	= Vitamin D_2	T	= α-Tocopherol
K_1	= Vitamin K_1	TQ	= α-Tocopherylquinone
Ph	= Phaeophytin	X	= Xanthophylls

The tested compounds were either bought commercially when available or isolated from spinach chloroplasts. A sample of synthetic plastoquinone 9 was kindly supplied by Hoffmann-LaRoche & Co.

Solvents

The choice of the solvent depended on the particular separation to be undertaken. Cyclohexane was found to be convenient for the separation of K_1 and Q_{10} from β-carotene. For the separation of the other quinones and vitamins from one another a mixture of cyclohexane–benzene (3:7), pure benzene, chloroform–benzene (1:1), and chloroform were used successfully.

Application of samples to paper

In general, samples were applied to the paper from ethanol, cyclohexane or isooctane solution. Samples in acetone were also satisfactory if care was taken to keep the spot size small.

Development of chromatogram

Ascending chromatography was used routinely; for small quantities chromatostrips were used, but when larger amounts of lipid were to be separated, large sheets of paper were used as separation media. Circular chromatography worked equally well with the above solvents, and was found especially convenient when test substances were employed, as well as for the quantitative analysis of natural extracts, when distinct separation of compounds in single, not overlapping, bands was required. All chromatograms were run at room temperature and in the dark to avoid destruction of the lipids.

Detection methods

1. Examination in daylight and in ultra-violet light.

2. Spraying with saturated solution of $SbCl_3$ in chloroform (A, β-carotene, carotenoids). D_2 gave a distinct brownish yellow color.

3. Spraying with a mixture of equal volumes of α,α'-dipyridyl (0.5 %) and $FeCl_3$ (0.2 %) in 95 % ethanol (tocopherol).

4. Use of the neotetrazolium spray according to LESTER AND RAMASARMA[4]: quinones were first reduced by immersing the paper in 0.1% solution of sodium borohydride for 30 sec. Excess borohydride was destroyed by dipping the paper in 0.1 *N* HCl for 2 sec. The chromatogram was then immersed in a solution containing

0.25% neotetrazolium chloride in 0.25 *M* potassium phosphate, pH 7.0 (hydroquinones). Naphthoquinones gave immediately an intense red color (K_1, menadione, 1,4-naphthoquinone and phthiocol). Benzoquinones (TQ, Q_{10}, PQ_9, PQB) showed red colored spots only after heating at 100° for 60 sec. Thus the neotetrazolium reaction allows a distinction between derivatives of naphtho- and benzoquinones. TQ gave an intense spot of deep violet, while PQ's showed only a pale red. Therefore care has to be taken not to miss the PQ bands when the method is applied to natural extracts.

RESULTS AND DISCUSSION

Chromatography of pure compounds

In the procedure described here, all known fat-soluble vitamins and various naturally occurring quinones have been separated successfully. The efficiency of the Schleicher and Schüll filter paper No. 288 for the separation of lipid-soluble quinones and vitamins is due to its content of alumina. On ordinary filter paper, quinones, vitamins and β-carotene are separated only poorly or not at all. Reverse phase chromatographic techniques using vaseline, paraffin, or silicone as the primary phase are limited by the small amount of material which can be applied to the paper and by the impossibility of eluting the separated compounds for further analysis without simultaneous extraction of the impregnated oil. Alumina paper, however, has an obvious advantage in its higher absorption capacity. It has the additional advantage of absorbing quinones and vitamins more strongly than β-carotene. Moreover, the separated compounds can be eluted uncontaminated. Quinones isolated from natural extracts by this new technique showed absorption spectra in the ultraviolet region which were in good agreement with the data reported in the literature.

Cyclohexane and a mixture of cyclohexane and benzene (7:3) were found to be convenient for the separation of vitamin K_1 from β-carotene and from other quinones. This procedure also produced the purest β-carotene. For the isolation of PQ_9, Q_{10} and T, a mixture of cyclohexane and benzene (3:7) or pure benzene was used. TQ was best separated from accompanying lipids with the benzene–chloroform mixture (1:1), and for vitamin D_2 and A pure chloroform provided good separation. Typical chromatostrips are represented in Fig. 1.

The adsorption sequence on the alumina paper follows the lipid character of the compounds being chromatographed. Molecules with a high lipid solubility (*e.g.*, hydrocarbons) are adsorbed to a much smaller extent than others with more hydrophilic groups. In the case of the quinones, one might assume that their lipid character, and consequently their order of migration, are determined by the length of the side chain in position 3. However, it will be seen that this is only partially true. Thus, PQ_9, a 5,6-dimethyl-3-solanesyl-benzoquinone with 9 isoprene units (C 45) in the side chain has a higher R_F than Q_{10}, a 5,6-dimethoxy-2-methylbenzoquinone with 10 isoprene units (C 50) in position 3. Thus, methoxy-groups in place of methyl-groups lower the R_F value considerably. The structure of the above mentioned compounds is shown in Fig. 2. TQ, a 5,6-dimethylbenzoquinone with a 3'-hydroxyphytyl side chain (C 20) compared with PQ_9 and Q_{10} has, as may be expected, a significantly lower mobility on the chromatogram. For vitamin K_1 (2-methyl-3-phytyl-naphthoquinone) which has a phytyl group (as does TQ) but no hydroxyl group, one could expect a chromatographic mobility somewhat greater than that of TQ.

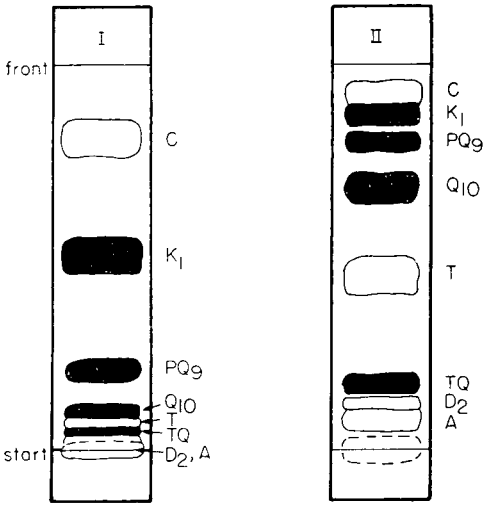

Fig. 1. Paper chromatogram of lipid-soluble vitamins and quinones. Solvent for I, cyclohexane-benzene (7:3); for II, benzene. Dark spots indicate quinones.

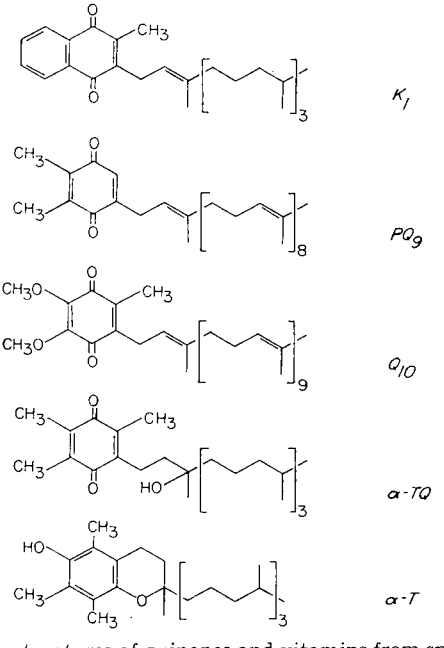

Fig. 2. Comparison of the structures of quinones and vitamins from spinach leaves.

Its R_F value, however, is even higher than those of the long side chain quinones PQ_9 and Q_{10}. This fact must be ascribed to the possession by the napthoquinone ring of a lipid character apparently greater than that of benzoquinone. In spite of its phenolic hydroxyl and its short side chain of only 15 C atoms, T, the chromanol from of TQ,

shows a higher R_F than TQ. This behavior, and that of vitamin K_1, prove that double ring structures possess a higher lipid solubility than single ring systems. Thus, comparison of structure and R_F values makes it clear that apart from the length of the polyisoprenoid side chain, the arrangement of substituents in the quinone molecule is of essential significance for the position on the chromatogram.

Various other solvents such as isooctane, petroleum ether, carbon tetrachloride, and toluene, or mixtures thereof, were tested for separation of lipid soluble quinones and vitamins. They also provided good separation, but had no advantages over the solvents described. More polar solvents such as pyridine, methanol and acetone were of no value, since all fat-soluble substances tested had R_F values higher than 0.9 in these solvents.

In Table I the R_F values of quinones and vitamins, as well as some plant pigments, are listed for the different solvents. The values of the tested substances varied within narrow limits, especially when natural extracts were applied. There was, however, no change in the order of migration.

Normally, the walls of the chromatographic tanks were lined with filter paper moistened with solvent. If this was not done, higher R_F values were obtained. In Table I, in case (*a*), where the glass tank was saturated with the solvent, the solvent travelled much farther from the starting point per time unit than in case (*b*), where the solvent partially evaporated from the chromatogram. Thus, when calculating R_F's, higher values were obtained. This fact has to be taken into account when highly volatile solvents are used, and particularly when, as in the present instance, short development times are employed (1–2 h). Therefore, in all further experiments the walls were covered with filter paper in order to ensure saturation of the tank with the solvent and to obtain reproducible R_F values.

Chromatography of plant extracts

The method described here has been used successfully for examining various plant extracts. It might also be employed for animal tissue extract analysis, a possibility now under investigation. β-Carotene, the principal compound accompanying and often masking quinones and vitamins in natural extracts, runs almost with the solvent front, while the chlorophylls and xanthophylls remain practically on the starting line. Thus, these normally interfering substances do not affect the separation of the other lipids. Fig. 3 shows chromatograms of a freshly prepared acetone extract from spinach leaves.

Large amounts of extraneous fatty material in the extract, however, have a considerable influence on the R_F values of the quinones and may cause poor separation. This is particularly true for extracts from whole leaves. Therefore, purification by column chromatography should precede the separation on paper. Repeated paper chromatography is also advisable. The application of the new technique to plant extracts therefore quickly provides information as to whether quinones or tocopherols are present, before further purification and final isolation are carried out. When preliminary column chromatography is employed, the method is very useful in detecting and identifying the quinones in the various fractions eluted. Its application as a test of purity of the isolated compounds is also of great significance.

With the new method, several quinones and α-tocopherols were found in chloroplasts and quantasomes of spinach. Some of these quinones were identified as K_1,

TABLE I

R_F VALUES FOR NATURALLY OCCURRING QUINONES, FAT-SOLUBLE VITAMINS AND PLANT PIGMENTS ON SCHLEICHER AND SCHUELL NO. 288 PAPER

Compounds	*Cyclo-hexane*	*Cyclo-hexane–benzene (7:3)*		*Cyclo hexane–benzene (3:7)*	*Benzene*	*Benzene–$CHCl_3$ (1:1)*		*$CHCl_3$*	*Iso-octane*	*Pet. ether (b.p. 30–60°)*	*CCl_4*	*Toluene*	
		(a)	*(b)*	*(a)*		*(a)*	*(b)*					*(a)*	*(b)*
β-Carotene	0.51	0.81	0.98	0.84	0.92	0.87	0.95	0.94	0.13	0.32	0.79	0.86	0.90
K_1	0.20	0.50	0.90	0.78	0.87	0.85	0.94	0.91	0.08	0.10	0.53	0.79	0.87
PQ_9	0.08	0.18	0.23	0.77	0.82	0.85	0.94	0.91	—	0.05	0.18	0.78	0.87
Q_{10}	0.05	0.09	0.16	0.47	0.70	0.83	0.90	0.91	—	—	0.13	0.55	0.85
T	—	0.05	0.13	0.26	0.46	0.48	0.64	0.82	—	—	0.10	0.32	0.78
TQ	—	—	0.05	0.05	0.14	0.41	0.50	0.73	—	—	0.05	0.09	0.65
D_2	—	—	0.05	0.08	0.13	0.40	0.41	0.59	—	—	—	0.09	0.52
A	—	—	—	0.04	0.11	0.29	0.31	0.51	—	—	—	0.09	0.38
Chl a + b	—	—	—	—	—	0.04	0.04	0.08	—	—	—	—	—
X	—	—	—	—	—	—	—	0.08	—	—	—	—	—
Ph	—	—	—	—	0.17	0.47	0.58	0.77	—	—	—	0.09	0.74

(a) Walls of tank lined with solvent-wetted filter paper.
(b) Walls of tank not lined as in (a).

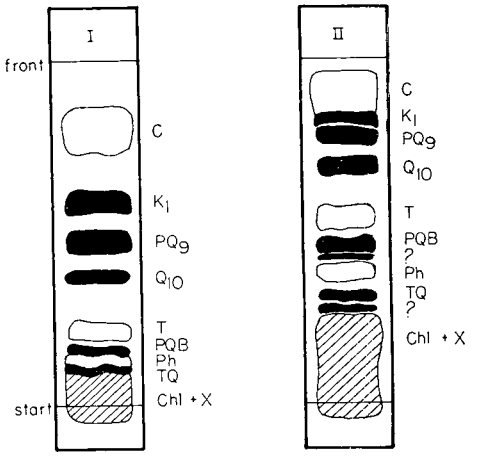

Fig. 3. Paper chromatogram of an acetone extract from spinach leaves. Solvent for I, cyclohexane–benzene (7:3); for II, benzene. Dark spots indicate quinones.

PQ_9, PQB and TQ. Q_{10}, present in the leaves of spinach, was not detected in chloroplasts and quantasomes. Further study is required for the identification of other quinones present in lower concentrations, as indicated on the chromatograms. These might correspond to plastoquinone C and other tocopherylquinones which recently have been shown to occur in chloroplasts[5].

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SUMMARY

A rapid method for separation and identification of naturally occurring quinones and fat-soluble vitamins on commercially available filter paper with alumina filler is described. The R_F values for different solvents are tabulated. The application of the method to natural extracts is discussed.

NOTE ADDED IN PROOF

When the chromatograms are immersed in the neotetrazolium chloride solution before they are taken into the sodium borohydride solution, the sensitivity of revealing the quinones as red spots is increased considerably. Heating is then to be omitted.

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THE DETECTION OF SOME INDOLES AND RELATED COMPOUNDS ON PAPER CHROMATOGRAMS*

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The condensation of pyrroles and indoles with aldehydes is a well known reaction which was studied extensively at the turn of the century and which aided HOPKINS AND COLE[1] in the discovery of tryptophan. Later on, the indole–aldehyde reaction was studied by BURR AND GORTNER[2] in connection with humin formation in protein hydrolysates. Since then, the reaction has been widely used to detect indoles, ureides and aromatic amines on paper chromatograms[3,4]. The detection of simple indoles has depended largely on the Ehrlich color reaction, using *p*-dimethylaminobenzaldehyde[4–6]. Recently HARLEY-MASON AND ARCHER[7] reported that *p*-dimethylaminocinnamaldehyde (DMCA) was ten times more sensitive for indole and tryptophan than *p*-dimethylaminobenzaldehyde (Ehrlich reagent).

The great interest in the biochemistry of naturally occurring indole derivatives in both plant and animal tissues, especially in connection with indole-3-acetic acid and serotonin, warrants further study on the methods of isolation and identification of these substances. The present report deals with the chromatographic behavior of twenty-seven indoles and eighteen related compounds. Ultra-violet fluorescence before spraying and the color reactions obtained after spraying with the two aldehyde reagents are reported and the relationship of structure to color reaction is discussed briefly.

MATERIALS AND METHODS

Materials

Indole, skatole, gramine, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, tryptophan, tryptamine, the aromatic amines, urea, thiourea, citrulline and quinaldic acid were obtained through the usual commercial sources. The 5-benzyloxyindoles, 1,2-dimethylindole, 2,3-dimethylindole, 7-azaindole, indole-3-aldehyde and indole-3-acetonitrile were obtained from Aldrich Chemical Co., Milwaukee, Wisc. 5-Hydroxytryptophan, 5-hydroxyindole-3-acetic acid (cyclohexylamine salt), kynurenine, kynurenic acid and xanthurenic acid were obtained from the California Foundation for Biochemical Research, Los Angeles, Calif. Serotonin or 5-hydroxytryptamine (creatinine sulfate complex) was kindly supplied by Vismara Terapeutici.

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The phenols were kindly supplied to us by Dr. H. MORITA of the Soils Research Institute, Canada Agriculture, Ottawa.

Indole-3-glyoxylic acid, indole-3-glyoxyl chloride, indole-3-glyoxylamide and methyl indole-3-glyoxylate were synthesized from indole by the methods of SHAW *et al.*[8]. 3-Cyanoindole, indole-3-aldoxime, indole-3-carboxylic acid and indole-3-acrylic acid were synthesized from indole-3-aldehyde by the methods of SHAW *et al.*

Indole-3-carboxylic acid, prepared by the carbonation of the Grignard reagent formed with ethyl magnesium bromide and indole[9] and recrystallized from aqueous ethanol was not chromatographically pure. On the other hand this acid prepared by hydrolysis of crude 3-cyanoindole by SHAW's method did not require further recrystallization and gave only one spot in both isopropanol–ammonia–water (8:1:1) and butanol–acetic acid–water (4:1:1). It decomposed at 246° (uncorr.). The product from the Grignard reaction decomposed at 220°–225°.

8-Hydroxyquinaldic acid was prepared by a four-step synthesis from *o*-anisidine and crotonaldehyde in good yield by the method of IRVING AND PINNINGTON[10,11].

Most of the substances from commercial sources were chromatographically pure and gave sharp melting points. 2,3-Dimethylindole, gramine and 5-benzyloxygramine yielded more than one spot which may have been due to decomposition during chromatography. Catechol and 3-methoxycatechol were unstable giving spots and streaks on the paper. The latter compound became discolored even when stored in the refrigerator in 95 % ethanol.

Methods

The chromatography was carried out on Whatman No. 1 paper using the ascending method in butanol–acetic acid–water (4:1:1) and isopropanol–ammonia–water (8:1:1). The spray reagents used were: (a) *Ehrlich aldehyde reagent*, prepared by dissolving 2 g *p*-dimethylaminobenzaldehyde in a mixture of 80 ml of 95 % ethanol and 20 ml of 6 *N* HCl[12]. (b) *p-Dimethylaminocinnamaldehyde* (DMCA) reagent, prepared according to HARLEY-MASON AND ARCHER[7] by dissolving 2 g of this aldehyde in a mixture of 100 ml of 95 % ethanol and 100 ml of 6 *N* HCl.

The chromatograms were equilibrated with the solvent for 2 h and developed overnight (16 h). After air-drying, they were sprayed with one or the other reagent, dried in front of a fan and then placed in an oven set at 70° for 5 min. After this treatment they were allowed to remain at room temperature for 48 h. Papers sprayed with DMCA turned very dark after 24 h, but the strong spots remained outlined on the paper. Papers sprayed with the Ehrlich reagent had very little background color. All papers were examined under ultra-violet light using a Blak-ray, long wave U.V. lamp before and after spraying with color reagent.

For chromatography, the substances were dissolved in a suitable solvent, generally 95 % ethanol or acetone and made up quantitatively to a concentration of 1 mg per ml, and 10 μl were spotted at the starting line with a micro-pipette. Because some of the substances did not react at this level some chromatograms were run using 20–30 μl per spot.

RESULTS AND DISCUSSION

The color reactions, R_F values, U.V. data, etc. for the indole derivatives and related metabolites are given in Tables I, II and III. Equations and formulae referred to throughout the text are found in Figs. 1 and 2.

TABLE I

DETECTION OF INDOLES

Compound	R_F*		Color with Ehrlich reagent**				Color with DMCA**				Misc. (U.V. reaction)
	Ip–NH_3	Bu–Ac acid	Color	Intensity	Heating and standing	Time	Color	Intensity	Heating and standing	Time	
Indole	0.91	0.92	Pi-R	3	R (fades)	F	G	4–3	B	F	
Skatole	0.92	0.92	P	3	P	F	P-B	4–3	—	F	
1,2-Dimethylindole	0.89	0.92	R	3	R-P	F	Pi	1	G	F	
2,3-Dimethylindole	—	—	Unstable, breakdown products in chromatography								
5-Benzyloxyindole	0.93	0.90	R-P	3	P	F	G	4–3	B	F	
7-Azaindole	0.83	0.83	—	—	—	—	—	—	—	—	B after spray
Indole-3-acetic acid	0.45	0.87	P-R	3	P	M	B	4	B	F	
Indole-3-propionic acid	0.50	0.86	P	3	P	M-F	B	4	B	F	
Indole-3-butyric acid	0.56	0.90	PB	3	B	F	B	4	B	F	
Indole-3-acrylic acid	0.43	0.86	G	4	B	M-F	B	3	B	F	
Indole-3-carboxylic acid	0.35	0.90	B-P	3	B-P	S	—	—	No reaction		
5-Benzyloxyindole-3-acetic acid	0.52	0.87	B	2	B	M	B	3	B	F	
Indole-3-aldehyde	0.90	0.86	—	1	lt B	SS	—		B	SS	
Indole-3-aldoxime	No immed. reaction										
Indole-3-acetonitrile	0.90	0.92	Pi	1	Br	SS	P-R	3	R-P	F	
3-Cyanoindole	—	—	No immed. reaction								
Tryptophan	0.36	0.42	P	2	Y (24 h)	M	P	3	P-B	F	
Tryptamine	0.78	0.69	P(R)	4	P-R	F	P	4	—	F	
5-Hydroxytryptophan	0.18	0.15	P	4–3	—	M	B	4–5	—	F	
5-Hydroxytryptamine	0.59	0.40	P	3	—	M	B	4–5	—	F	
5 Hydroxyindole-3-acetic acid	0.24	0.70	P	3	—	M	B	4–5	—	F	
Gramine											
5-Benzyloxygramine	—	—	—	—	—	—	—	—	—	—	
Indole-3-glyoxylic acid***	0.45	0.68	—	No reaction		—	—	No reaction		—	Y fluorescence
Indole-3-glyoxylchloride***	—	—	Y	1	—	SS	—	No reaction		—	
Indole-3-glyoxylamide***	0.81	—	Y	1		SS	Pi	3		S	
Methyl indole-3-glyoxylate***	—	—	Y	1	—	SS	—	No reaction		—	

* Ip–NH_3 = isopropanol–ammonia–water (8:1:1); Bu–Ac acid = butanol–acetic acid–water (4:1:1).

** B = blue; Br = brown; G = green; O = orange; P = purple; Pi = pink; R = red; Y = yellow; lt = light. Color intensity: 5 = very intense; 1 = very faint. Rate of color development: F = fast; M = medium; S = slow.

*** All the glyoxylic derivatives require at least 30 μg per spot to give a faint color with either reagent. The methyl ester and acid chloride are transformed to indole-3-glyoxylamide during chromatography in isopropanol–ammonia–water (8:1:1).

TABLE II

DETECTION OF AROMATIC AMINES, UREIDES AND QUINOLINES

Compound	*R_F**		*Color with Ehrlich***				*Color with DMCA***				
	Ip-NH_3	*Bu-Ac acid*	*Color*	*Intensity*	*Heating and standing*	*Time*	*Color*	*Intensity*	*Heating and standing*	*Time*	*U.V. reaction*
Anthranilic Acid	0.45	0.85	Y	5	Y	F	R-Br	4	R-P	F	B fluorescence
p-Aminobenzoic acid	0.20	0.80	Y	5	Y	F	P	5	R-P	F	No fluorescence
Urea	0.45	0.47	Y	2	—	M	R	3	—	F	
Thiourea	0.50	0.47	Y	1	—	S	P	1	—	S	
Citrulline	0.10	0.16	Y	2	—	M	R	2	—	M	
Kynurenine	0.28	0.30	Y	4	—	F	Gr	2	P	F	Strong fluorescence
8-Hydroxyquinaldic acid	0.67	0.14	Yellow color seen before spraying								R fluorescence
Quinaldic acid	—	—	No reaction				No reaction				No fluorescence
Kynurenic acid	0.45	0.45	No reaction				No reaction				B fluorescence
Xanthurenic acid	0.10	0.48	No reaction				No reaction				B fluorescence

* Ip-NH_3 = isopropanol–ammonia–water (8:1:1); Bu-Ac acid = butanol–acetic acid–water (4:1:1). B = blue; Br = brown; Gr = gray; P = purple; R = red; Y = yellow. Color intensity: 5 = very intense; 1 = very faint. Rate of color development: F = fast; M = medium; S = slow.

TABLE III

DETECTION OF PHENOLS

Compound	R_F*		Color with Ehrlich**				Color with DMCA*				U.V. reaction
	Ip-NH_3	*Bu-Ac acid*	*Color*	*Intensity*	*Heating and standing*	*Time*	*Color*	*Intensity*	*Heating and standing*	*Time*	
Eugenol		—	—	No reaction			—	No reaction			
Catechol		—	—	Decomposition				No reaction			
Gallic acid		—	—	No reaction			—	No reaction			
Syringic acid		—	—	No reaction			—	No reaction			
Phloroglucinol		0.60	R-P				P				
3-Methoxycatechol		0.77	—	Decomposed in refrigerator				— Decomposed in refrigerator			
2,5-Dihydroxyphenylacetic acid			—	No reaction				No reaction			
2,5-Dihydroxybenzoic acid	0.58	0.75	—								Strong blue fluorescence

* Ip–NH_3 = isopropanol–ammonia–water (8:1:1); Bu–Ac acid = butanol–acetic acid–water (4:1:1).
** P = purple; R = red.

In general the new aldehyde reagent, DMCA, reacted faster and gave more intense colors with indole compounds than the Ehrlich reagent. Indole-3-acetic acid could be detected on paper chromatograms in concentrations as low as 0.5 μg per spot. The reaction of DMCA with aromatic amines and ureides was disappointing .Red and purple colors were given which would make difficult their distinction from indole derivatives (Table II). One exception to the non-selective action of DMCA is the color reactions given by indole and skatole (Table I). Since these compounds have approximately the same R_F value in both solvents, they can be identified by the fact that indole gives a distinct green color with DMCA whereas skatole gives a purple spot. Indole-3-acrylic acid may be easily detected, if both sprays are used. This substance gives a green spot with the Ehrlich reagent and a blue spot with DMCA. Indole-3-acetonitrile reacts immediately and strongly with DMCA, but the reaction with the Ehrlich reagent is very slow and rather weak. Indole-3-carboxylic acid does not react with DMCA but gives a clear blue-purple spot with Ehrlich reagent after drying for about 10–15 min.

The chromatographic behavior of the indole-3-glyoxylic acid derivatives is interesting. The acid itself does not react with either aldehyde but does fluoresce under ultra-violet light (Table I). Under the conditions of chromatography in isopropanol–ammonia–water (8:1:1) the methyl ester and acid chloride are transformed to the amide which gives a faint pink spot with DMCA and a faint yellow spot with the Ehrlich reagent.

Of the phenols tested, only phloroglucinol reacts with the aldehyde reagents and gives a red-purple color with the Ehrlich reagent and a purple color with DMCA. The quinolines did not react but fluoresced under ultra-violet (Table III).

The most significant observation in this particular study is that many 3-substituted indoles either do not react with these aldehydes or react so poorly that the color reactions cannot be used with confidence in their identification. 3-Substituted indoles in which a carbonyl or nitrile group is attached directly to the β-carbon atom of the pyrrole ring are poor reactors. If the carbonyl or nitrile group is separated by a methylene group the reaction is greatly improved. For example, 3-cyanoindole does not give a color with DMCA or Ehrlich reagent but indole-3-acetonitrile reacts rapidly with DMCA and slowly with Ehrlich reagent. Indole-3-glyoxylic acid (two carbonyl groups) does not react but the reaction occurs to some extent when $-NH_2$ is substituted for –OH (indole-3-glyoxylamide). Indole-3-aldehyde and indole-3-carboxylic acid react faintly with both aldehydes but indole-3-acetic, indole-3-propionic and indole-3-butyric acids react strongly. Worthy of mention is the fact that in this homologous series the longer the chain the stronger and more rapid is the reaction.

On the basis of the above observations, these differences in reactivity can be explained by reference to the structural and electronic formulae of these indoles (Fig. 1). If the condensation takes place at the 2-position the electron-attracting CN and C=O groups tend to decrease the electron density around the 2-carbon atom, making that position positive and repelling the attack of the electrophilic aldehyde. When these electrophilic groups are separated by methylene groups there is no longer a conjugated system and therefore no electromeric effect. The presence of tertiary nitrogen in the gramines and 7-azaindole may explain their failure to react, because under the acid conditions of the test, the nitrogen becomes quaternary due to salt formation and becomes an electron-attracting group as well.

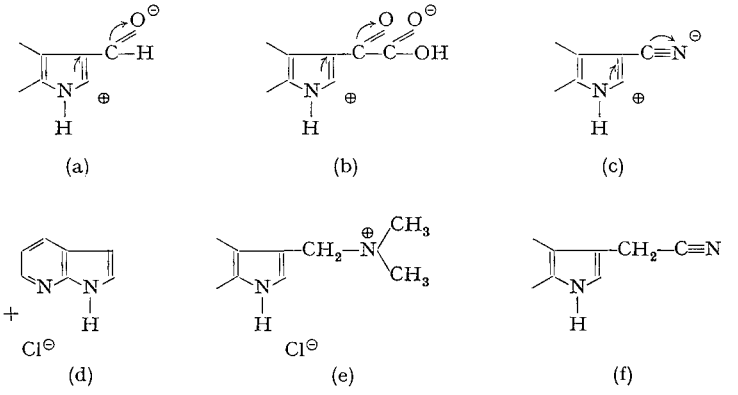

Fig. 1.

Substituents on the benzene ring in the 5-position had very little effect on the color reaction except perhaps to enhance it. Indole and 5-benzyloxyindole gave different colors with both aldehydes than the 3-substituted derivatives. The former gave a green color with DMCA and red-purple with Ehrlich reagent and the 3-substituted derivatives were mostly purple with Ehrlich reagent and blue with DMCA. FEIGL[13] suggests that indole and pyrrole react with the aldehyde in the ratio of 1 mole for 1 mole to form a colored complex of the indolidene-methane structure (B) (Fig. 2) and BURR AND GORTNER[2] have isolated colored salts of several 2-substituted indoles with this structure.

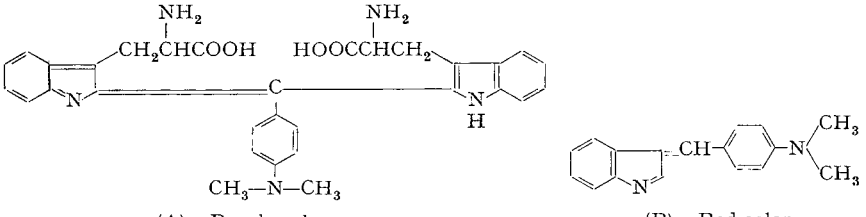

Fig. 2.

GHIGI[14] studied the reaction of *p*-dimethylaminobenzaldehyde with tryptophan and assumed that this reaction took place in the ratio of 2 moles of the indole for 1 mole of aldehyde to form a colored complex of the type (A) (Fig. 2).

It is probable that indoles with no substituents in the 3-position react 1 mole for 1 mole according to FEIGL and to BURR AND GORTNER whereas the 3-substituted compounds react in the manner described by GHIGI and that the condensation takes place on the pyrrole ring at position 2. The difference in color mentioned above and the decreased reactivity of 3-substituted indoles with carbonyl and nitrile groups attached directly to the 3-carbon atom lend support to the hypothesis that condensation takes place at the 2-position and that the colored compounds are different. However, the reaction mechanism requires further study before the nature of the colored products can be readily explained.

SUMMARY

1. *p*-Dimethylaminocinnamaldehyde (DMCA) is a more sensitive reagent, in most instances, than the Ehrlich reagent but is less selective in its color reaction and would be rather a poor chromatographic spray when both indoles and aromatic amines are present.

2. DMCA should be used if skatole or indole is assumed to be present on the chromatogram and in conjunction with the Ehrlich reagent if indole-3-acetonitrile and indole-3-acrylic acid are thought to be present.

3. The decreased reactivity of substituted indoles with carbonyl or nitrile groups attached directly to the 3-carbon atom suggest that the condensation takes place at the 2-position.

4. The differences in color of indole and benzyloxyindole from the 3-substituted derivatives, indole-3-acetic acid and tryptophan, etc., suggest that their condensation products are of different structure.

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PAPIERCHROMATOGRAPHISCHE TRENNUNG VON FUROCUMARINEN

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In der Pflanzenwelt weit verbreitet ist die Stoffklasse der Cumarine[1]. Neben dem einfachen Cumarin und seinen Derivaten finden sich besonders in der Familie der Umbelliferen und Rutaceen zahlreiche Verbindungen, die sich vom Furocumarin ableiten. Meistens kommen mehrere Verbindungen dieses Typs nebeneinander vor. Daher bereitet die Isolierung reiner Stoffe oft erhebliche Schwierigkeiten. Es wäre daher besonders vorteilhaft, durch eine geeignete Mikromethode die Reinheit und Einheitlichkeit eines Stoffes feststellen zu können. Als Methode der Wahl empfahl sich uns auch hier die Papierchromatographie unter der Voraussetzung, dass ein Lösungsmittel zur Anwendung kommt, welches eine hinreichende Trennung dieser Substanzen erlaubt.

Trotz des häufigen Vorkommens dieser Stoffklasse im Pflanzenreich sind vergleichsweise wenig Arbeiten in dieser Richtung erschienen. Die Untersuchungen wurden im wesentlichen mit zwei Typen von Lösungsmitteln angestellt. Einerseits verwendete man wässrig-alkoholische Systeme mit oder ohne Zusatz von Säure oder vereinzelt auch Basen, sowie Mischungen von Kohlenwasserstoffen und Alkohol als Fliessmittel, indem Wasser als stationäre Phase diente. Andererseits wurden die Papiere mit wässrigen Glykollösungen imprägniert und Petroläther als mobile Phase benutzt. Eine Übersicht über die brauchbarsten Systeme verschiedener Autoren ist in Tabelle I gegeben.

In einphasigen Systemen treten dabei oft Schwanzbildungen auf; die Trennung mehrerer Cumarine ist nach den angegebenen R_F-Werten nur in einigen Fällen möglich. Im allgemeinen liegen die R_F-Werte recht hoch, weil die Furocumarine in Wasser nahezu unlöslich sind. Es wurden daher wiederholt auch Versuche unternommen, von zweiphasigen Systemen die wässrige Phase zum Chromatographieren zu verwenden (SWAIN[6], JASTRZEBSKI[11]), wobei mittlere R_F-Werte zu erreichen sind. Aber auch in extrem hydrophoben Lösungsmitteln ist ihre Löslichkeit nur gering, so dass die Substanzen nur wenig mit ihnen wandern. Verheissungsvoller erscheinen die Ergebnisse, die bei "reversed-phase-Papierchromatographie" unter Verwendung von acetyliertem Papier erzielt wurden (Tabelle I, Zit. 4).

Wie aus der Tabelle hervorgeht, ist eine vergleichende papierchromatographische Untersuchung der Furocumarine bisher nur von RIEDL UND NEUGEBAUER[2] sowie von GRUJIC-VASIC[14] durchgeführt worden, ohne dabei jedoch eine befriedigende Trennung der Mehrzahl der Furocumarine erreichen zu können. Die eigenen Versuche sollten

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TABELLE I

LITERATURÜBERSICHT ÜBER PAPIERCHROMATOGRAPHISCHE TRENNUNGEN VON FUROCUMARINEN

System*	$R_F \times 100$													Autor (Zit. Nr.)
	Psoralen	*Angelicin*	*Imperatorin*	*Isoimperatorin*	*Pimpinellin*	*Isopimpinellin*	*Xanthotoxin*	*Bergapten*	*Isobergapten*	*Oxypeucedanin*	*Peucedanin*	*Sphondin*	*Xanthotoxol*	
A	—	58	—	—	—	—	35	—	—	63	88	—	—	2
B	07	07	21	47	03	12	05	00–16	00–22	—	—	—	—	
A	—	—	—	—	—	—	—	40	—	—	25	—	—	3
C	—	—	—	—	—	—	—	50	—	—	62	—	—	4
D	—	—	—	—	85	74	—	69	81	—	—	—	—	5
E	—	—	91	—	—	—	87	88	—	—	—	—	—	6
F	—	—	90	—	—	—	83	81	—	—	—	—	—	
G	—	—	50	—	—	—	67	59	—	—	—	—	—	
H	—	—	47	—	—	—	58	45	—	—	—	—	—	
J	54	—	—	—	—	—	60	45	—	61	—	—	—	7
H	—	15–20	45–48	—	—	—	58–60	43–46	—	—	—	—	67–72	8
E	—	90–93	90–92	—	—	—	85–90	88–90	—	—	—	—	—	
H	—	—	—	—	64	—	—	45	—	—	—	—	—	9
K	—	—	—	—	51	—	—	36	—	—	—	—	—	
L	—	—	—	—	72	—	—	60	—	—	—	—	—	
M	—	—	—	—	42	—	—	26	—	—	—	—		
N	—	—	—	—	68	—	—	46	—	—	—	—	—	
O	—	—	—	—	81	68	—	—	76	—	—	57	—	10
P	—	—	—	—	58	59	—	56	—	—	—	48	—	11
Q	—	—	—	87	—	—	82	75	—	—	—	—	—	12
R	—	—	80	—	—	—	75	70	—	—	—	—	—	13
S	73	76	—	—	81	76	73	74	77	78	—	—	—	14

* Lösungsmittelsysteme:

A: Impr. 20%ige wässrige Glykollösung/Lfm. Benzin 65–70°.
B: 20%ige wässrige Propylenglykollösung/Lfm. Benzin 65–70°.
C: Acetylcellulose/Lfm. Methyläthylketon–Aceton–Wasser (3:1:5).
D: Petroläther–Benzol–Methanol (5:4:2).
E: Butanol–Eisessig–Wasser (4:1:5).
F: Isopropanol–Wasser (2:3).
G: Benzylalkohol–Eisessig–Wasser (4:1:5) (wässrige Phase).
H: Essigsäure 10%ig.
J: Butylenglykol–Eisessig–Wasser (6:10:86).
K: Äthylenglykol–Wasser (1:24).
L: Pyridin–Wasser (3:97).
M: Wasser.
N: Wasser–Dioxan (9:1).
O: Benzin–Benzol–Methanol (30:15:10).
P: *n*-Pentanol–Eisessig–Wasser (4:1:5) (wässrige Phase).
Q: Methanol–Wasser (4:6).
R: Petroläther–Methanol (1:1) (26°).
S: Propanol–Wasser (9:1).

klären, inwieweit die Verwendung von Formamid bzw. Dimethylformamid als stationärer Phase eine Trennung der Furocumarine ermöglicht und inwieweit sich Beziehungen innerhalb dieser Stoffklasse zwischen Struktur und Verteilungsverhalten ableiten lassen.

Aus den Ergebnissen der eigenen Versuche (Tabelle II) ist ersichtlich, dass sich die Furocumarine im System I Formamid und im System II Dimethylformamid und Heptan–Benzol gut trennen lassen.

Für die Chromatographie mit Formamid bewährte sich eine Mischung von Heptan–Benzol (8:2), während bei Verwendung von Dimethylformamid zur Imprägnierung eine Erhöhung des Benzolanteils im Laufmittel von Vorteil war. Besonders hervorzuheben für dieses System ist dabei, dass auch Bergapten, das sonst leicht zu Schwanzbildung neigt, wohlausgebildete Flecke liefert. Durch Variation der Trockenzeit nach der Imprägnierung kann man den Grad der Polarisierung der stationären Phase verändern und dadurch eine allgemeine Erhöhung oder Erniedrigung der R_F-Werte erreichen.

Die beiden Grundkörper der Furocumarine sind das 2',3'; 7,6-Furocumarin (Psoralen) und das 2',3'; 7,8-Furocumarin (Angelicin). Diese zeigen in dem von uns benutzten System ein deutlich unterschiedliches Verhalten (R_F Psoralen in System I: 0.17, in II: 0.29; R_F Angelicin in I: 0.29, in II: 0.39). Die Eigenschaften der Grundkörper offenbaren sich gleichfalls in ihren Derivaten: Verbindungen mit linear ankondensiertem Furanring (Psoralentyp) haben durchgehend einen niedrigeren R_F-Wert als die isomeren Verbindungen mit angular ankondensiertem Furanring (Angelicintyp), wie die Beispiele Isopimpinellin < Pimpinellin sowie Bergapten < Isobergapten erkennen lassen. Diese Regel gilt auch für das Isomerenpaar Xanthotoxin–Sphondin, obgleich es hier weniger augenfällig ist.

Eine Verlängerung der Seitenkette verschiebt die Löslichkeit zugunsten der organischen Phase, wie es generell für die Verteilung der Glieder homologer Reihen erkannt wurde (Beispiel: Xanthotoxin–Imperatorin).

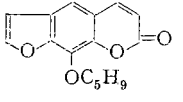
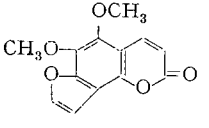
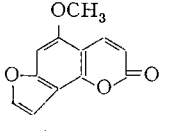
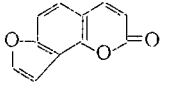
Die Entmethylierung und damit das Auftreten einer freien phenolischen Hydroxylgruppe erhöht die Löslichkeit in der polaren Phase so stark, dass die Verbindung am Start verbleibt (Beispiel: Xanthotoxol).

Während die einfachen Methyl- und Dimethyläther in beiden Systemen ein gleiches Verhalten zeigen, macht der Isopentenyläther Imperatorin eine Ausnahme. Infolge seiner besseren Löslichkeit in Dimethylformamid wandert er in diesem Lösungsmittelsystem weniger weit als Isobergapten und Pimpinellin, während er im System mit Formamid über diesen beiden Verbindungen liegt. Zur Abtrennung des Imperatorins von den übrigen Furocumarinen ist daher das Formamidsystem wegen seiner grossen Selektivität besser geeignet.

Zum Nachweis der Furocumarine empfiehlt sich die Beobachtung ihrer Fluoreszenz im U.V.-Licht, die nach Behandlung mit Alkali sich teilweise verändert oder besonders leuchtend hervortritt (Tabelle II). Bemerkenswert ist dabei, dass die Grundkörper Psoralen und Angelicin primär nur eine ganz schwache oder gar keine Fluoreszenz—wie auch Cumarin selbst—zeigen, sondern erst nach Behandlung mit Alkali unter allmählicher Aufspaltung des Lactonringes im U.V.-Licht sichtbar werden. Der Nachweis der Furocumarine unter der Quarzlampe ist die empfindlichste aller Methoden. Zur weiteren Differenzierung der einzelnen Verbindungen kann Diazoreagens herangezogen werden. Damit geben alle Furocumarine charakteristische

TABELLE II

PAPIERCHROMATOGRAPHISCHES VERHALTEN DER FUROCUMARINE

Name	*Formel*	R_F		*Nachweisreaktionen*			
		System I	*System II*	*U.V.*	*KOH/U.V.*	*Diazoreaktion*	*Emersonreaktion*
Imperatorin		0.56	0.48	gelb	tiefgelb	violettrot	—
Pimpinellin		0.50	0.54	gelb	gelbbraun	rosarot	schwach violett
Isobergapten		0.44	0.51	bläulich	fahlgelb	gelborange	violett
Angelicin		0.29	0.39	—	grünlichgelb	orange	rotviolett

Bergapten		0.23	0.35	bläulich	fahlgelb	rotviolett	--
Psoralen		0.17	0.29	bläulich	grünlichgelb	violett	(gelbbraun)
Isopimpinellin		0.15	0.31	gelb	braun	bräunlich	(gelb)
Sphondin		0.13	0.21	bläulich	fahlgelb	schwach violett	rotviolett
Xanthotoxin		0.12	0.20	gelb	tiefgelb	blauviolett	—
Xanthotoxol		0.00	0.04	bräunlich	braun	rot	blau

Färbungen, deren deutliche Nuancierung jedoch nur bei grösseren Mengen wahrzunehmen ist. Wie die vergleichende Betrachtung der Färbung zeigt, treten mit den Derivaten des Psoralens im allgemeinen violettrote, kräftige Farben auf, während die Abkömmlinge des Angelicins nur zarte Tönungen liefern.

Neben diesen bekannten Nachweisverfahren erprobten wir das Reagens nach EMERSON[15], das allgemein zum Nachweis von Phenolen dient. Dabei konnten wir die Feststellung machen, dass dieses Reagens nur mit Furocumarinen vom Angelicintyp reagiert, und zwar unter Bildung einer deutlichen und empfindlichen Rot- bis Rotviolettfärbung. Diese generelle Beobachtung lässt den Schluss zu, dass der Eintritt dieser Reaktion an ein nicht furanringgebundenes C-Atom C_6 (*p*-Stellung zur phenolischen Hydroxylgruppe) geknüpft ist. Dabei ist die Substitution durch eine Methoxylgruppe an dieser Stelle ohne Einfluss (Sphondin).

Eine Sonderstellung unter den untersuchten Furocumarinen nimmt das Xanthotoxol ein, das einzige Phenol dieser Reihe. So wie sein Verteilungsverhalten deutlich von dem anderer Verbindungen absticht, so ist auch die Reaktion mit Emerson-Reagens anders als bei den Äthern: mit dem Xanthotoxol tritt eine Blaufärbung auf. Die Untersuchungen zur Klärung dieser Reaktion werden fortgesetzt.

EXPERIMENTELLER TEIL

Auf Chromatographiepapier Schleicher & Schüll 2043b, 30 × 30 cm, werden etwa 10 μl einer 0.1 %igen Lösung der Substanzen in Aceton aufgetragen. Anschliessend werden die Bogen bis etwa 1 cm oberhalb der Startlinie durch das Imprägnierbad gezogen (System I: Formamid–Äthanol (40:60), System II: Dimethylformamid–Äthanol (40:60) Gew./Gew.). Der Bogen wird zwischen Filtrierpapier trockengepresst und die nichtimprägnierte Zone mit der Formamid- bzw. Dimethylformamidlösung besprüht. Man lässt die Bogen 15 Min. an der Luft hängen, heftet zu einem Zylinder und entwickelt ohne vorherige Sättigung mit Heptan–Benzol (System I (8:2), System II (7:3) Vol./Vol.).

Zur Identifizierung der einzelnen Cumarine dienen folgende Verfahren:

(a) Betrachtung im U.V.-Licht.

(b) Besprühen mit 0.5 *N* alkoholischer Kalilauge und Betrachtung im U.V.-Licht.

(c) Besprühen mit 0.5 *N* alkoholischer Kalilauge, 5 Min. langes Erwärmen auf 80° und anschliessendes Besprühen mit Diazoreagens. Lösung I: 1.0 g Natriumnitrit werden in 250 ml Wasser gelöst; Lösung II: 1.6 g Natriumsulfanilat werden in 240 ml Wasser gelöst und 10 ml conc. Salzsäure zugegeben. Die Lösungen werden im Kühlschrank aufbewahrt und vor Gebrauch gleiche Teile von I und II gemischt.

(d) Besprühen mit 0.5 *N* alkoholischer Kalilauge, Trocknen wie unter (c) und aufeinander folgendes Besprühen mit Lösungen I und II. Lösung I: 1.0 g Aminoantipyrin wird in 100 ml Wasser gelöst; Lösung II: 1.0 g Kaliumhexacyanoferrat (III) wird in 100 ml Wasser gelöst.

ZUSAMMENFASSUNG

Es wurde das Verhalten der wichtigsten Furocumarine vom Psoralen- und Angelicintyp bei der Papierchromatographie unter Verwendung des Systems Formamid bzw. Dimethylformamid und Heptan–Benzol als Laufmittel studiert. Die Beziehungen zwischen Struktur und chromatographischem Verhalten werden diskutiert. Zum

Nachweis der Verbindungen wird neben der Beobachtung der Fluoreszenz die Diazoreaktion herangezogen. Um festzustellen, welchem Grundkörper eine fragliche Verbindung zuzuordnen ist, wird die Reaktion nach EMERSON vorgeschlagen, die nur bei Derivaten des Angelicintyps positiv ausfällt. Nach dem angegebenen Verfahren lassen sich alle untersuchten isomeren Furocumarine durch ihr Verteilungsverhalten und ihre spezifischen Farbreaktionen eindeutig identifizieren.

SUMMARY

The behaviour was studied of the most important furocoumarins of the psoralene and angelicin types on paper chromatography, using the system formamide or dimethylformamide and heptane–benzol as solvent. The relationships between structure and chromatographic behaviour are discussed. The compounds were detected by their fluorescence and by means of the diazo reaction. In order to determine to which group an unknown compound belongs, the reaction of EMERSON is recommended, which reaction is only positive in the case of compounds belonging to the angelicin class. With the method described it was possible to identify all the isomeric furocoumarins investigated, by their chromatographic behaviour and their specific colour reactions.

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CHROMATOGRAPHY ON PAPER IMPREGNATED WITH ION EXCHANGE RESINS

IX. THE ADSORPTION OF METAL IONS FROM HCl ON ANION-EXCHANGE RESIN PAPER, DEAE PAPER AND AMINOETHYL-CELLULOSE PAPER

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INTRODUCTION

In recent years it has become increasingly apparent that the adsorption of metal ions on anion-exchange resins of the type of Dowex-1 depends not only on the so-called exchange groups (trimethylbenzylammonium groups) but also on the styrene–divinylbenzene network.

The main evidence for adsorption in which the quarternary ammonium groups are not involved is:

(1) The adsorption of chloro-complexes of the type $AuCl_4^-$ on sulphonic resins[1,2].

(2) The adsorption of neutral organic substances on ion-exchange resins (usually considered under the term "salting-out chromatography")[3].

(3) The adsorption of anions which are held very strongly on Dowex-1, on neutral substances such as cellulose[4].

(4) The desorption of strongly held anions from anion-exchange resins with organic solvents such as acetone[5].

We thought that a better picture of the effect of the resin network could be obtained, if it were possible to study anion exchange in presence and absence of the resin network. Such a comparison is possible at least in part, since anion exchangers of the type of diethylaminoethyl-cellulose and aminoethyl-cellulose exist where the network consists only of cellulose. In HCl solutions these substituted amines must be considered as completely ionised. Since it is possible to correct for adsorption on cellulose, the differences in behaviour between exchangers can thus only be due to the resin network and the substituents on the ammonium group.

In this paper we shall describe an extensive study of adsorption on various anion-exchange papers. Only a few data are at present available for the cellulose–anion exchangers[6–8], although it has been shown that for a few selected ions[9,10] equilibrium data can be used for resin papers and vice-versa. However, more experimental data were desirable both for the resin paper and for analytical applications.

EXPERIMENTAL

Usually four paper strips (each a different paper) were developed simultaneously in tightly closed jars (26 cm × 15 cm diam.) by the ascending method. For this work the

concentration of R.P. concentrated HCl (Carlo Erba) was assumed to be 12 N and all dilutions (namely 0.5 N, 1 N, 2 N, 4 N, 6 N and 8 N) were made from this acid.

Table I lists the papers employed and their relevant properties. The ratio A_L/A_S was obtained by weighing the dried and developed paper strips. The capacities are those claimed by the manufacturers. With exception of the Whatman No. 1 paper, the papers were washed twice with 2 N HCl and distilled water and air dried before use.

TABLE I

PROPERTIES OF ANION EXCHANGE PAPERS USED

Paper	*Ion exchange capacity mequiv. per gram*	*Ratio amount of 0.5 N HCl to amount of paper (factor A_L/A_S)*
Whatman No. 1	negligible	about 1.0
Amberlite SB-2 paper	1.13	0.88
Whatman DEAE paper DE 20	0.4	2.5
Whatman aminoethylcellulose paper AE 30	0.6	2.3

Solutions of metal ions were placed on the paper in HCl solution, usually 6 N HCl. The usual reagents such as 8-hydroxyquinoline or ammonium sulphide were used for the detection of most ions. Both cellulose ion exchange papers disintegrated in acid above 8 N and the aminoethylcellulose gave already rather indifferent chromatograms with 8 N HCl.

Fig. 1 shows the R_F values of most of the metal ions which adsorb on anion exchangers. A number of ions were not available at the time (TcO_4^-, $RuCl_6^{2-}$), and some others have a tendency to form double spots (*e.g.* Sb (V)). It is intended to deal with these at a later date.

DISCUSSION

(a) *Correlation between the data on SB-2 papers and data obtained by column and equilibrium experiments*

To obtain the conversion of R_F values to D values (distribution coefficients) the equation

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

may be used as has been shown previously[10]. The factor A_L/A_S was calculated by comparison with equilibrium data[10] and was found to be 1.9; it may also be obtained from the ratio

$$\frac{\text{mg resin/cm}^2}{\text{mg 0.5 } N \text{ HCl/cm}^2} \qquad (2)$$

and was found to be 1.95. The agreement is excellent considering the assumptions made and the relative irregularity of the paper.

On the resin paper D values of more than 200 correspond to R_F values below 0.01 and D values below 0.2 correspond to R_F values above 0.90. For practical reasons these are the limits in which comparisons can be made. Although KRAUS AND NELSON[11] carried out their work with Dowex-1 and the SB-2 paper does not contain the same

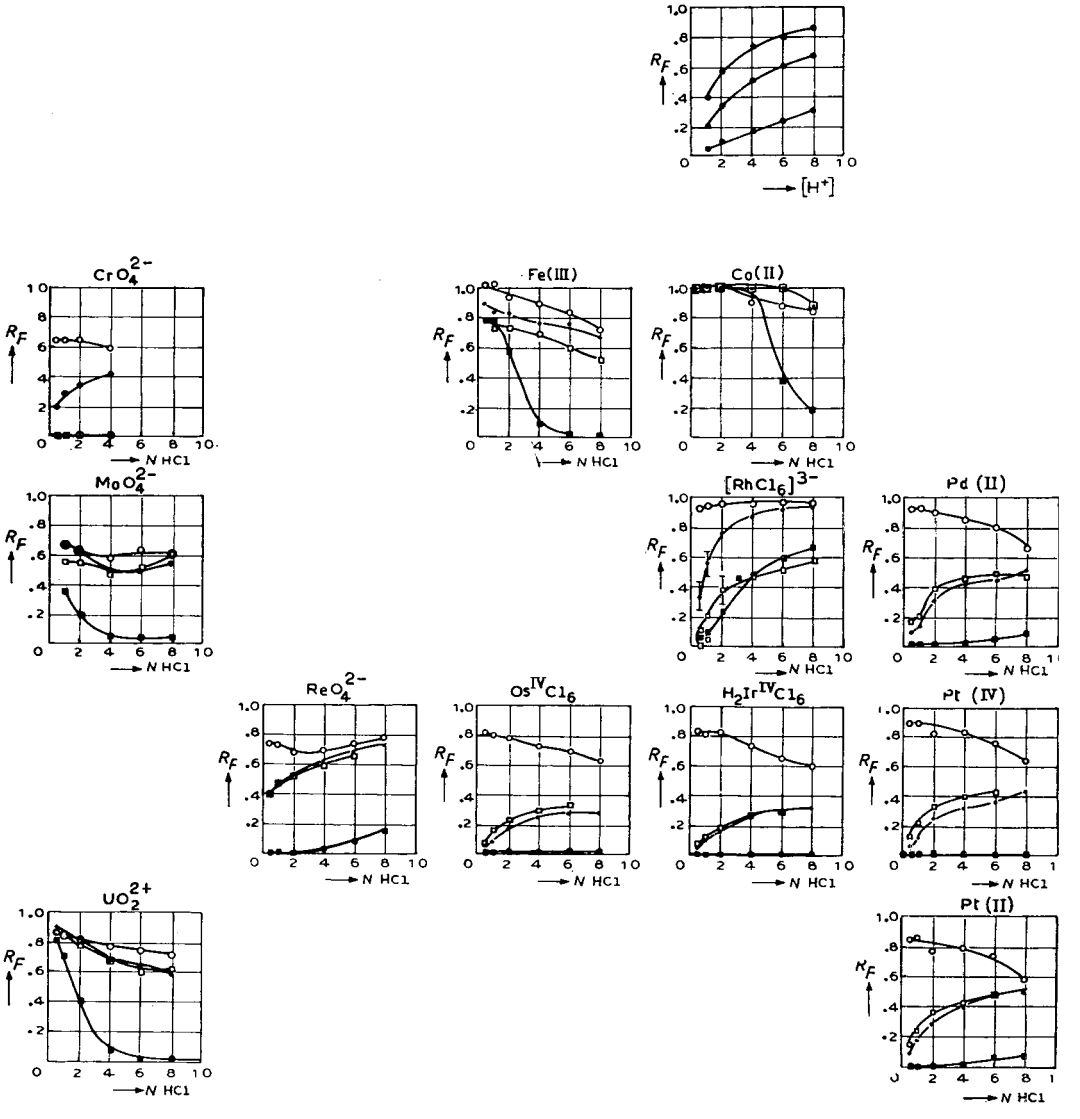
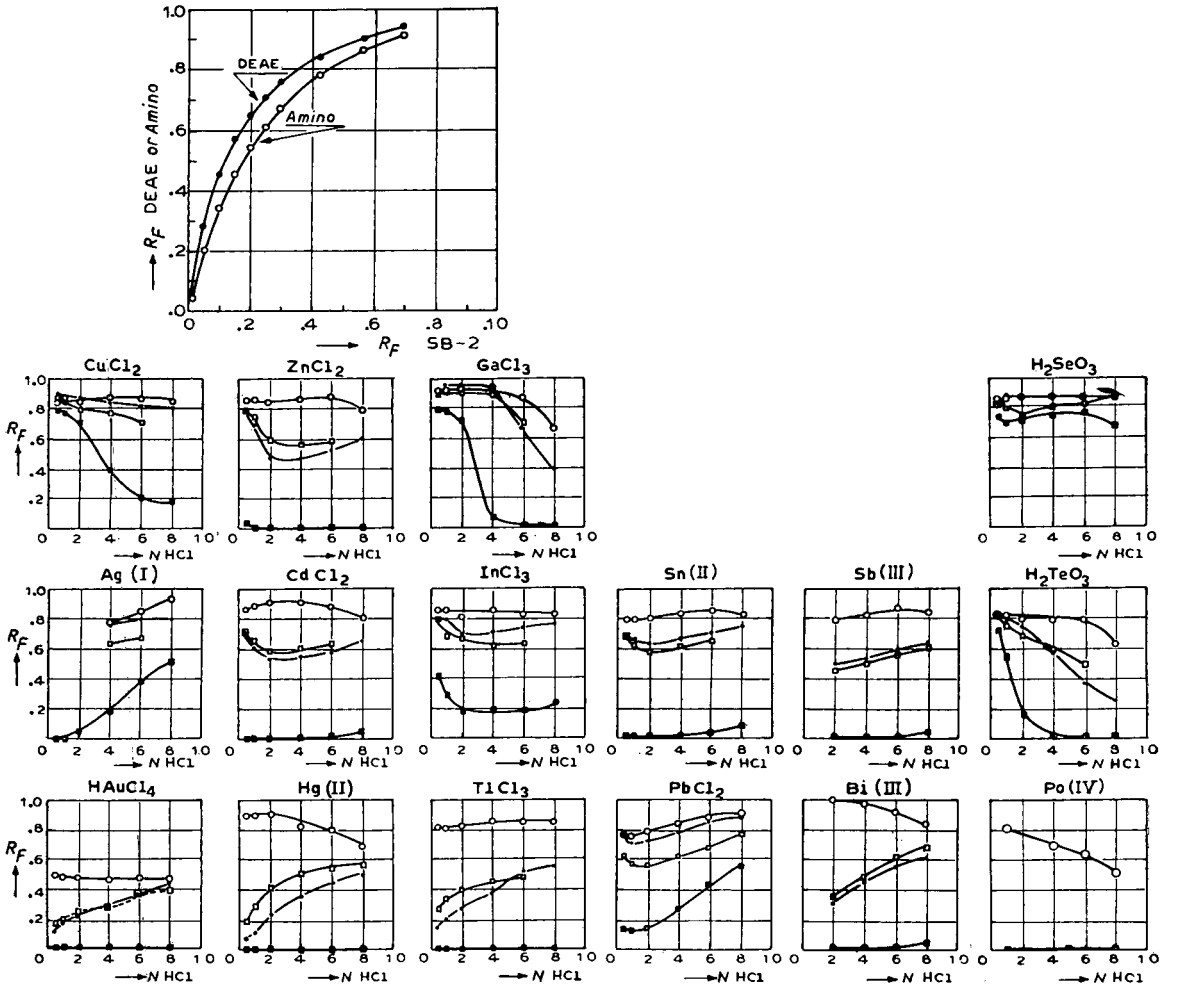


Fig. 1. Top, left: the theoretical R_F–H^+ curve of a monovalent anion obeying the law of mass action (calculated for three different R_F values at 1N H^+). Top, right: an alignment chart for converting R_F values on SB-2 paper to R_F values on AE-30 or DE-20 paper (or vice-versa). In this alignment chart only the differences in water/paper ratios and exchange capacities were considered.



Below: R_F values of metal ions on Whatman No. 1 paper (○——○), SB-2 resin paper (■——■), DE-20 cellulose anion-exchange paper (●——●), and AE-30 cellulose anion-exchange paper (□——□) are plotted against the concentration of HCl. With the exception of Pt (II) which was placed below Pt (IV) the metal ions are placed in their positions in the periodic table.

but only a similar resin, namely Amberlite IR-400, the agreement between D values calculated from Fig. 1 with those of KRAUS AND NELSON[11] is very satisfactory except for H_2TeO_4, which had already been studied and corrected by KLEEMANN AND HERRMANN[12]. Our results agree with those of KLEEMANN AND HERRMANN[12] and the adsorption of Te (IV) on Dowex-1 was further checked by DR. DOBICI of this Institute and found to agree essentially with these authors.

(b) *Comparison of the adsorption on resin and cellulose exchangers*

Fig. 1 (top, right) gives an alignment chart for converting R_F values on SB-2 paper to R_F values on DE 20 and AE 30 papers. In this chart only the differences in exchange capacity and water/paper ratios were taken into account (conversion factors for equation (1) being 7.75 for DE 20 and 4.9 for AE 30 paper). Thus the R_F value obtained by converting either way assumes that no interaction between the polymer (resin or cellulose) and the metal ion occurs. Rather good correlations are obtained with Pb (II) and Ag (I) where the R_F values are all of the right order of magnitude as shown for Pb (II) in Table II. We also want to emphasise that since the A_L/A_S ratios for the two cellulose exchangers are very similar, an ion held merely by ion exchange

TABLE II

R_F VALUES OF Pb (II) ON CELLULOSE EXCHANGERS CALCULATED FROM R_F VALUES ON SB-2 PAPER, AND OBTAINED EXPERIMENTALLY

Conc. of HCl	*R_F value on SB-2 paper*	*R_F value on DE 20 paper*		*R_F value on AE 30 paper*	
		calculated	*measured*	*calculated*	*measured*
2N	0.13	0.54	0.72	0.42	0.55
4N	0.27	0.74	0.78	0.64	0.62
6N	0.43	0.85	0.86	0.79	0.67
8N	0.57	0.91	0.88	0.87	0.79

should always have a higher R_F value on the DE 20 paper than on the AE 30 paper since the capacity of the DE 20 paper is about 30 % higher.

However, if the results for most of the strongly adsorbed anions are examined, it is evident that there is no correlation between the various papers if calculations on the basis of their relative capacities are made and also that, in most instances, there is a stronger adsorption on the DE 20 paper than on the AE 30 paper.

This can be explained if we consider that in addition to ion exchange there is adsorption on the organic network. The resin paper would have the strongest adsorption, then the DE 20 paper which contains two ethyl groups more on each exchange group than the AE 30 paper. Such small differences in the nonpolar nature seem to be sufficient to reverse the order of adsorption but the order of magnitude of the R_F values is essentially the same.

(c) *Some comments on the adsorption of the very strongly adsorbed anions of the type $AuCl_4^-$*

KRAUS AND NELSON[11] noted that a number of anions such as $AuCl_4^-$, $HgCl_4^{2-}$, $TlCl_4^-$, $FeCl_4^-$ and $GaCl_4^-$ have extremely high distribution coefficients on Dowex-1, of the order of 10^5 to 10^6, in contrast to anions such as ReO_4^-, Br^- etc. which have maxima

of less than 10^4. These strongly adsorbed anions were also found to adsorb strongly on cation exchangers (such as Dowex-50) and cellulose, and extract into ether and other organic solvents. On cellulose anion exchangers they are adsorbed to the same extent as the less strongly adsorbed anions, such as ReO_4^-. Actually very little difference exists between the R_F value curves of most of the anions. In Fig. 1 (top, left) we have also calculated the type of R_F–$[H^+]$ concentration curve for several R_F values assuming a monovalent anion, the validity of the law of mass action and ignoring the corrections necessary due to the activity coefficient of the acid. It is remarkable that many anions, whether strongly adsorbed or otherwise, follow this "theoretical curve" very closely.

We believe that the above is evidence that on cellulose anion exchangers the adsorption is mainly one of anion exchange with relatively small adsorption effects (except perhaps for $AuCl_4^-$) while for most anions the contrary seems to occur on anion-exchange resins. A similar conclusion was reached by KNIGHT[13] for amino acids in a study of the adsorption behaviour on cationic resin and cellulose exchange papers.

The results given in Fig. 1 also contain numerous analytical possibilities. Several mixtures of analytical interest which showed R_F differences on the cellulose exchange papers were separated and the R_F values in mixtures were identical with those when chromatographed alone.

ACKNOWLEDGEMENTS

Thanks are due to DR. F. DOBICI for the equilibrium studies with Te(IV) and to Dr. S. ALLULLI for the R_F values of Po(IV) on SB-2 paper.

SUMMARY

The adsorption of metal ions from HCl onto SB-2 resin paper, DE-20 and AE-30 cellulose anion-exchange papers was studied and the mechanism of adsorption discussed considering the differences observed between the various exchangers.

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CHROMATOGRAPHY ON ION EXCHANGE PAPERS

X. SOME DATA FOR CELLULOSE ION-EXCHANGERS WITH ORGANIC SOLVENTS

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The influence of organic solvents on the ion exchange behaviour of several metal ions on cation and anion resin papers and on zirconium phosphate paper was recently reported by us[1].

Cellulose ion exchange papers have already been employed for two-dimensional chromatography of metal ions using an organic solvent mixture in one direction[2]. However, no systematic work similar to our study with resin papers[1] has been reported so far and this seemed to us of interest both for analytical purposes and for the understanding of the effect of organic solvents on ion exchange.

The work described here was thus undertaken and its aim was to study a large number of ions in some solvent systems where the amount of organic solvent can be increased gradually (*i.e.* systems in which the solvents are completely miscible). For this purpose $2N$ HCl containing varying quantities of either ethanol or acetone was selected.

The following four papers were chromatographed together in one container by the technique already described[1]:

Whatman No. 1 pure cellulose paper,
Whatman cellulose phosphate paper P 20,
Whatman diethylaminoethyl-cellulose (DEAE) paper DE 20,
Whatman aminoethyl-cellulose paper AE 30.

The results are given in Fig. 1–4 where the metal ions are grouped roughly according to their complexing properties.

DISCUSSION

Fig. 1 shows the behaviour of Ni(II), Al(III), La(III) and Th(IV) which all are known not to form complexes with $2N$ HCl in any of the mixtures examined.

Al, La and Th adsorb to some extent on cellulose phosphate probably due to the formation of phosphate complexes, otherwise the R_F curves for the metal ions on the exchange papers are almost identical with that on Whatman No. 1 paper *i.e.* a partition mechanism seems to be responsible for the retention on the paper.

Fig. 2 shows the behaviour of several metal ions which form anionic complexes

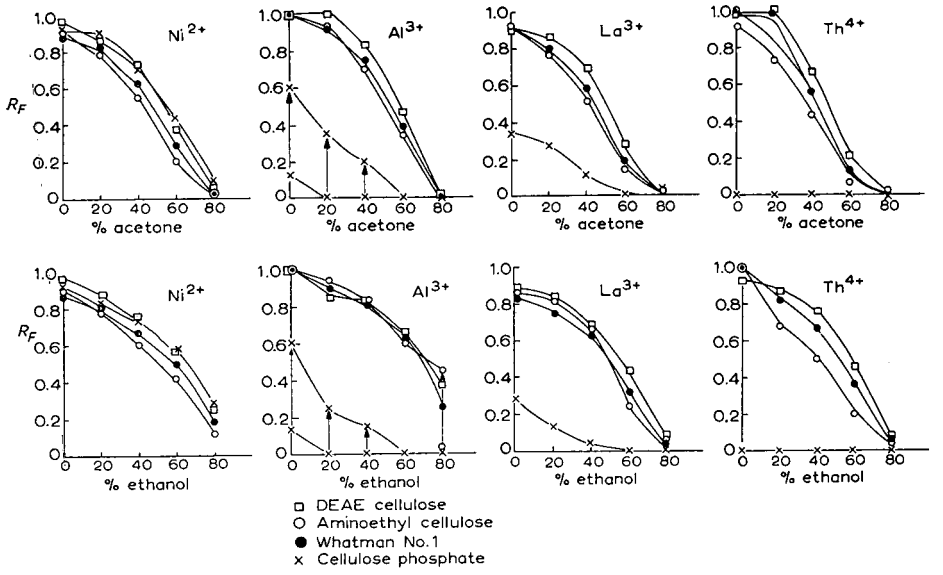

Fig. 1. R_F values of some non-complexing cations on various cellulose exchangers with acetone–HCl and ethanol–HCl mixtures (the concentration of HCl is maintained constant at 2 N).

reversibly with HCl. Again it is best to consider the cellulose phosphate paper separately. Both UO_2^{2+} and Fe(III) are adsorbed strongly on P20 paper and only eluted with 80% acetone–2 N HCl. It seems worthy of note that the adsorption, which undoubtedly is one due to complex formation, can be reversed by a non-polar organic solvent such as acetone but not by ethanol.

For UO_2^{2+}, Cu(II), Co(II) and Fe(III) there is little difference in behaviour between pure cellulose and anion exchange cellulose papers, lowering of R_F values due to partition effects occurring in most cases.

Zn(II) and Cd(II) both show an increased retention on DEAE and aminoethyl-cellulose papers. Two phenomena are interesting: both ions are more strongly adsorbed on the DEAE paper than on the aminoethyl-cellulose paper although the latter has a higher capacity. This was also discussed when comparing adsorption from various concentrations of HCl[3], and appears to be due to the more non-polar nature of the DEAE paper, thus suggesting an adsorption mechanism rather than ion exchange proper. The second point of interest is that the adsorption increases with the concentration of ethanol and decreases with the concentration of acetone. This difference between ethanol and acetone was not noted in our study of resin papers[1] because most anions are too strongly held. We would like to suggest the explanation that ethanol decreases the hydration of the complex anion and thus permits stronger adsorption on the paper but is sufficiently polar to permit adsorption, while acetone is sufficiently non-polar to effect a desorption from the more polar cellulose exchanger.

Fig. 3 shows the behaviour of several typical stable chloro-anions. The differences between ethanol and acetone can be explained in the same manner as for Cd(II) and Zn(II) above.

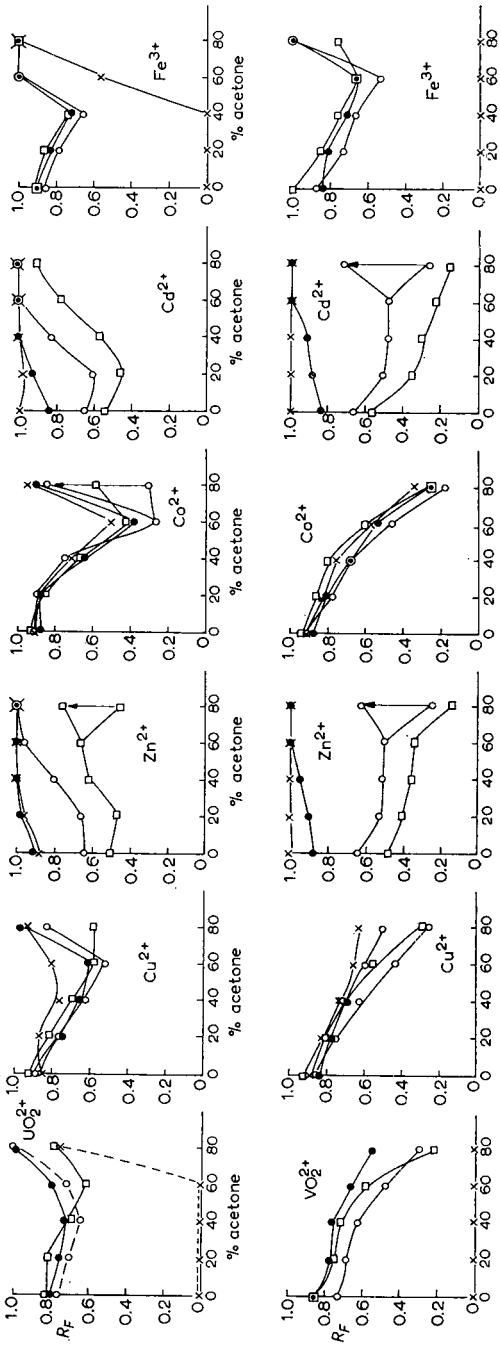

Fig. 2. R_F values of some transition elements on various cellulose exchangers with acetone–HCl and ethanol–HCl mixtures (as in Fig. 1).

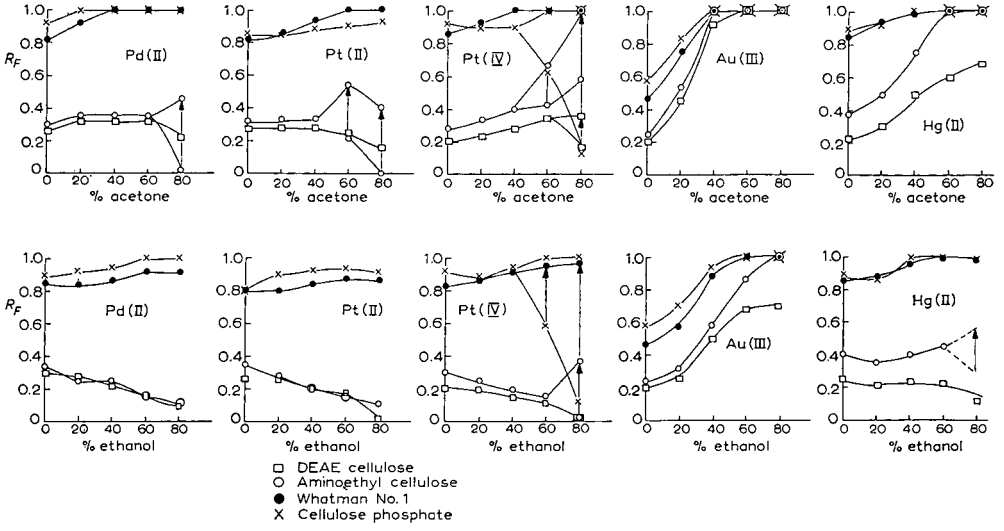

Fig. 3. R_F values of some anionic chloro-complexes on various cellulose exchangers with acetone–HCl and ethanol–HCl mixtures (as in Fig. 1).

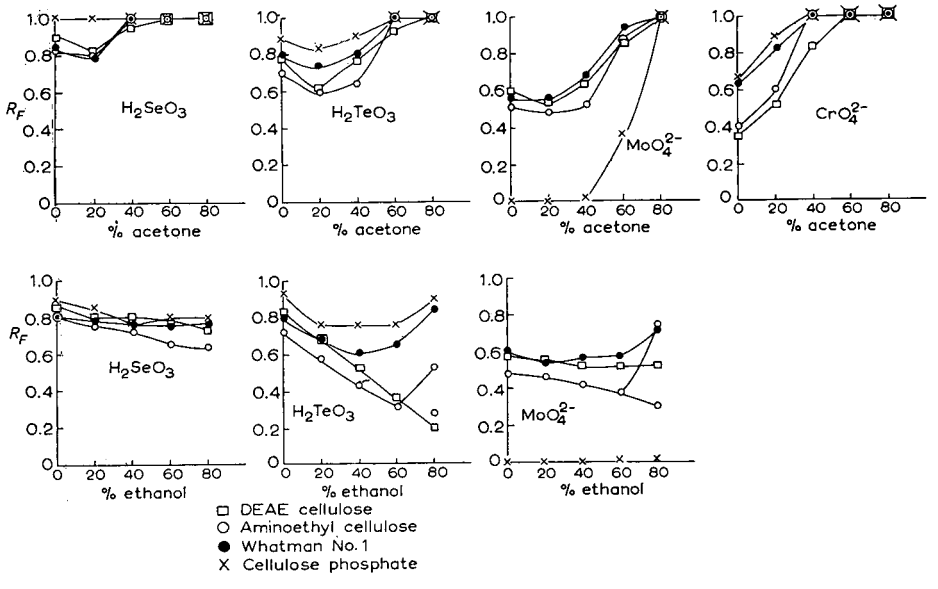

Fig. 4. R_F values of some oxy-anions on various cellulose exchangers with acetone–HCl and ethanol–HCl (as in Fig. 1).

A discussion on the desorption of $AuCl_4^-$ is merited. Cellulose phosphate absorbs it least, then pure cellulose, then ethylamino-cellulose and strongest adsorption is found on the DEAE paper. This sequence is that of the polarities of the various materials, cellulose phosphate naturally being more polar than pure cellulose and it has nothing to do with anion exchange capacities. $AuCl_4^-$ is the only anion which is not more strongly absorbed with an increase in the ethanol concentration; perhaps the hydration is already very poor.

The behaviour of a few oxy-anions is shown in Fig. 4. Selenite is little adsorbed on any paper. Tellurite forms an anionic chloro-complex in $2N$ HCl which is more strongly adsorbed with an increase in the ethanol concentration and desorbed by acetone. Molybdate is strongly adsorbed on cellulose phosphate and it is tempting to attribute this to a formation of a complex of the phosphomolybdate type. If this is correct, such a complex is reversibly decomposed by acetone. Chromate could only be studied in acetone as it is completely reduced in ethanol and shows an adsorption similar to $AuCl_4^-$.

CONCLUSIONS

The behaviour of ions on the cellulose exchangers can be explained in a manner analogous to that employed for resin papers[1]. The only new property observed concerns the increased adsorption of some anionic complexes from ethanol solutions on anion exchangers. The use of organic solvents in conjunction with cellulose exchangers can have numerous analytical possibilities as is indicated by the examples studied.

SUMMARY

The adsorption of numerous inorganic ions on various cellulose exchangers from mixtures of HCl and organic solvents was studied.

As on ion exchange resins the adsorption depends on various factors and can not be interpreted in terms of true "ion exchange".

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THE ISOLATION OF PEROXOMONOPHOSPHORIC ACID BY ANION-EXCHANGE CHROMATOGRAPHY

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Previous attempts to separate peroxomonophosphoric acid from associated orthophosphoric acid have been unsuccessful, partly because no suitable selective precipitant has been found[1,2] and partly because the strongly oxidizing peroxo-acid is readily decomposed.

In the analogous peroxomonosulphuric acid–sulphuric acid system[1], sulphuric acid can be removed as barium sulphate, leaving the peroxomonosulphuric acid in solution, but it has been confirmed that aqueous peroxomonophosphates decompose in the presence of barium ions and molybdate ions, the commonest precipitants used in mixed phosphate systems.

BOYLAND AND MANSON[3] reported that peroxomonophosphoric acid could be separated from its acetone complex and phosphoric acid by paper chromatography, but all the active oxygen was subsequently lost from the chromatograms.

Orthophosphates, pyrophosphates, and the more highly condensed polymetaphosphates can be separated by precipitation techniques[2,4] but a more effective and more widely applicable method of separation is anion-exchange chromatography[5–8]. We now report the use of an anion-exchange method for the separation of peroxomonophosphoric acid.

A chloride eluant is normally employed for phosphate separations; the pH of the eluant is varied over a wide range depending on the composition of the phosphate mixture.

Preliminary experiments showed reaction between peroxomonophosphoric acid and polystyrene resins to be very slow below 20°. Peroxomonophosphates decompose rapidly in alkaline solution, but only very slowly in neutral or weakly acid chloride solution or in acetate buffer solution. When aqueous peroxomonophosphoric and orthophosphoric acids were run on to a small column of strong-base anion-exchange resin in its chloride form, orthophosphate ion was eluted by a neutral chloride solution significantly before peroxomonophosphate ion, and a partial separation of the two anions was achieved. Solutions of peroxomonoposphoric acid in aqueous sodium chloride were concentrated by freeze drying, without significant loss of peroxo-oxygen, but neither chloride nor peroxomonophosphate could be precipitated from the solution without loss of oxygen. Peroxomonophosphoric acid rapidly oxidized hydrochloric acid, even at 0°C, when the solution was acidified.

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Peroxomonophosphoric acid decomposes only slowly in dilute acetic acid at 20°C. With the resin column in its acetate form, effective separation of a peroxomonophosphate/orthophosphate mixture is achieved when an acetate buffer solution is used as eluant. The eluate can be concentrated by vacuum distillation and the metal ions removed by passing the solution through a cation-exchange resin in its hydrogen form; only slight (5%) loss of peroxo-oxygen occurs during these operations. Acetic acid can be removed from the solution containing the peroxomonophosphoric acid, by further vacuum distillation and freeze drying, to leave a viscous, involatile residue of peroxomonophosphoric acid.

More than 25% of the peroxo-oxygen was lost during the final concentration of the peroxo-acid. Attempts to precipitate salts of the acid or to obtain crystalline salts from solutions of the acid were unsuccessful.

Although it has not been possible to isolate a pure salt of peroxomonophosphoric acid, the procedure described overcomes several difficulties experienced by other workers. We have shown that it is possible to separate peroxomonophosphoric acid from orthophosphoric acid and to estimate each. Peroxomonophosphoric acid prepared from the acetate eluate is suitable for further study since its passage through both cation and anion-exchange resins removes all ionic impurities, among them the heavy metal ions which are likely to cause catalytic decomposition. Apart from the orthophosphoric acid formed by self-decomposition, the only contaminant is a small amount of involatile organic material.

EXPERIMENTAL

Preparation of peroxomonophosphoric acid

Peroxomonophosphoric acid was prepared by adding hydrogen peroxide (Laporte "80% plus" peroxide) to a suspension of phosphorus pentoxide in acetonitrile, according to the method of TOENNIES[9]. This method gave a mixture of orthophosphoric and peroxomonophosphoric acids; the yield of peroxo-acid (*ca.* 60%) was lower than previously reported, but in the earlier work the product may have been contaminated by hydrogen peroxide. When the acetonitrile solution was run on to an anion-exchange column the phosphorus acid anions were absorbed whilst the solvent and the excess of hydrogen peroxide could be washed from the column with water.

Analytical methods

Peroxomonophosphoric acid reacted rapidly and quantitatively with neutral potassium iodide solution to give iodine which was estimated spectrophotometrically by the absorption due to the I_3^- ion at 360 mμ[10]. Aliquots of the eluate (0.05 ml) were added to 2 ml of 1% potassium iodide and the light absorption measured using 1 cm cells in a Unicam SP 600 spectrophotometer. The iodine equivalent to H_3PO_5 had a molar extinction coefficient of 23,700.

Phosphoric acid was estimated spectrophotometrically. Aliquots of the eluate (0.05 ml) were added to 2 ml of ammonium vanadomolybdate reagent[11] and the absorption measured against a reagent blank at 375 mμ, using the SP 600 Under these conditions the phosphate complex had a molar extinction coefficient of 5,250. In the presence of peroxomonophosphoric acid, orthophosphoric acid readings were too high since the peroxo-acid was catalytically decomposed to orthophosphoric acid by

the reagent. A correction was applied to obtain the actual orthophosphate concentration.

Chromatographic separation

Preliminary experiments were made with a 10 ml column packed with Deacidite FF strong-base (quaternary ammonium) polystyrene resin. Peroxomonophosphate could be recovered from the column in better than 95% yield (chloride elution). Subsequent experiments were carried out using a larger column (80 ml). To reduce the decomposition of the peroxo-anion the resin was maintained at 5°C by the circulation of cold glycol between double walls.

With the column in its acetate form *ca.* 5 ml of the prepared phosphorus acid mixture in acetonitrile was run on to the column and eluted with an acetate buffer solution, pH 5.0, at 3 ml/min. Corrected analysis figures for a typical separation are shown in Table I and the corresponding elution curve in Fig. 1.

TABLE I

THE CHROMATOGRAPHIC SEPARATION OF PEROXOMONOPHOSPHORIC ACID AND ORTHOPHOSPHORIC ACID

Sample: 6 ml of H_3PO_4/H_3PO_5 prepared solution in acetonitrile. Eluant: 0.5 *M* KOAc/HOAc buffer solution, pH 5.0. Elution rate: 3 ml/min. Column: 22 cm × 2.1 cm. Packing: Deacidite FF resin.

Total eluate (ml)	*Phosphorus concentration (mmole/l)*	
	as H_3PO_4	*as* H_3PO_5
350	0.16	0.03
450	4.88	0.03
550	10.27	0.28
600	11.75	0.40
650	10.37	0.65
800	3.88	1.27
1,000	0.41	2.02
1,200	0	2.24
1,400	0	1.92
1,600	0	1.33
1,800	0	0.80
2,000	0	0.45

Treatment of eluate

The eluate was collected for peroxo-acid recovery after the phosphorus present in the peroxo-form exceeded 95%. The maximum peroxomonophosphate concentration was about 0.005 *M*; eluate was collected until the concentration fell to 0.0005 *M* (total volume 1–2 l). The combined eluate was vacuum distilled below 28°C to reduce its bulk (to *ca.* 150 ml). Some potassium acetate generally crystallized from the solution at this stage and was filtered off. The remaining peroxomonophosphate solution (*ca.* 0.1 *M*) in concentrated potassium acetate was run through a large column of cation-exchange resin (Amberlite IR 120, hydrogen form) to give a solution of peroxomonophosphoric acid in aqueous acetic acid. This solution was again vacuum distilled to leave a colourless, viscous mass. Peroxomonophosphoric acid was estimated in this residue iodometrically, total acid by a pH titration using sodium hydroxide (equiv-

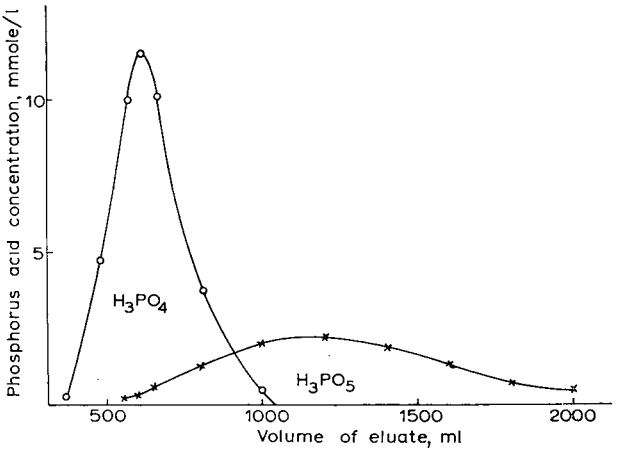

Fig. 1. The chromatographic separation of H_3PO_4 and H_3PO_5.

alent point pH 5.0, corresponding to $NaH_2PO_4 + NaH_2PO_5$). When first analysed the mixture contained 80 % peroxomonophosphoric acid but it slowly lost oxygen.

Peroxomonophosphates were obtained by addition of one equivalent of alkali (LiOH, NaOH, KOH) to the cold, concentrated aqueous acid. The solutions so obtained were concentrated *in vacuo* over phosphorus pentoxide; no crystalline salts separated but the solutions slowly yielded deliquescent, amorphous, white solids, which contained up to 70 % of the active oxygen required for a salt of formula MH_2PO_5. When these salts were stored over phosphorus pentoxide at 0°C, 40 % of their active oxygen was lost in eight weeks.

ACKNOWLEDGEMENT

We wish to acknowledge the gift of hydrogen peroxide from Laporte Industries Limited.

SUMMARY

The separation of peroxomonophosphoric acid from orthophosphoric acid by anion-exchange chromatography is described; also the recovery of peroxomonophosphoric acid from the acetate buffer solution used as eluate.

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DOSAGE SIMULTANÉ DU COBALT, DU ZINC, DU NICKEL ET DU CUIVRE PAR CHROMATOGRAPHIE DE DÉPLACEMENT SUR ÉCHANGEURS D'IONS

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(Reçu le 16 mai 1963)

Des travaux récents[1-3] exécutés au Laboratoire de chimie minérale et analytique de l'Université de Lausanne, ont montré qu'il est possible de déterminer avec une précision satisfaisante les éléments individuels de mélanges de terres rares, à l'aide d'un procédé de chromatographie de déplacement sur échangeurs d'ions. Des procédés analogues pour la détermination d'autres séries de métaux seraient très utiles (p. ex.: analyses d'alliages, dosages d'oligo-éléments dans les engrais et les fourrages). Nous exposons ci-dessous une méthode permettant le dosage simultané du cobalt, du zinc, du nickel et du cuivre.

PRINCIPE DE LA MÉTHODE

Les cations M^{2+} (M^{2+} = Co^{2+}, Zn^{2+}, Ni^{2+} et Cu^{2+}) sont séparés dans un tube capillaire de section uniforme et connue, par un procédé de chromatographie de déplacement. Comme échangeur d'ions, nous utilisons une résine sulfonique pratiquement incolore, sous forme H^+, et comme éluant, une solution-tampon à base de glycocolle. La résine H^+ joue le rôle de "barrière": les complexes $[MG]^+$ (G = glycocollate) qui rencontrent cette couche sont décomposés et les cations M^{2+} se fixent sur la résine, tandis que le glycocolle, très soluble dans l'eau, s'écoule à travers la colonne. Simultanément, les éléments séparés s'ordonnent d'après la stabilité croissante de leurs complexes[4,5] en zones adjacentes bien délimitées. Il faut obtenir une séparation complète des divers éléments M, puis on mesure la longueur de chaque bande. Les zones du cobalt, du nickel et du cuivre sont colorées respectivement en rouge carmin, vert et bleu vif; ces colorations sont celles des complexes $[MG]^+$ superposées à celles des cations M^{2+} fixés sur la résine. Par contre, les ions Zn^{2+} et $[ZnG]^+$ étant incolores, la zone occupée par le zinc est incolore. Cependant, la bande du zinc, intercallée entre celles du cobalt et du nickel (Fig. 1A), ou entre celles du cobalt et du cuivre lorsque le nickel est absent (Fig. 1B), peut se mesurer sans autre. La Fig. 1C montre un exemple de chromatogramme obtenu en l'absence du zinc (séparation Co^{2+}– Cu^{2+}).

Le calcul des teneurs en cations M^{2+} de la solution analysée se fait en comparant les volumes des zones obtenues avec les volumes des bandes correspondantes d'une chromatographie-étalon.

APPAREILLAGE

L'appareillage utilisé est représenté dans la Fig. 2. Comme colonne de séparation, on utilise un tube capillaire A en verre "Pyrex" (environ 2 mm de diamètre interne;

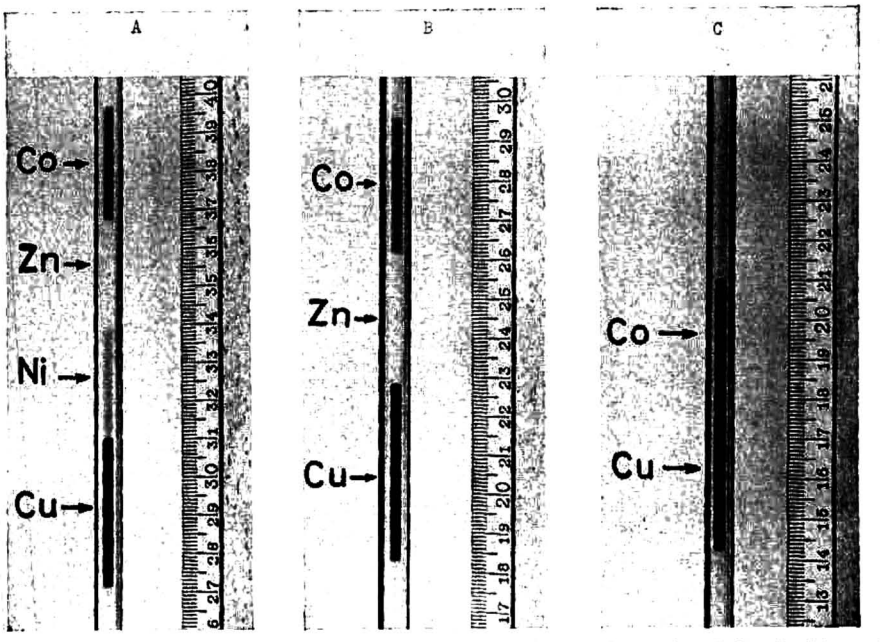

Fig. 1. A: Séparation Co^{2+}– Zn^{2+}– Ni^{2+}– Cu^{2+}. B: Séparation Co^{2+}– Zn^{2+}– Cu^{2+}. C: Séparation Co^{2+}– Cu^{2+}.

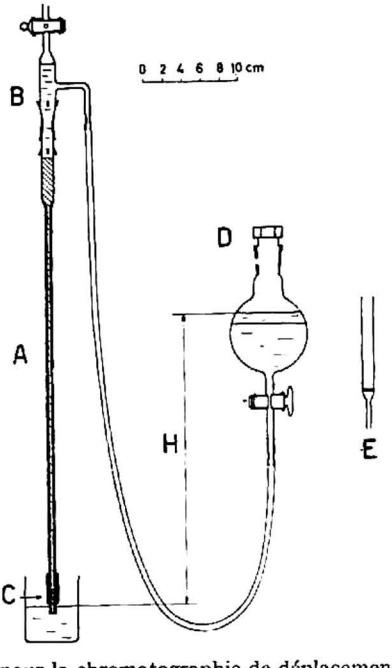

Fig. 2. Appareillage pour la chromatographie de déplacement sur échangeurs d'ions.

400 mm de longueur), prolongé par un tube de 10 mm de diamètre intérieur, et qui est terminé par un rodage (NS-K 12,5/21, DIN 12242). Chaque tube est numéroté (p. ex. à l'aide d'une fraise électrique à diamant) et la section interne de chaque tube capillaire est déterminée par pesée d'eau distillée (pour exécuter ces déterminations, on munit la colonne, à son extrémité inférieure, d'un petit robinet de verre). Les colonnes de séparation sont complétées par une fritte C^2 et par une pièce à robinet B munie d'un rodage (NS-H 12,5/21, DIN 12242). Le réservoir de l'éluant est une ampoule à robinet D de 250 ml, en "Pyrex". Nous utilisons encore un petit tube E avec fritte (Jena 12 G 1) et une colonne de chromatographie (environ 500 × 40 mm) avec fritte de grande porosité (P 1). L'ampoule D est reliée à la pièce B à l'aide d'un tuyau de caoutchouc "para". Il faut encore noter que les tuyaux de PCV ne conviennent pas, car ils sont attaqués par l'éluant.

PARTIE EXPÉRIMENTALE

Préparation de l'éluant

On dissout 40.00 g de glycocolle puriss. dans environ 750 ml d'eau déminéralisée et on additionne, sous agitation mécanique, une solution d'ammoniaque env. 2 *M* ou d'hydroxyde de sodium env. 2 *M*, jusqu'à obtention d'un pH de 8.80 (mesuré à 24° à l'aide d'un pH-mètre à électrode de verre). Finalement, on complète à 1000 ml avec de l'eau. Le pH des éluants doit être fréquemment contrôlé et, au besoin, réajusté. Afin d'éviter une variation trop rapide du pH de la solution éluante au cours de la chromatographie (par perte d'ammoniac ou par carbonatation), on recouvre l'éluant d'une couche de kérosène d'environ 1.5 cm d'épaisseur.

Résine échangeuse d'ions

Après quelques essais, nous avons adopté la résine Dowex 50 W X2 (200–400 mesh). La résine fraîche est introduite dans une colonne de chromatographie (500 × 40 mm) et abondamment lavée à l'eau. On élimine ensuite les traces de métaux lourds en faisant passer à travers la résine une solution 0.5 *M* de citrate biammonique. On lave à l'eau, puis on met la résine sous forme H^+ en passant dans la colonne une solution d'HCl puriss. (1 vol. HCl 25 % + 3 vol. d'eau). Finalement, on lave à fond avec de l'eau. On conserve la résine sous l'eau dans un flacon fermé à large col.

Remplissage des colonnes de séparations

On plonge la tête de la colonne dans un mélange d'eau et de résine, puis on aspire à l'aide d'une trompe à eau ou d'une pompe électrique (p. ex. pompe Réciprotor, type 406 G). On interrompt l'aspiration lorsque tous les grains de résine sont immobiles dans la colonne (examen à contre-jour!); on laisse une couche de résine H^+ de 1.0–1.5 cm de hauteur dans la tête de la colonne et, après quelques minutes de décantation, on aspire l'excès d'eau.

Préparation de l'échantillon à analyser

La solution à analyser doit être faiblement acide. On place de la résine sous forme H^+ dans la fritte E (couche d'environ 2.5 cm de hauteur) et on verse un volume connu de solution à analyser (contenant environ 0.8 méquiv.-g M^{2+}) sur la résine; finalement, on lave à l'eau. Seule la moitié environ de la couche de résine doit être chargée de

cations M^{2+}. Le contenu de la fritte E est ensuite transvasé quantitativement (à l'aide de petites portions d'eau) dans la colonne de chromatographie préparée à l'avance. Lorsque la résine s'est déposée, on aspire le surplus d'eau, on enduit toutes les parties rodées d'une pâte de silicone de viscosité moyenne, on place la pièce à robinet B sur la colonne et on la maintient en place à l'aide d'un élastique. Afin d'éviter la cristallisation du glycocolle par évaporation de l'éluat, on fait plonger la fritte C dans l'eau.

Développement du chromatogramme

On remplit délicatement la pièce B d'éluant provenant du réservoir D, en évitant de provoquer une mise en suspension de la résine. Ensuite, on place le réservoir à une hauteur telle que la différence de niveau H soit d'environ 100 cm. Lorsque les cations M^{2+} ont entièrement passé dans le capillaire, on abaisse H à 6 cm; on mesure la longueur des zones de Co^{2+}, de Zn^{2+}, de Ni^{2+} et de Cu^{2+} quand les fronts sont nets et horizontaux. On répète les mesures après avoir fait progresser les bandes de 4–5 cm dans la colonne. La durée totale de la chromatographie est de l'ordre de 40 heures.

Étalonnage et calcul des résultats

Avec un lot de résine déterminé (lot No. 1) et un éluant composé de glycocolle et d'ammoniaque (40.00 g glycocolle/l; pH = 8.80 à 24°), nous avons exécuté une série de chromatographies de quantités connues de cations M^{2+}. Dans ce but, nous avons utilisé des solutions de sels purs, dont les titres ont été obtenus par titrages complexométriques au moyen d'une solution de Na_2H_2–édta·$2H_2O$ 0.05 *M* (édta = éthylènediaminetétraacétate), en milieu ammoniacal et en présence d'un indicateur d'ions métalliques (murexide pour le titrage de Co^{2+}, Ni^{2+} et Cu^{2+}, noir ériochrome T pour le titrage de Zn^{2+})[6]. Les quatre solutions obtenues avaient les titres suivants:

$CoCl_2$ à 1.000 méquiv.-g Co^{2+}/10 ml

$ZnSO_4$ à 1.173 méquiv.-g Zn^{2+}/10 ml

$NiSO_4$ à 0.901 méquiv.-g Ni^{2+}/10 ml

$CuSO_4$ à 1.000 méquiv.-g Cu^{2+}/10 ml

Les résultats des chromatographies sont consignés dans le Tableau I. De plus, ils sont représentés graphiquement dans la Fig. 3: en ordonnée, nous avons reporté les quantités de cations M^{2+} introduites dans la colonne (en méquiv.-g) et en abscisse le volume des zones M^{2+} (en mm^3), après développement des chromatogrammes. On obtient 4 droites passant par l'origine; elles sont donc caractérisées par leur pente p, laquelle peut être déterminée graphiquement ($p = 1.53 \cdot 10^{-3}$; $1.54 \cdot 10^{-3}$; $1.58 \cdot 10^{-3}$; $1.24 \cdot 10^{-3}$, pour Co^{2+}, Zn^{2+}, Ni^{2+} et Cu^{2+} respectivement). L'expérience montre donc que, pour un lot de résine et un éluant déterminés, les volumes des zones des cations M^{2+} sont proportionnels aux teneurs de la solution analysée. D'autre part, nous avons observé que la pente p varie un peu avec le lot de résine employé, ainsi qu'avec l'éluant utilisé; par conséquent, pour obtenir des résultats précis, il faut déterminer p pour chaque série d'analyses en effectuant en parallèle une chromatographie-étalon avec

TABLEAU I

CHROMATOGRAPHIES D'ÉTALONNEMENT

Éluant: glycocolle (40.00 g/l) + NH_4OH (pH = 8.80 à 24°).
Résine: Dowex 50 W X2, 200–400 mesh, lot No. 1, forme H^+.
Les volumes des zones sont donnés en mm^3.

M^{2+} (méquiv.-g)	Co^{2+}		Zn^{2+}		Ni^{2+}		Cu^{2+}	
	Zone	*Moyenne*	*Zone*	*Moyenne*	*Zone*	*Moyenne*	*Zone*	*Moyenne*
0.0250	16.5		—	—	—	—	19,9	
0.0500	34.0	33.7	32.7	32.4	32.3	32.1	39.5	40.2
0.0500	33.4		32.1		31.8		40.8	
0.1000	67.7		64.9		62.9		80.2	
0.1000	66.1	66.4	64.0	64.5	64.0	63.5	79.1	79.4
0.1000	65.3		—		63.6		78.8	
0.1500	98.0	98.6	98.5	98.3	94.9	95.4	122.0	121.6
0.1500	99.1		98.0		95.8		121.2	
0.2000	128.5		128.8		126.0		160.0	
0.2000	129.9	129.2	130.4	129.6	126.0	126.7	163.2	161.6
0.2000	—		—		128.0		—	

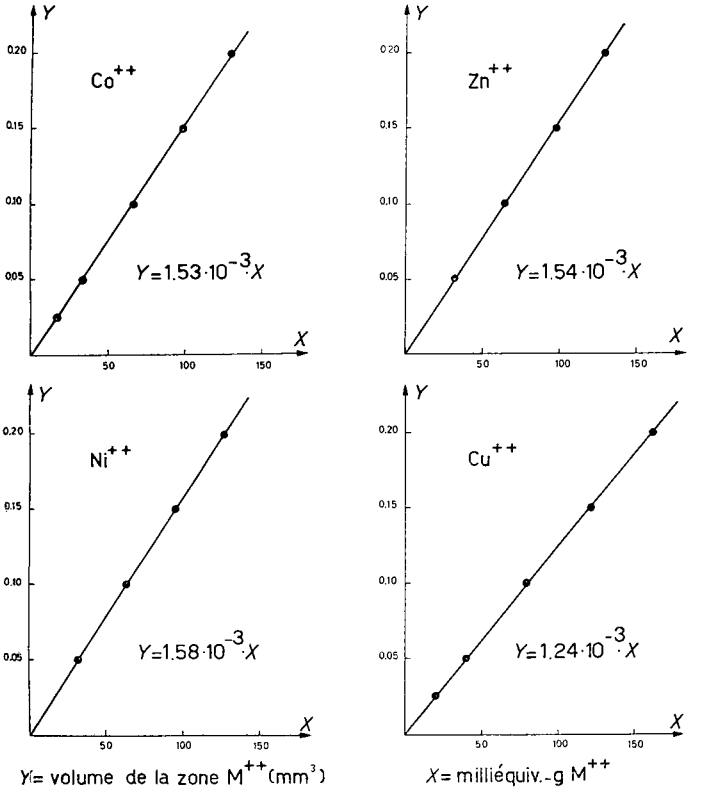

Fig. 3. Droites d'étalonnement (voir Tableau I).

TABLEAU II

ANALYSES CHROMATOGRAPHIQUES DE MÉLANGES SYNTHÉTIQUES DE CATIONS

Éluant: glycocolle (40.00 g/l) + NH_4OH (pH = 8.80 à 24°).
Résine: Dowex 50 W X2, 200–400 mesh, lot No. 2, forme H^+.
Les résultats sont donnés en méquiv.-g, les volumes des zones en mm^3, et les écarts en %.

M^{2+}	*Chromatographie- étalon E 1*		*Analyse 1*				*Analyse 2*			
	Donné	*Zone*	*Donné*	*Zone*	*Trouvé*	*Ecart*	*Donné*	*Zone*	*Trouvé*	*Ecart*
Co^{2+}	0.1300	90.3	0.1500	103.1	0.1485	−1.0	0.0900	62.4	0.0898	−0.2
Zn^{2+}	0.1300	88.1	0.1500	101.3	0.1496	−0.3	0.0900	61.0	0.0900	0.0
Ni^{2+}	0.1300	86.3	—	—	—	—	0.0900	59.8	0.0901	+0.1
Cu^{2+}	0.1300	117.5	0.1500	133.1	0.1473	−1.8	0.0900	80.1	0.0886	−1.5

TABLEAU III

ANALYSES CHROMATOGRAPHIQUES DE MÉLANGES SYNTHÉTIQUES DE CATIONS

Éluant: glycocolle (40.00 g/l) + NaOH (pH = 8.80 à 24°).
Résine: Dowex 50 W X2, 200–400 mesh, lot No. 2, forme H^+.
Les résultats sont donnés en méquiv.-g, les volumes des zones en mm^3, et les écarts en %.

M^{2+}	*Chromatographie- étalon E 2*		*Analyse 3*				*Analyse 4*			
	Donné	*Zone*	*Donné*	*Zone*	*Trouvé*	*Ecart*	*Donné*	*Zone*	*Trouvé*	*Ecart*
Co^{2+}	0.1500	105.2	0.0800	57.2	0.0815	+1.9	0.1100	78.8	0.1123	+2.1
Zn^{2+}	0.1500	104.3	0.0938	64.9	0.0933	−0.5	0.1760	120.9	0.1738	−1.2
Ni^{2+}	0.1500	101.6	0.1530	104.7	0.1545	+1.0	0.0721	49.0	0.0723	+0.3
Cu^{2+}	0.1500	148.0	0.1200	120.2	0.1218	+1.5	0.1700	167.7	0.1699	−0.1

des quantités connues de cations. Le calcul des résultats se fait alors à l'aide de la relation:

$$\text{méquiv.-g } M_x^{2+} = \frac{V_x \cdot M_e}{V_e} \qquad (1)$$

où V_x = volume de la zone de M_x^{2+}, V_e = volume de la zone correspondante de la chromatographie-étalon, M_e = quantité de cation M_x^{2+} dans la chromatographie-étalon.

Exemples d'analyses

Quatre exemples d'analyses de mélanges synthétiques de cations sont consignés dans les Tableaux II et III; on constate que la précision est satisfaisante.

Présence de cations étrangers

Nous avons exécuté six chromatographies de mélanges de Co^{2+}, de Zn^{2+}, de Ni^{2+} et de Cu^{2+} en quantités équivalentes (4 × 0.1500 = 0.6000 méquiv.-g M^{2+}), en présence d'un cation étranger (0.1000 méquiv.-g par chromatographie). A titre de comparaison, nous avons procédé à deux chromatographies (E 3 et E 4) avec les mêmes quantités de cations M^{2+} que ci-dessus, mais sans impuretés. Les résultats sont résumés dans le Tableau IV; on constate que de petites quantités de Mg^{2+}, de Ca^{2+}, de Mn^{2+}, de Fe^{3+}, de Cr^{3+} ou d'Al^{3+} ne diminuent pas la précision des analyses.

TABLEAU IV

CHROMATOGRAPHIES EN PRÉSENCE DE CATIONS ÉTRANGERS

Éluant: glycocolle (40.00 g/l) + NaOH (pH = 8.80, à 24°).
Résine: Dowex 50 W X2, 200–400 mesh, lot No. 3, forme H^+.

Les volumes des zones sont donnés en mm^3; les écarts entre les volumes moyens des zones des chromatogrammes de comparaison E 3 et E 4 et les volumes des zones correspondantes des chromatogrammes 5 à 10 sont indiqués en %.

	Chromatographies d'étalonnement			
	Zone Co^{2+}	*Zone Zn^{2+}*	*Zone Ni^{2+}*	*Zone Cu^{2+}*
E 3	104.0	101.6	101.0	150.1
E 4	104.0	102.7	102.0	147.9
Moyenne	104.0	102.1	101.5	149.0

	Analyses chromatographiques								
	Co^{2+}		*Zn^{2+}*		*Ni^{2+}*		*Cu^{2+}*		*Cation étranger*
	Zone	*Écart*	*Zone*	*Écart*	*Zone*	*Écart*	*Zone*	*Écart*	
5	103.6	−0.4	101.1	−1.1	101.0	−0.5	151.0	+1.3	Mg^{2+}
6	104.0	0.0	101.0	−1.1	101.4	−0.1	148.9	−0.1	Ca^{2+}
7	104.5	+0.5	101.3	−0.8	101.2	−0.3	150.0	+0.7	Mn^{2+}
8	105.1	+1.1	101.9	−0.2	102.0	+0.5	148.9	−0.1	Fe^{3+}
9	105.3	+1.3	101.0	−1.1	101.7	+0.2	151.7	+1.8	Cr^{3+}
10	104.2	+0.2	100.5	−0.6	101.0	−0.5	149.5	+0.3	Al^{3+}

Sensibilité

On peut encore déceler, dans une colonne de 1 mm de diamètre intérieur, des quantités de l'ordre donné dans le Tableau V.

TABLEAU V

Co^{2+}	$5 \cdot 10^{-4}$	méquiv.-g (= 15 γ)
Zn^{2+}	$5 \cdot 10^{-4}$	méquiv.-g (= 16 γ)
Ni^{2+}	$5 \cdot 10^{-4}$	méquiv.-g (= 15 γ)
Cu^{2+}	$1 \cdot 10^{-4}$	méquiv.-g (= 3 γ)

RÉSUMÉ

Un procédé de chromatographie de déplacement sur échangeur d'ions, permettant le dosage simultané du cobalt, du zinc, du nickel et du cuivre, est décrit en détail.

SUMMARY

A method of displacement chromatography on ion exchangers is described which permits a simultaneous quantitative analysis of cobalt, zinc, nickel and copper.

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REVERSED-PHASE CHROMATOGRAPHY OF ALKALI METALS AND ALKALINE EARTHS ON PAPER TREATED WITH DI-(2-ETHYLHEXYL) ORTHOPHOSPHORIC ACID IN ACETATE MEDIUM

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INTRODUCTION

Reversed-phase chromatography with paper supporting di-(2-ethylhexyl) orthophosphoric acid (HDEHP) was found useful for the separation of rare earths[1].

The investigation has now been extended to the separation of alkali and alkaline earth metals. Paper treated with organic solutions of HDEHP was used and R_F values were determined as functions of the HDEHP concentration and of the acidity of the eluent. Aqueous solutions of acetic acid at different molarities were used as the eluents.

EXPERIMENTAL

Reagents and equipment

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

The alkali and alkaline earth metals were used as RP grade chlorides, supplied by Carlo Erba (Milan). Stock 0.1 *M* solutions of pure chlorides, titrated with $AgNO_3$, were diluted to obtain 0.005*N* solutions. Since the drops applied to the paper were no more than 0.2 ml each, up to $1 \cdot 10^{-7}$ equivalent of each element was spotted.

Chromatographic paper Whatman No. 1, CRL/1 type, was used for multiple experiments, whilst ordinary 3 × 40 cm Whatman No. 1 paper strips were used for normal ascending and descending chromatograms.

For multiple experiments with CRL/1 type sheets, the chromatographic assembly shown in Fig. 1 was used. It consists of a Witt apparatus, in which a pyrex crystallizer is placed. A glass device on the crystallizer supports the sheet of paper. A long-stemmed separating funnel is fitted in the cover, and a stopcock is used to prevent increase of pressure in the apparatus when the eluting solution is transferred from the funnel to the crystallizer. A battery of five of these assemblies enabled us to perform up to fifteen different multiple developments a day. Ascending and descending chromatograms with single paper strips were carried out with ordinary glass ground-cover columns.

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Treatment of paper and chromatographic procedure

Treatment of chromatographic paper with HDEHP has been already described[1]. In normal experiments, HDEHP solutions in cyclohexane were equilibrated for 20 min with a 2.5*M* solution of acetic acid. Since the experiments being performed

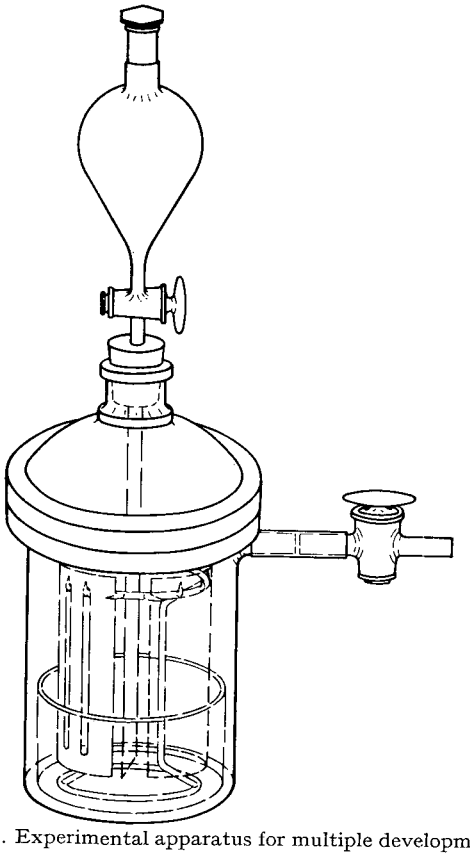

Fig. 1. Experimental apparatus for multiple development.

dealt with alkali and alkaline earth metals, paper sheets were carefully washed before HDEHP treatment with a 1 *M* solution of acetic acid, following the procedure described elsewhere in which 0.1*M* HCl was used[2]. After drying, the paper was immersed in the equilibrated HDEHP solution for 30 sec, then allowed to drip and dried in a current of warm air.

Spots were applied using 0.01 to 0.02 ml of solution (*i.e.* 0.5 — $1.0 \cdot 10^{-7}$ equiv.). Each sheet of the CRL/1 type paper was hung on the glass device and placed for ascending elution inside the assembly shown in Fig. 1. At first, a small amount of the acid was allowed to enter the apparatus to saturate the atmosphere; after 15 min its level was raised to touch the paper, and development began.

When the front had moved about 9 cm, the paper was quickly removed from the apparatus and, after the front had been marked, was dried first with warm air and then in an oven at 40 to 50° for several hours.

Much trouble was encountered in revealing the spots, because of the very small amount of ions used, together with a slight acidity retained by the paper even after prolonged drying. To detect Li^+ spots, an alcoholic solution of 0.1 g/100 ml of 1-amino-5-hydroxyanthraquinone was sprayed on the paper[3]. The spots became visible on exposing the strips to ammonia vapours. Better results were sometimes obtained by dipping the strips in a 0.1 *N* NaOH solution. Na^+ spots were detected as sodium zinc uranyl acetate, visible in U.V. light. Strips were dipped into the developing solution prepared according to BARBER AND KOLTHOFF[4]. Checks were also performed using ^{22}Na tracer. K^+, Rb^+ and Cs^+ spots were made visible as alkali lead cobaltinitrites; the paper was sprayed with the solution recommended by MILLER AND MAGEE[5]. Be^{2+}, Mg^{2+} and Ca^{2+} spots were clearly visible in U.V. light after spraying the strips with 0.1% 8-hydroxyquinoline solution in 50% alcohol, and exposing them to ammonia vapours[6]. To detect Sr^{2+} and Ba^{2+}, the strips were treated with a 0.5% sodium alizarinsulphonate solution in water[7]. The spots became visible a few minutes after spraying and exposing the strips to ammonia vapours.

Most of the work was performed with two different concentrations of HDEHP in cyclohexane solutions, namely 0.100 and 0.010*M*; experiments were also performed with 0.075, 0.050 and 0.025*M* HDEHP, with particular attention to 0.050*M*.

Elutions were made using different concentrations of acetic acid. When the concentration of the acid used was low, de-aerated water was used to prevent any effect of dissolved CO_2 on the pH determination. Each point was checked at least once under the same conditions, except in a few cases in which the relative position of results at various concentrations left no doubt as to their reliability. Reproducibility of the R_F values within $\pm$ 3% was considered satisfactory, because of the shortness of the chromatograms and of the difficulty often encountered in obtaining an exact view of the real shape of the eluted and developed spots. Where bigger discrepancies appeared, as in the case of very high acetic acid concentrations, several chromatograms were run and the average result was taken.

RESULTS AND DISCUSSION

General

Experimental R_F values for alkali and alkaline-earth ions are shown in Table I as functions of the molarity of the acid eluent for paper treated with 0.010 and 0.100 *M* HDEHP. In Fig. 2 results are plotted against the log of acetic acid concentration.

As already pointed out[1], the relationship between the extraction coefficient E_a° for a given element in a liquid–liquid extraction and the R_F value in a chromatographic system in which the same extractant is used as the stationary phase and the same inorganic solution is used as the eluent, can be written as:

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

where k is a constant that depends on the experimental conditions. Information can therefore be obtained on the extraction mechanism, responsible for the retention by the stationary phase of the cations involved, by studying the behaviour of the quantity $(1/R_F - 1)$ as a function both of the concentration of hydrogen ion in the aqueous phase (since HDEHP acts as a cationic exchanger) and of the concentration

TABLE I

R_F VALUES FOR ALKALI AND ALKALINE EARTH IONS AS FUNCTIONS OF THE MOLARITY OF THE CH_3COOH ELUENT

Paper treated with 0.010 and 0.100M HDEHP. Operating temperature 23° ± 1°

HDEHP molarity	Cations	R_F									
		CH_3COOH molarity									
		0.01	0.03	0.05	0.1	0.2	0.3	0.5	1	2.5	3
0.010	Li^+	0.64	0.72	—	0.86	0.91	0.87	0.93	0.90	0.88	0.90
	Na^+	0.91	0.90	—	0.95	0.94	0.92	0.95	0.95	—	0.95
	K^+	0.66	0.71	0.75	0.82	0.85	0.85	0.95	0.90	—	0.92
	Rb^+	0.56	0.67	0.70	0.80	0.87	0.86	0.84	0.87	—	0.89
	Cs^+	0.56	0.68	0.72	0.82	0.87	0.85	0.85	0.88	—	0.87
	Be^{2+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mg^{2+}	0.08	0.23	0.17	0.31	0.38	0.46	0.51	0.54	0.77	0.75
	Ca^{2+}	0.03	0.04	0.02	0.05	0.05	0.08	0.07	0.08	0.14	0.20
	Sr^{2+}	0.12	0.14	0.20	0.25	0.33	0.39	0.40	0.45	0.59	0.63
	Ba^{2+}	0.08	0.13	0.20	0.24	0.35	0.37	0.40	0.52	0.54	0.60
		CH_3COOH molarity									
		0.01	0.02	0.05	0.1	0.2	0.5	0.75	1	2	3
0.100	Li^+	0.30	0.47	0.61	0.65	0.73	0.75	0.70	0.80	0.81	0.83
	Na^+	0.95	—	0.95	—	0.95	0.95	—	0.93	0.92	0.92
	K^+	0.48	0.62	0.67	0.75	0.80	0.79	0.78	0.74	0.77	0.82
	Rb^+	0.46	0.62	0.65	0.73	0.76	0.78	0.78	0.74	0.77	0.82
	Cs^+	0.43	0.57	0.65	0.70	0.75	0.78	0.76	0.74	0.77	0.82
	Be^{2+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mg^{2+}	0.02	0.02	0.02	0.02	0.06	0.14	0.14	0.21	0.26	0.28
	Ca^{2+}	—	—	—	0.00	0.00	0.00	0.00	0.00	0.02	0.02
	Sr^{2+}	—	—	—	0.02	0.05	0.09	0.14	0.14	0.14	0.12
	Ba^{2+}	—	—	—	0.02	0.06	0.08	0.08	0.11	0.14	0.10

of the stationary phase on the chromatographic support. Thus the log $(1/R_F - 1)$ values were evaluated and plotted against the logarithm of the H^+ concentration. Owing to the dissociation equilibrium of CH_3COOH, the second-power of the H^+ concentration is proportional to the acetic acid concentration in the whole range of acetic acid solutions used as eluents.

After the first series of results with 0.010 and 0.100M HDEHP treated papers, additional experiments became necessary with paper treated with intermediate concentrations of HDEHP in cyclohexane between 0.100 and 0.010M. Such chromatograms were made with paper treated with 0.075, 0.050 and 0.025M HDEHP.

In correlating the quantity log $(1/R_F - 1)$ with log [HDEHP] it was decided to use for the concentration of the stationary phase its actual concentration on the paper and not the concentration of the extractant when in the organic solution during the paper treatment. This latter method had been used in correlating data for Dy, Gd and Sm in previous work[1], and though the expected linearity of log $(1/R_F - 1)$ *vs.* log M HDEHP was found, the slope was much less than the expected value of + 3. It was qualitatively explained on the basis of a difference between the behaviour of HDEHP when in the organic solution and when fixed on the cellulose. Further attention has now been given to this.

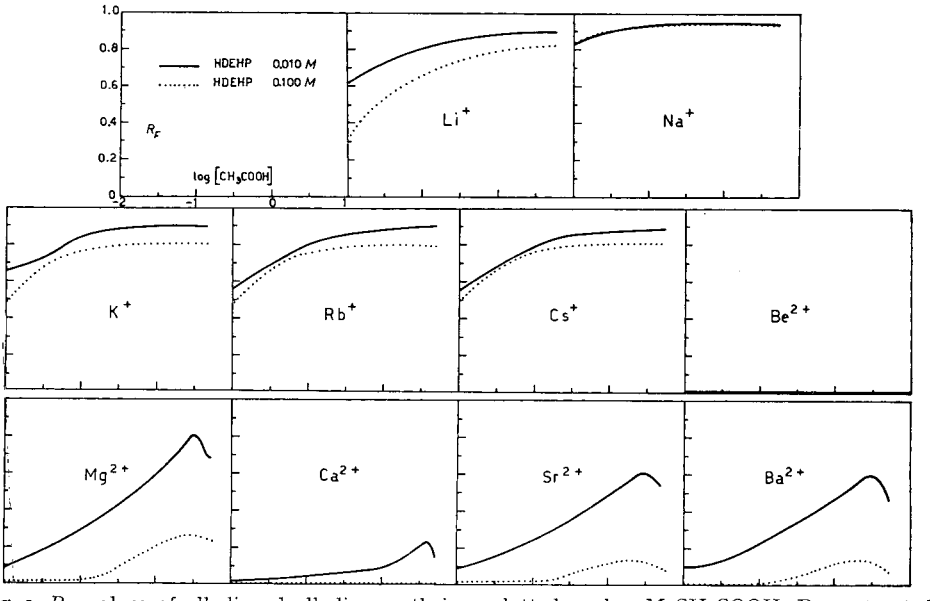

Fig. 2. R_F values of alkali and alkaline earth ions plotted *vs.* log *M* CH_3COOH. Paper treated with HDEHP.

Provided that the HDEHP concentration in the treatment solution is directly proportional to the "effective" concentration on the paper, the slope of log $(1/R_F - 1)$ = f(log [HDEHP]) does not change when the concentration in the organic solution is used for that on the paper. But if such a direct proportionality does not hold, the slope mentioned above can be affected to some extent. Experiments are therefore being performed in our laboratory to determine the "effective" concentration of extractant on the paper, $[HDEHP]_{eff}$, as a function of the concentration of the extractant in the cyclohexane solution, [HDEHP]. At present, preliminary experiments with beryllium ion, which is strongly retained by HDEHP on paper, have shown that a linear relationship exists between the two quantities. Paper strips (2.5 × 5 cm) treated with cyclohexane solutions having different concentrations of HDEHP were completely immersed in 20 ml of acidic solutions containing a few μg per ml of beryllium. The amount of beryllium retained per surface area unit was determined by measuring the ion loss in the respective solutions. Beryllium was determined by colorimetry. By assuming that the amount of Be^{2+} retained per cm^2 of paper is directly proportional to the "effective" HDEHP concentration, the following experimental relationship was found:

$$C_{Be} = k[HDEHP]_{eff} = 0.14 + 1.5[HDEHP] \quad (2)$$

in which C_{Be} is the concentration of Be^{2+} on the paper (μg/cm^2) and k is the proportionality constant between beryllium concentration and $[HDEHP]_{eff}$. Such an equation holds for [HDEHP] from 0.010 to 0.100*M* in cyclohexane.

From the results reported above it appears that when log $[HDEHP]_{eff}$ is needed for correlating data with log $(1/R_F - 1)$, the quantity log C_{Be} can be used, since log k

is an additional term which does not affect the slope obtained. On the other hand, where log [HDEHP] is used for log $[HDEHP]_{eff}$ the slope is actually affected. Thus, by correcting the results of the rare earth experiments referred to above, using this equation, the expected slope + 3 can be found. In the present work this correction has been applied when the quantity k $[HDEHP]_{eff}$ is indicated.

Alkaline earth ions

Plots reported in Fig. 2 show that all the alkaline earth ions, Be^{2+} excluded, present a maximum R_F at a given molarity of the acetic acid eluent.

Figs. 3 and 4 show log $(1/R_F - 1)$ values plotted against log $[H^+]$ up to $2M$ acetic acid (see below), for Mg^{2+} and Sr^{2+} respectively, at the five HDEHP concentrations considered. In Fig. 3 the behaviour of Ca^{2+} is also shown at the lowest HDEHP concentration, which is the only case in which this element presents R_F values higher than zero.

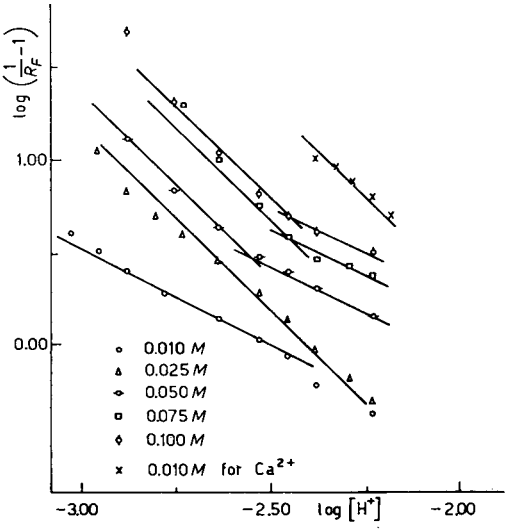

Fig. 3. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for magnesium. Paper treated with HDEHP at various concentrations. Calcium is also shown at 0.010 M HDEHP.

Straight lines are indicated in the two plots having slopes —2.0 and —1.0; such lines fit the experimental points with a sufficient agreement, and it appears that the quantity $(1/R_F - 1)$ (*i.e.* E_a°) is inversely proportional to the square of the H^+ concentration, if the concentration of HDEHP in cyclohexane is more than $0.025M$ and provided that the acetic acid concentration is kept low. In fact, when the acetic acid concentration is raised, the quantity $(1/R_F - 1)$ tends to become inversely proportional to the H^+ concentration.

At low HDEHP concentrations (0.010 and $0.025M$) Sr^{2+} presents an inverse first-power dependency and Mg^{2+} shows a slope of —1 at $0.010M$ and —2 at $0.025M$, which is quite unexpected. Experimental results for Ba^{2+} are similar to those of Sr^{2+}, whilst Be^{2+} never moves from the starting point.

In Fig. 5 the log $(1/R_F - 1)$ values for Sr^{2+} and Mg^{2+} are plotted as functions of the logarithm of the effective concentration of HDEHP on paper, $[HDEHP]_{eff}$. This

concentration is expressed as $\mu g\ Be^{2+}/cm^2$ (see above). Acetic acid of strengths 1.8 M and 0.2 M were chosen as the eluents, as these were considered representative of concentrated and dilute acid behaviour respectively. In both cases the slope + 3.0 shows that a third-power dependency holds between $(1/R_F - 1)$ and $[HDEHP]_{eff}$.

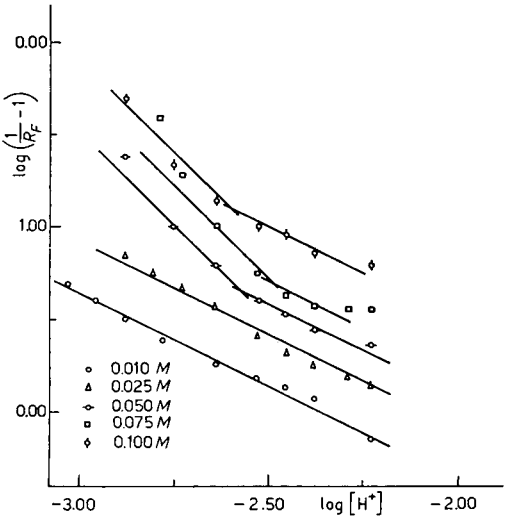

Fig. 4. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for strontium. Paper treated with HDEHP at various concentrations.

Both the slope —2.0 of Figs. 3 and 4 and the slope +3 of Fig. 5 can be explained in terms of the analogy between the liquid–liquid and the chromatographic systems. According to PEPPARD *et al.*[8] the extraction of calcium ion from an aqueous solution

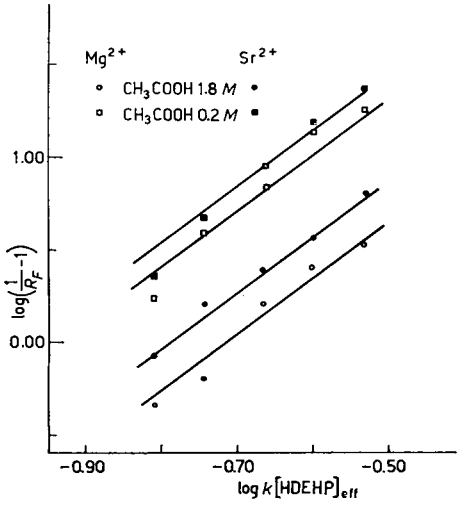

Fig. 5. Plot of log $(1/R_F - 1)$ *vs.* log k $[HDEHP]_{eff}$ for magnesium and strontium at two CH_3COOH concentrations.

by acid esters of phosphorous oxyacids in an organic phase follows the equation:

$$M^{2+} + 3(HDEHP)_2 \rightleftharpoons M(DEHP \cdot HDEHP)_2 \cdot (HDEHP)_2 + 2H^+$$

where M^{2+} is the calcium ion. In a more recent study on the extraction of strontium from acidic aqueous solutions with HDEHP, McDOWELL AND COLEMAN[9] suggest a reaction scheme which also leads to the same reaction coefficients without giving any special significance to hydrogen bonding between ligands within the complex. Such a scheme may be represented by the equation:

$$M^{2+} + 3(HDEHP)_2 \rightleftharpoons M(DEHP)_2 \cdot 4HDEHP + 2H^+.$$

In both cases the extraction coefficient is inversely proportional to the square of the hydrogen ion concentration in the equilibrated aqueous phase and proportional to the cube of the HDEHP concentration in the organic phase.

The slope -1.0 found in Figs. 3 and 4 where acetic acid is more concentrated may be explained by the formation of a different compound in the organic phase. The formation of a compound where the cation is surrounded both by CH_3COO^- and by HDEHP, may be the reason for this different behaviour toward hydrogen ions, while still maintaining the same dependency on HDEHP concentration. Following the scheme by McDOWELL AND COLEMAN cited above, the cation may form ionic bonds with both CH_3COO^- and $(DEHP \cdot HDEHP)^-$ whilst four HDEHP molecules are coordinated. Consequently the following reaction is suggested as a possible mechanism:

$$M(CH_3COO)^+ + 3(HDEHP)_2 \rightleftharpoons MCH_3COO \cdot (DEHP \cdot HDEHP) \cdot 4HDEHP + H^+$$

from which the dependency on the hydrogen ion concentration becomes first power.

PEPPARD *et al.*[10] in the case of Th-nitrate systems had also considered a partial combination of the cation with the aqueous phase acid and the extraction of a combined ion of this kind by the organic extractant. The peculiar effect of acetic acid on the extraction of Sr^{2+} with HDEHP was also pointed out by McHENRY AND POSEY[11] who showed that the distribution coefficients were affected not only by the H^+ concentration but by the acetic acid concentration as well. On the other hand, SCHULZ *et al.*[12] studying the extraction of Sr^{2+} with HDEHP from a $0.5N$ CH_3COONa solution acidified with HNO_3, report a plot of log E_a° *vs.* pH which has a slope of $+1.0$, that is, E_a° is inversely first power dependent on the H^+ concentration. In the same work, the log of the distribution coefficients of Sr^{2+} and Ca^{2+} from a $1M$ citric acid solution show almost a $+1$ slope *vs.* pH. A slope smaller than 2 was also found by WISHOW AND HORNER[13] for the first portion of the plot of log E_a° of Sr^{2+} *vs.* pH of an aqueous phase containing tartaric acid.

Furthermore, results reported by HARDY *et al.*[14] on the extraction of Be^{2+} with HDEHP from aqueous solutions containing HCl, H_2SO_4 and HNO_3 show that E_a° values at high acid concentrations are greater than expected from the inverse second power dependency on the acid concentration shown with more dilute solutions. They suggest that at high aqueous acid concentrations complexes containing the anion of the aqueous acid are probably also extracted.

Alkali ions

The results obtained with alkali ions are quite different from the results with alkaline-earth ions. Since the extraction coefficients are very low, the R_F values of alkali ions tend to unity. In fact Na^+ consistently runs with the front of the eluent, except at the lowest acetic acid concentrations; K^+, Rb^+, Cs^+ and Li^+ show a retention somewhat higher than Na^+, which increases slightly from K^+ to Li^+.

An attempt was made to obtain some information about their behaviour, by calculating the $(1/R_F - 1)$ function along the ascending portion of the alkali ion curves of Fig. 2. Thus in Fig. 6 log $(1/R_F - 1)$ is plotted against log $[H^+]$ for K^+ at the five HDEHP concentrations studied; Rb^+, Cs^+, and Li^+ behave similarly.

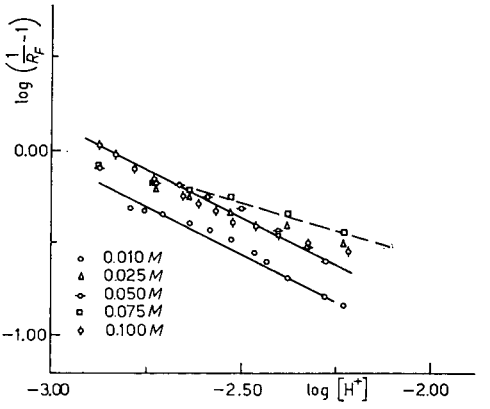

Fig. 6. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for potassium. Paper treated with HDEHP at various concentrations.

It is evident that the dependence of $(1/R_F - 1)$ or E_a° upon the hydrogen ion concentration is less than that shown by alkaline earths. In Fig. 6 two lines (solid) of slope −1.0 are drawn, by analogy with the results obtained by McDOWELL AND COLEMAN[9] for the extraction of Na^+ with HDEHP in benzene. Whilst points obtained with 0.010*M* HDEHP follow this slope quite closely the same cannot be said for the other HDEHP concentrations, where lower negative slopes fit better (broken line in Fig. 6).

SCHULZ *et al.*[12] report a plot of the extraction coefficient for Na^+ with HDEHP from an aqueous phase containing CH_3COONa, against the acidity of the aqueous solution, which shows an inverse square root dependency upon the hydrogen ion concentration, that is a +0.5 slope in the log E_a° *vs.* pH plot.

Nothing can be said about the dependency of $(1/R_F - 1)$ on the HDEHP concentration. It is interesting, however, that in Fig. 6 only points at 0.010*M* HDEHP are separated from the others, and this shows that the extraction coefficient is practically independent of the HDEHP concentration in the portion of the curve considered. The absence of relation between R_F and the HDEHP concentration, may be explained by recalling results reported by BLAKE *et al.*[15] on the effective solubility in water of sodium salts of HDEHP. Thus R_F values would depend solely upon the solubility of the organic salts in the acid solution.

Systems containing concentrated acetic acid

Particular attention was given to what happens when elutions are performed with very concentrated acetic acid. It was found that R_F values for alkaline earth ions present a maximum at acetic acid concentrations near $3M$ (see Fig. 2), while reproducibility of results was good enough up to $4M$ acetic acid. For higher concentrations it was not possible to obtain reliable results. Development of paper strips with 8-oxyquinoline and sodium alizarinsulphonate showed strange spots all over the paper which had been in contact with the acid. It was therefore decided to investigate the behaviour of the HDEHP treated papers with concentrated acetic acid. Since from various experimental results it looked as if HDEHP had lost its retention capacity after an elution with concentrated acid, three possibilities were taken into account, (i) the HDEHP somehow reacting with the acetic, acid (ii) the paper being damaged and (iii) the HDEHP being eluted away from the paper.

HDEHP (0.100M) equilibrated with 10M CH_3COOH was prepared and paper sheets were washed with acid of the same concentration. A series of elutions with Mg^{2+} spots was made, with 1M and 10M CH_3COOH, and results showed that while every development made with 10M acetic acid gave unsatisfactory results, experiments carried out using HDEHP and/or paper which had been previously in contact with 10M acetic acid, gave R_F values in agreement with results obtained under normal conditions.

These results pointed to the HDEHP being eluted away from the paper by the concentrated acetic acid as the most probable possibility. Drops of 0.1M HDEHP on paper strips were eluted with acetic acid at various high concentrations and the spots were detected as phosphomolybdate complexes[2]. Although the HDEHP spot was found at the starting point, a faint blue spot immediately behind the front of the solvent appeared at acid concentrations higher than $3M$, this spot increasing in dimension and intensity as the acid concentration was raised. When the latter exceeded $7M$, the original HDEHP spot disappeared. Fig. 7 shows elutions with $6M$, $7M$ and $8M$ acetic acid.

The partial elution of HDEHP by concentrated acetic acid was considered re-

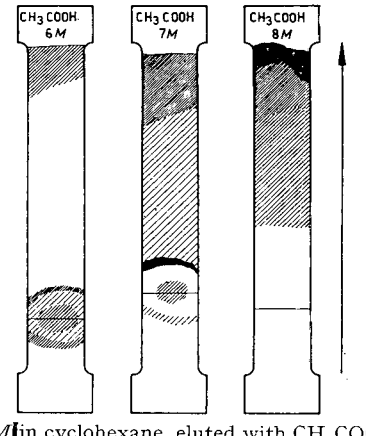

Fig. 7. Spots of HDEHP 0.1 M in cyclohexane, eluted with CH_3COOH at various concentrations. The shading is proportional to the intensity of the blue colour shown after development as phosphomolybdate complex.

sponsible for the bad results obtained when dealing with acid concentrations higher than $2M$. It should be noted that the decrease of R_F values for alkaline earths after their maximum cannot be put down to a phenomenon of this kind, since such a hypothesis would lead to an increase rather than a decrease of R_F values. In addition, an increase of E_a° values with the acid concentration in extraction experiments with HDEHP was also found by HARDY *et al.*[14] for Be^{2+} and HNO_3. MCHENRY AND POSEY[11] found this also in their extraction of Sr^{2+} from acetic acid solutions.

Application to chromatographic separations

From the experimental results described above it appears that some separations are possible of alkali and alkaline earth ions. Some ascending and descending chromatograms were carried out with paper strips (3 × 40 cm) cut from Whatman No. 1 paper sheets. The strips were treated with 0.050M and 0.100M HDEHP in cyclohexane. Elutions with 3M and 0.2M acetic acid were performed.

TABLE II

R_F VALUES OBTAINED IN ASCENDING AND DESCENDING CHROMATOGRAPHY ON PAPER TREATED WITH HDEHP

Paper strips 3 × 40 cm. Each column refers to an individual chromatogram. Operating temperature 23° ± 1°

HDEHP 0.050 M	CH_3COOH 2 M							CH_3COOH 0.1 M		
	R_F *from Fig. 2*	R_F						R_F *from Fig. 2*	R_F	
Li^+	0.90	n.d.	n.d.	0.89	n.d.	n.d.	n.d.	0.76	n.d.	n.d.
Na^+	0.94	n.d.	0.98	n.d.	0.94	0.94	n.d.	0.94	0.92	n.d.
K^+	0.90	0.82	n.d.	n.d.	n.d.	n.s.	0.88	0.82	0.81	n.d.
Rb^+	0.90	0.82	n.d.	n.d.	n.d.	n.s.	0.88	0.82	0.81	n.d.
Cs^+	0.90	0.82	n.d.	n.d.	n.d.	0.88	n.s.	0.82	0.81	n.d.
Be^{2+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mg^{2+}	0.46	0.34	0.32	0.41	0.44	0.46	0.46	0.14	0.14	0.14
Ca^{2+}	0.00	0.00	0.02	0.03	0.04	0.03	0.03	0.00	0.01	0.00
Sr^{2+}	0.30	0.17	0.18	0.23	0.27	0.29	n.s.	0.10	0.11	0.12
Ba^{2+}	0.30	0.17	0.18	0.23	0.27	n.s.	0.23	0.10	0.11	0.12
Run (cm) ascend.				29.4	25.8	31.0	26.1		32.1	32.6
descend.		33.1	34.0							
HDEHP 0.100 M										
Li^+	0.81	n.d.	0.82	n.d.	n.d.	0.84		0.70	n.d.	
Na^+	0.94	n.d.	0.95	0.93	0.93	0.92		0.94	n.d.	
K^+	0.80	0.78	n.d.	n.s.	n.s.	0.85		0.78	0.73	
Rb^+	0.80	0.78	n.d.	0.81	n.s.	n.s.		0.78	0.73	
Cs^+	0.80	0.78	n.d.	n.s.	0.80	n.s.		0.78	0.73	
Be^{2+}	0.00	0.00	0.00	0.00	0.00	n.s.		0.00	0.00	
Mg^{2+}	0.26	0.27	0.22	0.24	0.28	0.34		0.05	0.07	
Ca^{2+}	0.00	0.01	0.00	0.02	0.01	0.02		0.00	0.00	
Sr^{2+}	0.14	0.12	0.09	0.14	0.14	0.18		0.04	0.07	
Ba^{2+}	0.14	0.12	0.09	0.14	0.14	n.s.		0.04	0.07	
Run (cm) ascend.				29.6	29.2	26.6			32.2	
descend.		32.0	35.0							

n.d. = element not developed, n.s. = element not spotted.

The results are reported in Table II. It was not possible to detect Li^+ and K^+ (Rb^+, Cs^+) together on the same chromatogram, because of the closeness of the spots and the incompatibility of the developing reagents. Amongst the many types of separations which can be carried out under the conditions reported in Table II, can be cited Ca–Sr, Mg–Ba–Ca, Mg–Ca, Na–Ca–Sr, Cs–Sr, Cs–Ca–Sr.

From the curves in Fig. 2 it is also possible to choose better conditions under which to perform these and other separations. Most alkali and alkaline earth ions can be separated from each other by means of relatively short elutions with appropriate HDEHP and CH_3COOH concentrations.

CONCLUSIONS

The experimental results reported in this paper show the great similarity between the extraction properties of HDEHP in liquid–liquid systems and the chromatographic behaviour of paper treated with HDEHP which holds also in systems involving alkali and alkaline earth ions.

As in the case of paper chromatography or column separation of rare earths with cellulose[1,16,17] or Kel-F powder[18] treated with HDEHP, the influence of the H^+ concentration in the aqueous phase and of the effective concentration of HDEHP on the support can be related to hypotheses derived from liquid–liquid extraction investigations.

The use of more concentrated acetic acid gives rise to peculiarities in the chromatographic behaviour, which are here discussed. The behaviour of paper treated with HDEHP towards very concentrated acetic acid solutions limits the concentration of the eluent which can be used.

Within a considerable range of acidities, from an upper limit ($3M$ CH_3COOH) to reasonable dilutions ($0.1M$ CH_3COOH), many interesting separations of alkali and alkaline earth metals can be carried out.

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SUMMARY

The chromatographic behaviour of alkali and alkaline earth metals on paper treated with di(2-ethylhexyl) phosphoric acid (HDEHP) has been investigated, using acetic acid as the eluent in the range of concentrations from $2M$ to $0.01M$.

The quantity $(1/R_F - 1)$ has been related to the hydrogen ion concentration and to the effective HDEHP concentration on the paper.

A behaviour similar to that shown in liquid–liquid extraction with HDEHP solutions was found, provided that the acetic acid concentration was kept low. The peculiar behaviour shown with concentrated acetic acid has been discussed.

No direct proportionality has been obtained between the HDEHP molarity in the solution used to treat the paper and the effective concentration of HDEHP on the paper which acts during elution.

The instability of HDEHP on paper with very concentrated acetic acid has been also investigated.

Some chromatographic separations of alkali and alkaline earth metals have been carried out, and others have been suggested.

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QUANTITATIVE INORGANIC CHROMATOGRAPHY

XI. THE CHROMATOGRAPHIC SEPARATION AND AUTOMATIC FLAME SPECTROPHOTOMETRIC DETERMINATION OF THE ALKALINE EARTH METALS USING α-HYDROXYISOBUTYRIC ACID

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A previous communication[1] described the chromatographic separation and automatic flame spectrophotometric determination of the alkaline earth metals using ammonium lactate as eluting agent in the ion exchange procedure. The disadvantage of this method was that the separation took twelve hours, and the present separation was studied to give a more rapid determination. The technique has been developed so that magnesium, calcium, strontium, and barium may be separated and determined in under five hours.

WISH[2] found that calcium, strontium and barium could be separated by cation exchange chromatography, eluting with α-hydroxyisobutyric acid. As this separation did not include the separation of magnesium, a slightly modified procedure has been devised to separate all four alkaline earth metals.

EXPERIMENTAL

This paper describes the recommended procedure for the quantitative separation and estimation of sub-milligram quantities of all four alkaline earth metals using conventional cation exchange chromatography and automatic flame spectrophotometric detection. The separation was achieved by batch elution technique with ammonium α-hydroxyisobutyrate and the estimation was achieved by direct aspiration of the eluate into the flame after constant dilution. The area of the elution peaks so obtained was a measure of the total alkaline earth metal loaded on to the column. The accuracy of the method was found to be within 4 % at sub milligram levels.

Apparatus and general technique

The cation exchange material used for the separation was Dowex 50W resin with 8 % divinylbenzene cross-linking, 100–200 mesh. The resin bed was 9 cm long and 0.4 cm diameter. A glass wool plug was placed at the top of the resin so that in loading samples on to the column, the upper layers of the resin remained undisturbed. A 250 ml dropping funnel was fitted to the top of the column so that there was an approx. 10 cm pressure head of eluting agent above the column bed. The dropping rate of the column was 0.65 ml/min.

In the initial investigations of the separation, a Shandon automatic fraction collector (balance type) was used to collect 5 ml samples of eluate to be analysed.

For the automatic procedure, the constant volume delivery burette, previously described[1], was used to enable the column effluent to drop into a constant volume of water. This was achieved by balancing the aspiration rate of the flame with the total flow from the column and the constant volume delivery burette.

Eluting agents

The magnesium was eluted with 0.8 *M* α-hydroxyisobutyric acid pH 4.15. Calcium and strontium were eluted with 1 *M* α-hydroxyisobutyric acid pH 5.00 and barium with 2 *M* solution pH 6.20.

The solutions were prepared as follows:

0.8 *M* solution was prepared by dissolving 83.3 g of the acid in water and adding 0.880 ammonia (AnalaR) until the solution reached pH 4.15, and water was added to 1 l.

1 *M* solution required 104.1 g acid dissolved in water, ammonia was added until pH 5.0 and made up to 1 l.

2 *M* solution required 208.2 g acid in water, ammonia was added until pH 6.20, and made up to 1 l.

Most of the α-hydroxyisobutyric acid used was prepared from acetone and potassium cyanide to form acetone cyanohydrin as suggested by WELCH AND CLEMO[3], and this was hydrolysed and extracted to give the α-hydroxyisobutyric acid as done by RULE AND HARROWER[4]. The acid was recrystallised from 60°–80° petroleum ether.

Purity of water and reagents

All water used was deionised by passing distilled water from an all-glass apparatus, through a mixed Zeocarb resin column. The water was stored in hard glass or plastic aspirators, and the alkaline earth content of the water was below the detection limit of the flame spectrophotometer. All alkaline earth metal solutions were prepared from AnalaR reagents.

The alkaline earth metal content of the α-hydroxyisobutyric acid was found to be negligible, whether commercially or laboratory prepared. The laboratory prepared acid had a slight sodium impurity, but this did not affect results obtained, and so no further purification was deemed necessary. The effect of organic matter in the eluant gave slight enhancement of emission of the alkaline earths, and also an increase in background emission during automatic detection. Thus when determining fraction concentrations, prior to the automatic runs, standard solutions of the alkaline earth metals were prepared containing a similar quantity of α-hydroxyisobutyric acid to that in the sample fraction being examined.

Solutions

(*a*) *For chromatography*: Standard barium solution: AnalaR barium chloride $BaCl_2 \cdot 2H_2O$ in water containing 1 mg/ml of barium.

Standard calcium solution: AnalaR calcium carbonate $CaCO_3$ in 0.01 *N* hydrochloric acid containing 1 mg/ml calcium.

Standard magnesium solution: AnalaR magnesium carbonate $MgCO_3$ in 0.01 *N* hydrochloric acid containing 1 mg/ml magnesium.

Standard strontium solution: AnalaR strontium carbonate $SrCO_3$ in 0.01 N hydrochloric acid containing 1 mg/ml strontium.

The column was loaded with 0.25 ml, 0.5 ml, 1.0 ml or 1.5 ml of each solution for standard runs.

(*b*) *For flame spectrophotometric analysis of fractions.* The above solutions were diluted for comparison standards when initial runs were carried out, by collecting fractions and analysing them. Each solution contained the appropriate quantity of α-hydroxyisobutyric acid solution for exact comparison with fraction samples. The working solutions used were:

Calcium and strontium: 0–2 μg/ml of alkaline earth metal.

Barium and magnesium: 0–5 μg/ml of alkaline earth metal.

The quantitative chromatogram

The resin was generated in the ammonium form by the passage of 3 M ammonium chloride down the column followed by copious washing with water. The column was loaded with the alkaline earth metal bearing solution, followed by further washing with water. The dropping funnel was then filled with 0.8 M α-hydroxyisobutyric acid which was passed down the column for 13 fractions when using the fraction collector or until the magnesium had been eluted when using automatic detection. This solution was then replaced by 1 M acid in the funnel. The passage of 1 M α-hydroxyisobutyric acid was continued until fraction 28 when collecting fractions, or until both the calcium and strontium were removed automatically. The elution was then continued replacing the 1 M acid by 2 M until fraction 35 or barium had been eluted.

The column was washed with water and regenerated with ammonium chloride. If quantities of alkaline earth metals much greater than 1–2 mg are used for separation, then a longer column will be necessary.

The detection of the alkaline earth metals

(*a*) *Using a fraction collector.* 1 ml aliquots of the 5 ml fraction were diluted until the concentration of the alkaline earth metal in the sample solution was within the standard range. The quantity of the alkaline earth metal in each fraction was then determined by comparison with standard calibrations in the normal manner.

(*b*) *For automatic detection.* The column effluent was run into 10 ml of water which was continuously stirred. The resulting solution was aspirated directly into the flame of the instrument. As the flame aspiration rate was greater than the flow rate of the column, a constant volume delivery burette was required to keep the volume of water, into which the effluent fell constant. The use of this technique has previously been described in detail[1]. The flame spectrophotometer wavelength settings were the same as used for the ammonium lactate procedure, as these wavelengths gave good peaks. The concentration of alkaline earth metal in the 10 ml of water was found to be proportional to the concentration of the ion in the column effluent, and so the elution curve for each metal was directly recorded by coupling the output of the flame spectrophotometer's photomultiplier to a potentiometric recorder with a chart speed of 2 in. per h.

In order to overcome day to day variations in conditions, it was found most practicable to fix the gain settings of the instrument and vary the pressure of acetylene daily. This was done by passing a 1 μg/ml solution of calcium with standard

instrument settings, and vary the acetylene pressure until the scanned line gave the same deflection as on previous days.

Calibration curves were obtained using synthetic mixtures containing 0–1.5 mg amounts of individual alkaline earth metals by plotting area of elution curve against quantity of alkaline earth loaded on to the column. Using these calibration curves, mineral ore and biological samples were successfully analysed.

Flame spectrophotometry

The Unicam SP 900 was used operating under the following conditions:

B.O.G. cylinder gases with approximately 3 in. of acetylene indicated on the dibutyl phthalate manometer, and 30 lb./sq. in. of compressed air.

(*a*) *With fraction collector.* Samples and standards were aspirated under the conditions shown in Table I.

TABLE I

CONDITIONS FOR ANALYSIS OF FRACTIONS

Electrical bandwidth 4. Photomultiplier Mazda 27M3 Sunvic RSP 2 high speed potentiometric recorder. Chart speed 1 in./min.

	Magnesium	*Calcium*	*Strontium*	*Barium*
Slit (mm)	0.08	0.04	0.04	0.06
Wavelength (mμ)	284.5–286.0	420–427	457–467	554–561.0
Amplifier gain	4.3	4.5	4.3	4.3
Recorder f.s.d. (mV.)	1	10	10	2.5

The amplifier gain is only approximate in these cases, and varies slightly from day to day. Peak heights recorded in these conditions are measured. Barium and magnesium were determined by allowing the recorder to "settle" after scanning as described earlier[5]. The elution pattern for separation with a fraction collector is shown in Fig. 1.

(*b*) *Automatic detection.* In this procedure, the gain settings were kept constant, day to day variations were minimised by adjustment of acetylene pressure. Previously

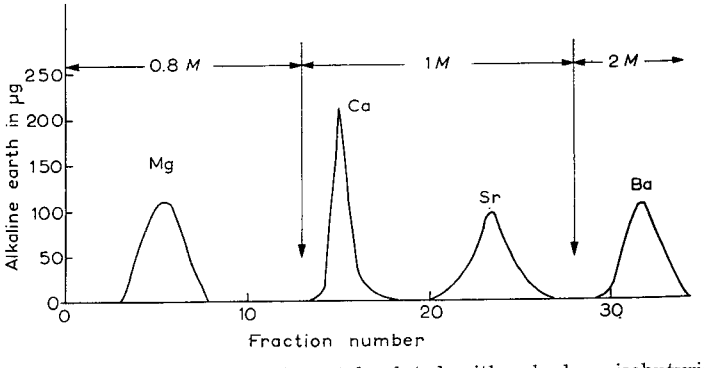

Fig. 1. Elution curve of alkaline earth metals eluted with α-hydroxyisobutyric acid using a fraction collector and analysing fractions. 5 ml fractions collected.

the most critical factor affecting determinations was found to be the wavelength setting[1]. The wavelengths used for the previous ammonium lactate process gave good results with this present separation, and no further investigation of wavelength settings was deemed necessary.

The settings used for analysis by automatic flame spectrophotometry are shown in Table II.

A constant volume of 10 ml of solution was used.

TABLE II

CONDITIONS FOR AUTOMATIC ANALYSIS

Electrical bandwidth 4. Sunvic RSP 2 high speed potentiometric recorder 10 mV f.s.d. and chart speed 2 in./h. Photomultiplier Mazda 27M3.

	Magnesium	*Calcium*	*Strontium*	*Barium*
Slit (mm)	0.08	0.04	0.04	0.06
Wavelength (mμ)	285.5	423.0	461.0	560.0
Amplifier gain	4.7	2.5	2.5	4.5

The shapes of the elution curves recorded automatically are shown in Fig. 2.

Magnesium was found to give a slightly flatter shaped elution curve compared with the other alkaline earth metals, and also a lower sensitivity as was found in the previous ammonium lactate procedure[1]. Also the calibration curve for magnesium is non-linear, unlike the other metals, and these differences appear to be due to the phenomenon reported by DEAN[6] that the wavelength of maximum intensity for magnesium varies with concentration of magnesium in solution. Before the actual peaks of magnesium and barium, a raising of background level of emission appears,

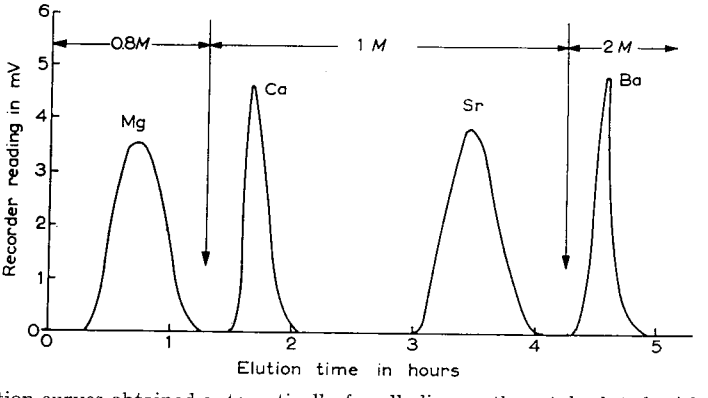

Fig. 2. Elution curves obtained automatically for alkaline earth metals eluted with α-hydroxyisobutyric acid.

which does not disappear after elution of the metal. This phenomenon was noticed with elution of barium with ethylenediaminetetraacetic acid (EDTA)[1] and the explanation would seem to be the comparatively sudden increase of organic matter

reaching the flame causing a rise in flame temperature, and hence of flame background. All areas of peaks drawn were measured with a planimeter.

Analysis of synthetic mixtures

(a) During initial investigations of the separation, the use of a fraction collector was employed to demonstrate the quantitative nature of the chromatography. The figures given in Table III illustrate this and serve to show that this method may be used where a flame spectrophotometer cannot be used to record directly.

TABLE III

ANALYSIS OF SYNTHETIC SAMPLES IN FRACTIONS

Loaded on to the column (μg)				*Found by analysis of fractions (μg)*			
Magnesium	*Calcium*	*Strontium*	*Barium*	*Magnesium*	*Calcium*	*Strontium*	*Barium*
300	300	300	300	309	294	282	293
300	500	500	500	311	505	504	502

These results showed that the separation was indeed quantitative and all further results were recorded automatically.

(b) Results of automatic separation of standard synthetic mixtures for calibration purposes are given in Table IV.

These results were used to obtain the calibration curves shown in Fig. 3.

TABLE IV

AUTOMATIC ANALYSIS OF SYNTHETIC SAMPLES FOR CALIBRATION CURVES

	Magnesium			*Calcium*			*Strontium*			*Barium*		
Quantity of metal (mg)	0.5	1.0	1.5	0.25	0.5	1.0	0.25	0.5	1.0	0.25	0.5	1.0
Peak area	0.70	1.41	1.62	1.46	2.97	6.16	0.71	1.55	3.09	0.83	1.58	3.20
(sq. in.)	0.71	1.36	1.60	1.47	2.94	6.25	0.73	1.53	3.17	0.85	1.62	3.10
	0.70	1.34	1.59	1.52	3.00	6.20	0.73	1.56	3.19	0.80	1.65	3.10
	0.73	1.32	1.61	1.46	3.12	6.13	0.72	1.55	3.06	0.85	1.56	3.30
						6.30						
Mean area	0.71	1.36	1.61	1.48	3.01	6.21	0.72	1.55	3.12	0.83	1.60	3.17

Analysis of mineral ores by automatic detection

Samples of mineral ores were taken up into solution and aliquots of these solutions loaded on to the column and analysed for their alkaline earth metal content. The area of the elution peaks for each sample was measured, and the amount of alkaline earth metal in each sample determined from the calibration graph in Fig. 3.

The results obtained are given in Table V.

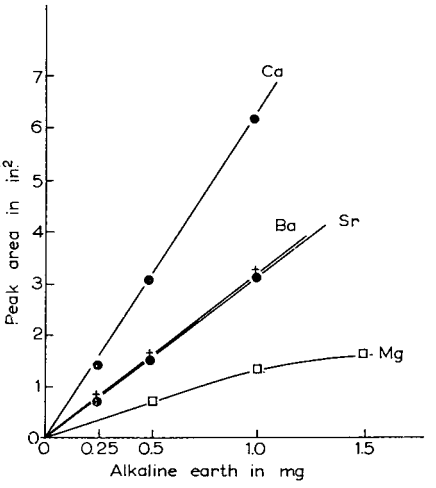

Fig. 3. Calibration curves for alkaline earth metals determined automatically when eluted with α-hydroxyisobutyric acid.

TABLE V

RESULTS OF AUTOMATIC ANALYSIS OF MINERAL ORE SAMPLES

Ore	*Metal*	*% found*	*% found by previous automatic analysis*[1]	*% by chemical analysis*
Dolomite	Magnesium	5.99	5.80	5.78
	Calcium	31.2	30.3	31.1
Celestine	Strontium	46.9	47.3	45.6
Barytes	Calcium	16.5	16.7	16.6
	Barium	33.3	34.7	35.4

* All chromatographic analyses of these barytes have given low results between 32.2 %–34.7 %.

Analysis of biological samples

Samples of blood serum and bones were obtained from a rat and ashed with concentrated nitric and sulphuric acids in the ratio of 10:1 respectively. The blood sample was diluted to a standard volume as the ash remained in solution in even a very small volume of remaining sulphuric acid after evaporating to almost dryness. The bone ash was fused with sodium carbonate, leached with water and the resultant residue dissolved in dilute hydrochloric acid and made up to a standard volume.

These solutions were analysed chemically and chromatographically. The chemical calcium analysis was carried out according to the CLARK-COLLIP method[7] by precipitating it as oxalate and, after acidification, titrating with standard potassium permanganate.

The magnesium analysis was carried out colorimetrically using Eriochrome Black T as directed by VOGEL[8].

The results by automatic chromatographic and chemical analyses are given in Table VI.

TABLE VI

RESULTS OF AUTOMATIC ANALYSIS OF BIOLOGICAL SAMPLES

	Found chromatographically %	*Found chemically* %
Calcium in blood	0.0053	0.0055
Magnesium in bone	0.034	0.032
Calcium in bone	17.6	17.1

Thus this cation exchange process with automatic flame spectrophotometric detection has been used successfully for synthetic, mineral ore and biological samples containing alkaline earth metals.

ACKNOWLEDGEMENTS

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SUMMARY

An ion exchange separation of submilligram quantities of alkaline earth metals has been developed using α-hydroxyisobutyric acid as eluting agent and automatic flame spectrophotometric determination.

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Short Communications

Chromatography on ion-exchange papers

XI. The salting-out effect in the adsorption of inorganic anions

The adsorption of anions (such as $AuCl_4^-$) on ion exchangers usually increases with the concentration of LiCl or other chlorides but decreases with the concentration of HCl.

This increased adsorption with LiCl was observed on both anion exchange resins[1] and cation exchange resins[2,3] and seems contrary to what would be expected in ion exchange equilibria which are supposed to obey the law of mass action.

By comparing the adsorption of numerous metal ions on various anion exchangers (resins and cellulose exchangers) we concluded[4] that at least two mechanisms are involved, namely electrostatic attraction (or ion exchange) and a "physical" adsorption akin to extraction with liquid solvents.

As no data were available for the effect of salts such as LiCl on cellulose exchangers we have now carried out a preliminary investigation taking perrhenate and chloroaurate as typical anions and observing the change of adsorption with the change in the LiCl concentration on a range of neutral, cationic and anionic supports. From the results given below it appears that the salting-out effect is quite independent of possible electrostatic attraction and occurs on all supports. Indeed it seems that anion exchange and salting-out are competitive on anion exchangers.

Experimental

The various papers given in Table I were developed by the ascending technique with solutions being 1, 2, 4 and 8 *N* with respect to LiCl and 0.1 *N* to HCl (the latter to prevent hydrolysis of $AuCl_4^-$). Ion exchange papers were all washed twice with 2 *N* HCl and water, and air dried.

Perrhenate was detected by spraying with a solution of $SnCl_2$ and KCNS in 5 *N* HCl and the gold by spraying with a solution of $SnCl_2$ in HCl.

The papers in Table I are in the order of decreasing R_F values and this order is approximately the same for both ions.

Perrhenate does not change its R_F values appreciably on neutral or acid supports with an increase in the LiCl concentration and decreases them on anion exchangers as would be expected from an adsorption obeying the law of mass action.

Chloroaurate decreases its R_F values on all supports with increasing LiCl concentration, the least decrease occurring with aminoethylcellulose which also in our studies with HCl[4] proved to combine the least adsorption affinity with anion exchange properties. DEAE cellulose already shows a stronger salting-out effect presumably due to the presence of the non-polar ethyl groups on the nitrogen atoms.

The sequence of adsorption differs for the two ions only for the two cellulose anion exchangers. The aminoethylcellulose has a higher exchange capacity (and

TABLE I

R_F VALUES OF ReO_4^- AND $AuCl_4^-$ ON VARIOUS PAPERS DEVELOPED WITH SOLUTIONS OF LiCl

Paper	*Perrhenate*				*Chloroaurate*			
	1N LiCl	*2N LiCl*	*4N LiCl*	*8N LiCl*	*1N LiCl*	*2N LiCl*	*4N LiCl*	*8N LiCl*
Whatman paper P20 (cellulose phosphate)	diffuse zone at liquid front				0.61	0.50	0.48	0.32
Whatman No. 1 paper (pure cellulose)	0.67	0.66	0.64	0.67	0.43	0.36	0.30	0.23
Amberlite WA-2 paper (containing 45% of Amberlite IRC-50, an acrylic resin with COOH groups)	0.59	0.55	0.50	0.56	0.16	0.15	0.11	0.08
Whatman paper DE 20 (diethylaminoethylcellulose)	0.40	0.47	0.50	0.60	0.13	0.12	0.11	0.06
Whatman paper AE 30 (aminoethylcellulose)	0.40	0.43	0.46	0.55	0.15	0.15	0.13	0.12
Amberlite SA-2 paper (containing 45% of Amberlite IR-120, a nuclear sulphonic resin on styrene base)	0.32	0.36	0.36	0.32	0	0	0	0
Amberlite WB-2 paper (containing 45% of Amberlite IR 4B, a phenolic weak base resin)	0.04	0.06	0.07	0.07	0	0	0	0
Amberlite SB-2 paper (containing 45% of Amberlite IRA-400, a strong base styrene resin)	0	0	0	0.02	0	0	0	0

hence adsorbs ReO_4^- stronger) and the additional ethyl groups increase the adsorption of $AuCl_4^-$ on DEAE-cellulose even at lower LiCl concentrations.

Cellulose phosphate adsorbs anions less than cellulose which could be expected since highly polar groups are present in the first. The acrylic (carboxylic) resin Amberlite WA-2 adsorbs less strongly than cellulose anion exchangers and even less than a nuclear sulphonic resin. Evidently the network plays a considerable role in the adsorption on these resins.

The low R_F values of $AuCl_4^-$ on anion exchange and sulphonic resins does not permit any conclusions, however, for these resins equilibrium studies have already been recorded[1-3] and confirm the general picture here presented.

Work on a large number of metal ions with various exchangers and LiCl as solvent is in progress and will be described later.

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Chromatography on a column of Raney cobalt

Platinum, palladium, nickel, and other catalysts prepared from transition metals are readily poisoned by the presence of small quantities of organo-sulphur compounds, and it has been established that the sulphur compounds are chemisorbed onto the metal via the lone pair of electrons[1]. On the other hand, when an organo-sulphur compound is treated with a large excess of a hydrogen-rich catalyst, such as Raney nickel, the initial chemisorption may be followed by desulphurisation[2]. The transition metals differ in their ability to desulphurise compounds, just as they differ in their ability as hydrogenation catalysts. Cobalt, copper and iron, for example, have some activity as desulphurisation catalysts, but are much less effective than nickel[3].

It therefore seemed likely that a weak catalyst could be used, under mild conditions, to chemisorb an organo-sulphur compound without effecting desulphurisation, and that this might be adapted as a chromatographic procedure for the separation of organo-sulphur compounds.

A column of Raney cobalt mixed with sand was found to be suitable for this purpose. The mixture containing the sulphur compound was applied to the column, which was then eluted with methanol. In this way, isoeugenol, geraniol, geranyl acetate and butyl acetate were readily eluted, but 2,5-dimethylthiophen and ethyl thiophen-2-carboxylate were completely retained, and were recovered only by prolonged Soxhlet extraction of the adsorbent. No desulphurisation or hydrogenation took place. In another investigation a column of Raney cobalt prepared in this way has been found useful for the separation of a mixture of dibenzothiophen and biphenyl[4].

Preparation of Raney cobalt. A solution of sodium hydroxide (80 g) in water (300 ml) was cooled with ice and cobalt–aluminium alloy (Light's 30% Co; 65 g) added with vigorous stirring as quickly as frothing allowed. After digesting the mixture on the water bath at 100° for 1 h the supernatant liquid was decanted and the cobalt washed by decantation with water (6 × 100 ml) and twice with methanol.

Chromatography. The freshly-prepared Raney cobalt (*ca.* 7.5 g) was mixed with clean sand and packed into a chromatographic column (1.2 cm × 10 cm). A mixture of isoeugenol (0.5 g) and 2,5-dimethylthiophen (0.5 g) was applied to the column and eluted with methanol (a 3-ft. head of liquid was required). Evaporation of the first fraction (30 ml) gave sulphur-free isoeugenol (0.477 g). Subsequent fractions contained only trace amounts of isoeugenol and were also sulphur-free. The dimethylthiophen was subsequently recovered by Soxhlet extraction of the cobalt with methanol.

In another experiment a column of Raney cobalt retained ethyl thiophen-2-carboxylate, but allowed butyl acetate, geraniol and geranyl acetate to pass through.

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Notes

Kieselgel-Dünnschichtchromatographie von Flavonoiden

Die Chromatographie von Flavonoiden wurde bisher fast ausschliesslich auf Papier durchgeführt. Besonders brauchbar erwiesen sich die von BATE-SMITH UND WESTALL[1], ROUX UND EVELYN[2], HERGERT[3], HÖRHAMMER, WAGNER UND GÖTZ[4] sowie von EGGER[5] und neuerdings von WONG UND TAYLOR[6] vorgeschlagenen Laufmittel. Die ersten Versuche, Flavonoide auch dünnschichtchromatographisch zu trennen, stammen von STAHL UND SCHORN[7] sowie von PARIS[8]. Zur Auftrennung der Citrus-Bioflavonoide[9], Kamillenflavone[10] und zum Nachweis von Isoflavonen in Pflanzenextrakten[11] wurde in eigenen Arbeiten die Kieselgel-Dünnschichtchromatographie mit folgenden Systemen verwendet: Butanol–Eisessig–Wasser (4:1:5)[9] und wassergesättigtes Chloroform–Eisessig (2:1.5)[10] bzw. (2.5:1)[11].

Da die Dünnschichtchromatographie sehr selektive Trennungen erlaubt, nur kurze Zeit beansprucht und daher für systematische Untersuchungen bestens geeignet ist, haben wir unsere Versuche auch auf andere Flavonoide ausgedehnt.

Mit dem Laufmittel Benzol–Pyridin–Ameisensäure (36:9:5) erreichten wir eine ausgezeichnete Trennung folgender Flavonoide:

1. Flavone: Apigenin, Acacetin, Luteolin, Pectolinarigenin, Genistein.
2. Flavanone: Naringenin, Hesperetin.
3. Flavonole: Kämpferol, Kämpferid, Quercetin, Isorhamnetin, Rhamnetin, Centaureidin.

Das Chromatogrammschema (Fig. 1) gibt eine Übersicht über den Grad der Auftrennung und die Laufhöhe der einzelnen Aglykone bei der Chromatographie

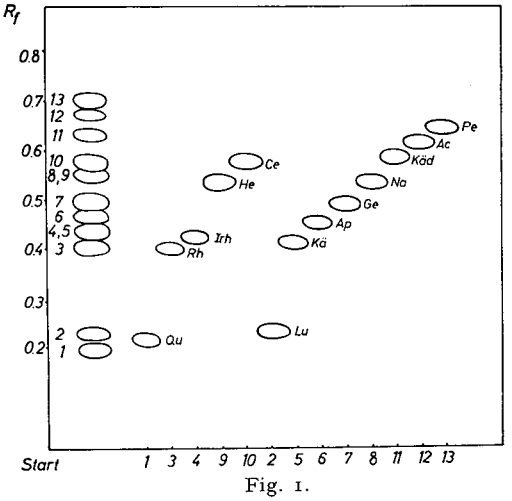

Fig. 1.

über eine Wegstrecke von 15 cm. Die Aglykone sind mit 1–13 beziffert und in Tabelle I und II in ihrer Struktur und ihrem chromatographischen Verhalten näher erläutert.

Diskussion der R_F-Verteilung

Die Laufhöhe der Aglykone vergrössert sich mit abnehmender Polarität der Verbindungen. So findet man die Polyhydroxyflavone im R_F-Bereich von 0.0–0.25, die weniger hydroxylierten und methoxylierten Flavonoide im mittleren R_F-Bereich von 0.3–0.5 und zwischen 0.5–0.75 die Flavanone einschliesslich der stark methoxylierten Aglykone. In Tabelle I sind die Aglykone nach zunehmenden R_F-Werten angeordnet.

TABELLE I

SUBSTITUTION DER UNTERSUCHTEN FLAVONOIDE

Nr.	*Aglykon*	*OH-Substitution*	*OCH_3-Substitution*
1	Quercetin	5, 7, 3, 3′, 4′	—
2	Luteolin	5, 7, 3′, 4′	—
3	Rhamnetin	5, 3, 3′, 4′	7
4	Isorhamnetin	5, 7, 3, 4′	3′
5	Kämpferol	5, 7, 3, 4′	—
6	Apigenin	5, 7, 4′	—
7	Genistein	5, 7, 4′	—
8	Naringenin	5, 7, 4′	—
9	Hesperetin	5, 7, 3′	4′
10	Centaureidin	5, 7, 3′	4′, 3, 6
11	Kämpferid	5, 7, 3	4′
12	Acacetin	5, 7	4′
13	Pectolinarigenin	5, 7	4′, 6

Die Trennung lässt sich auf gipshaltigem und gipsfreiem Kieselgel mit dem gleichen Ergebnis durchführen. Bei systematischen Untersuchungen kann die organische Phase nach dem Ausschütteln der Hydrolyselösung direkt ohne weitere Vorbehandlungen zur Chromatographie gebracht werden.

Mit dem neu entwickelten Laufmittel erreichten wir eine Trennung auch solcher Aglykongemische, die papierchromatographisch bisher nur schwer trennbar waren. Die Isomerenpaare Genistein und Apigenin sowie Rhamnetin und Isorhamnetin sind scharf voneinander getrennt und können auch farblich nach Behandlung mit Sprühreagenzien gut voneinander unterschieden werden. Als Sprühmittel bewährten sich basisches Bleiacetat und Antimon-(III)-chlorid[12]. Diese geben auch bei den übrigen Flavonoiden so starke Farbunterschiede, dass bereits eine gewisse Charakterisierung möglich ist. Tabelle II gibt die Fluoreszenzfarben wieder, die nach dem Besprühen im U.V.-Licht auftreten.

Methodik

Zur Herstellung der Dünnschicht-Platten kann Kieselgel "Woelm" oder Kieselgel G "Merck" verwendet werden. 20 g Kieselgel rührt man mit 40 ml Wasser an (ausreichend für vier Platten 12 × 20 cm) und streicht in bekannter Weise[13] aus. Anschliessend gibt man die Platten zur Aktivierung 30 Min. in den Trockenschrank bei 100°. Nach dem Ausgiessverfahren von HÖRHAMMER und Mitarb.[14] werden 4 g Kieselgel "Woelm" mit 16 ml Äthylacetat angeschüttelt und gleichmässig auf vier

TABELLE II

FLUORESZENZFARBEN DER FLAVONOIDE

Nr.	Aglykon	Sprühreagenzien	
		Bas. Bleiacetat	Antimon-(III)-chlorid
1	Quercetin	braun	gelb
2	Luteolin	braungelb	gelb
3	Rhamnetin	braun	gelb
4	Isorhamnetin	gelb	grün
5	Kämpferol	leuchtend gelb	grün
6	Apigenin	hellgelb	fahlgelb
7	Genistein	weissgelb	gelbbraun
8	Naringenin	fahlblau	gelbbraun
9	Hesperetin	mattblau	schwarzbraun
10	Centaureidin	braun	braungelb
11	Kämpferid	grüngelb	grüngelb
12	Acacetin	fahlgelb	fahlgelb
13	Pectolinarigenin	schwarzbraun	schwarzbraun

Platten der Dimension 12 × 20 cm verteilt. Um Schwanzbildung bzw. Überdecken anderer Aglykone zu vermeiden, trägt man pro Fleck ca. 0.5 μg Aglykon auf. Für die Herstellung des Laufmittels Benzol–Pyridin–Ameisensäure (36:9:5) wurden Benzol mit einem Siedepunkt von 79–82°, Pyridin pur. von Riedel de Haen und Ameisensäure 98–100%ig von Merck verwendet. Die Glaskammer muss vor der Chromatographie 12 bis 15 Stunden mit dem Laufmittel gesättigt werden. Dieses kann ohne Nachlassen der Trennfähigkeit bis zum völligen Verbrauch verwendet werden. Die Laufzeit beträgt bei einer Temperatur von 20° ± 2° und bis zu einer Laufhöhe von 15 cm 1.5 Stunden. Als Sprühreagenzien wurden eine etwa 25%ige wässrige Lösung von basischem Bleiacetat und eine 10%ige Antimon-(III)-chloridlösung in Chloroform verwendet. Vor dem Besprühen müssen die Platten eine Stunde mit warmem Föhn behandelt werden, um sie von der Ameisensäure und dem Pyridin zu befreien. Für die Auswertung der Fluoreszenzfarben benutzten wir eine U.V.-Lampe mit einem Maximum von 366 mμ.

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Separation of actinomycins by thin-layer chromatography

Actinomycins are chromopeptides with antibiotic and cytostatic activity produced by species of Streptomycetes[1]. Chemically, actinomycins are characterized by a chromophoric phenoxazinone group, which is identical for all known actinomycins, and two pentapeptidic chains. The variation in the amino acid composition of the peptide chains gives rise to the different actinomycins[2].

The separation of actinomycins has been achieved by countercurrent distribution[3], column chromatography[4] and paper chromatography[4–6].

In the course of studies on the biosynthesis of actinomycins it became necessary to separate rapidly the actinomycins of the C group[2] from those of the F group[7]. (Actinomycin C_1 has two molecules of D-valine in the peptide chain, while in actinomycin C_2 one molecule of D-valine is substituted by one molecule of D-alloisoleucine and in actinomycin C_3 two molecules of D-alloisoleucine are present. Actinomycins F_1 to F_4 are identical to those of the C group, but for the presence of three or four sarcosine molecules instead of two as in the case of the C group.).

Separation has been achieved (Table I) by chromatography on layers of alumina (Merck, G grade) or silica gel (Merck, G grade). Localization of the antibiotic is easily accomplished since actinomycins show a bright orange color (E_{max} 440 to 450 mμ) and strongly absorb under U.V. light (E_{max} 240 mμ). Identification of each actinomycin was achieved by semi-quantitative analysis of the amino acid content of the peptide chains[8].

The actinomycins may be recovered from the plates by elution with methanol; as determined colorimetrically, 74% and not more than 50% of the substance applied was recovered from the silicagel and alumina layers, respectively.

If the chromatographic run and the elution of the antibiotic from the plates were performed in the dark or in dim light, the recovery of actinomycins from both types of layer became almost quantitative.

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TABLE I

SEPARATION OF ACTINOMYCINS C AND F

Layer	*Solvent*	*Distance* travelled cm*	R_F						
			C-group	C_1	C_2	C_3	*F-group*	F_1	F_2
Alumina	Ethyl acetate-*sym*-tetrachloroethane–water (3:1:3, v/v, bottom layer)	12.5	—	0.44	0.51	0.58	—	0.21	0.35
	Ethyl acetate–di-*n*-butyl ether–water (3:1:3, v/v, top layer)	15	—	0.40	0.46	0.53	—	0.23	0.29
	Ethyl acetate–di-*n*-butyl ether–water (2:1:2, v/v, top layer)	17	—	0.28	0.30	0.33	—	0.10	0.13
Silica gel	Benzene–ethyl acetate–methanol (10:2.5:1, v/v)	15	0.24	—	—	—	0.13	—	—
	Benzene–ethyl acetate–methanol (6:4:1, v/v)	16	0.43	—	—	—	0.33	—	—
	Butan-1-ol–methanol–water (6:1:3, v/v)	14.5	0.63	—	—	—	0.53	—	—
	Butan-1-ol–acetic acid–water (10:1:3, v/v)	15	0.70	—	—	—	0.50	—	—
	Ethyl acetate–propan-2-ol–water (5:2:1, v/v)	15.5	0.95	—	—	—	0.75	—	—

* Migration time ranged from 30 to 60 min.

The thin-layer chromatographic characterization of some oxidation products of vitamin E

The chemistry of vitamin E, *dl*-α-tocopherol, is of increasing interest in view of its important role in metabolism and the intensified research on its metabolic products. Methods for the separation and identification of the tocopherols, their metabolites, and oxidation products in the past have largely been confined to column and coated-paper chromatography. An interesting review covering these earlier methods and some more recent data on the use of thin-layer chromatography with the isomeric tocopherols was presented by KOFLER *et al.*[1]. The petroleum jelly, silicone or liquid paraffin impregnated paper chromatographic systems that have been used for the tocopherols and their oxidation products are time-consuming to use, and are difficult to prepare so that uniform results are obtainable.

In recent years, the various isomeric tocopherols or their acetates have been characterized by SEHER[2], DÁVIDEK AND BLATTNÁ[3] and BOLLIGER[4], using silica gel or alumina thin-layer chromatography. The latter workers also used secondary magnesium phosphate thin-layer plates.

In connection with our studies on the oxidation of the tocopherols, we have found the use of silica gel thin-layer chromatography as indispensable for separation and establishment of purity of the various oxidation products produced. Table I summarizes the results of the use of silica gel G thin-layer chromatography for the separation of *dl*-α-tocopherol and some of its oxidation products and derivatives. Optimum separation of these compounds was obtained using cyclohexane–chloroform (2:1) as

TABLE I

SILICA GEL THIN-LAYER CHROMATOGRAPHY OF *d,l*-α-TOCOPHEROL AND ITS OXIDATION PRODUCTS

dl-α-Tocopherol derivative	*Relative mobility (R_F × 100)*			*Detection sprays*			
	Benzene	*Chloroform*	*Cyclohexane–Chloroform (2:1)*	*A*	*B*	*C*	*D*
Tocopheryl quinone[a]	01–07	17–25	01–06	blue	tan	—	blue
Toco Red[b]	01–02	14–16	02–04	blue	tan	orange	blue
Tocopherol[c]	37–43	80–87	23–29	blue	yellow	pink	blue
Keto ether dimer[d]	59–63	96–98	40–45	blue	grey	pink	blue
Compound B[d]	80–90	96–99	59–65	blue	brown	red	blue
Compound A[d]	92–96	96–99	78–85	blue	brown	red	blue
Tocopheryl acetate[c]	41–54	91–96	40–51	blue[f]	yellow	yellow	blue
Toco Purple[e]	01–06	15–27	01–06	blue	tan	—	blue
Dihydroxy dimer[d]	05–08	70–75	03–07	blue	grey	tan	blue

A: 5% aqueous K_3FeCN_6 followed by 5% aqueous $FeCl_3$.
B: 60% H_2SO_4 with heating to 150°
C: 20% $SbCl_5$ in $CHCl_3$.
D: 10% ammonium molybdate in 10% H_2SO_4 with heating to 150°.

[a] Prepared by $FeCl_3$ oxidation of *dl*-α-tocopherol[5].
[b] Prepared by HNO_3 oxidation of *dl*-α-tocopherol[6].
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[e] Prepared by $FeCl_3$ oxidation of *dl*-α-tocopherol[7].
[f] After 10 min.

solvent. Results are presented on the use of four spray reagents (5% aqueous potassium ferricyanide followed by 5% aqueous ferric chloride, 60% sulfuric acid with heating to 150°, 20% antimony pentasulfide in chloroform, and 10% ammonium molybdate in 10% sulfuric acid with heating to 150°). The most useful spray reagent for these compounds was the Turnbull Blue reagent (K_3FeCN_6–$FeCl_3$).

Experimental

The thin-layer plates (8 in. × 8 in.) were prepared from "Silica-Gel G. acc. to Stahl" (Brinkmann Instruments Co., Great Neck, Long Island, N.Y.) by mixing 30 g of the dry powder with 60 ml of distilled water and applying to the glass plates with a 250-micron spreader. After drying in the air the plates were baked in an oven for one hour at 110°.

All chromatograms were prepared by the ascending method at 20° in a solvent-saturated atmosphere. The compounds (2 γ from ethanol solution) were placed 2 cm from the bottom of the thin layer which was submerged in solvent to a depth of 3–5 mm and the solvent allowed to run a distance of 15 cm.

Purity of the oxidation products utilized in this study was established by comparison of their infrared spectra and thin-layer chromatographic behavior with that of authentic samples.

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An improved method for collecting gas-chromatographically separated estrogens*

In recent years, the gas chromatographic separation of synthetic mixtures of estrogens as well as their isolation from biological material have been reported[1–6]. The further characterization and verification of the fractions obtained, in many instances, would be extremely helpful.

A technique, employed in our laboratory, for collecting the effluent fractions at the exit port of the gas chromatograph has facilitated the collection of the fractions, representing the individual estrogens, for further characterization.

Experimental

The estrogens are separated employing a Barber-Colman model 10 gas chromatograph equipped with a ^{90}Sr ionization detector[3,6].

The effluent fractions, representing the individual compounds, are collected at

TABLE I

RECOVERY EFFICIENCY OF COLLECTION METHOD FOLLOWING INJECTION OF STANDARD

Injected (μg)	*Collected (μg)*	*Collected (%)*
	17β-Estradiol	
3	2.7	91
3	2.5	83
3	2.7	91
6	5.0	83
6	5.5	91
6	4.6	76
	Average	86
	Estrone	
3	2.5	83
3	2.7	90
3	2.4	85
6	4.8	80
6	5.6	93
6	5.2	87
	Average	86
	Estriol	
5	4.7	94
5	4.6	91
5	4.7	94
10	8.7	87
10	8.4	84
10	9.1	91
	Average	90

* This investigation was supported in part by a research grant from The Heart Association of Southeastern Pennsylvania and the American Medical Association Education and Research Foundation.

the exit port of the gas chromatograph by heating the exit line with an electric heating coil. Temperature of the line is controlled employing a 7.5 A variable transformer (powerstat). The fraction is collected by immersing the exit line (a 2.5 cm area not covered by the heating coil) directly into a 7 cm long, conical bottom pyrex glass tube containing 1.0 ml of redistilled dioxan. Each collection tube was cooled in a refrigerator at about 7°C for 10–15 min before collecting the samples. The resulting dioxan solution is then used to further characterize the compounds.

Result and discussion

In Table I may be observed the recovery efficiency of the collecting method following the injection into the gas chromatograph of a synthetic mixture of estrone, 17β-estradiol and estriol. The recoveries are based on the observed maxima upon determining the ultraviolet absorption spectra of the compounds prior to and following passage through the gas chromatograph.

Previously published methods of collection have employed electrical precipitation[7], Volman traps[8], or traps packed with defatted cotton, glass wool, glass beads, or silica. None of these methods were found to be adaptable to the microgram concentrations of estrogen employed in our experiments or known to be present in biological material.

Employing the method described in this communication, will provide a means of collecting gas chromatographically separated fractions for further characterization.

The efficiency of the method permits recovery of the fractions, after passage through the column, in sufficient concentration so that microgram quantities of material may be conveniently handled. It may be possible to adapt this method to the collection of compounds other than estrogens.

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A micro-fraction collector for gas chromatography*,**

A number of fraction collectors are available commercially for the collection of gas chromatographic peak compounds. However, none of them is suitable for the collection of micro-amounts of fractions of a complicated gas chromatogram. The present non-automatic fraction collector is designed for samples such as flavor compounds isolated from foods. These compounds usually can only be obtained in micro-amounts and yield gas chromatograms of imperfect resolution with more than a dozen peaks.

Construction of the fraction collector

The main portion of the present fraction collector (Fig. 1) consists of one distributor (A) and three manifolds (B). They are constructed of borosilicate glass capillary tubings (6 mm O.D., 1 mm I.D.) and 1 mm bore three way stopcocks (Kontes Glass Company, Vineland, N.J.). The distributor directs the effluent gas from the chromatograph, according to the position of the stopcock (C), either to one of the three manifolds or to an exit port. The latter can be used to eliminate the effluent gas when no peak is indicated by the recorder. Each manifold consists of five stopcocks which can be arranged to direct the effluent gas to any one of the six collection ports. The three manifolds are connected to the distributor through S/J 12/1 joints. The 16 stopcocks are so arranged that the effluent gas can be directed to one collection port or to the exit port by turning only one stopcock.

The distributor and the three manifolds are each imbedded between two aluminum blocks. The upper block (D) is 1 in. in thickness and the lower one (E), 2 in. Channels, 0.1 in. larger in diameter than the capillary tubing, are engraved into each of the aluminum blocks. The clearance between the capillary tubing and the channel is to prevent breakage of the borosilicate glass tubing due to its difference in thermal expansion coefficient from that of aluminum. A well, $^5/_8$ in. diameter and $12^1/_2$ in. long, is drilled into the center of each lower aluminum block. An immersion heater (F) (Blue M Electric Co., No. TH 3012, Blue Island, Ill.) is fitted into each well. The distributor and the three manifolds are independently maintained at constant temperatures by connecting one variable transformer to each immersion heater. A thermocouple is inserted into each of the aluminum blocks at a position close to the capillary tubing. The thermocouples are connected to a pyrometer (G) through a five position rotary switch (H).

Connection of the fraction collector to the gas chromatograph

The distributor is extended to 4 in. from the sample exhaust port of the gas chromatograph through a piece of borosilicate glass capillary tubing with S/J 12/1 joints on both ends. This tubing is connected to the sample exhaust tube of the gas chromatograph through a piece of stainless steel tubing ($^1/_8$ in. O.D., $^1/_{25}$ in. I.D.). The latter is silver soldered on one end to a stainless steel S/J 12/1 joint which fits to the borosilicate capillary tubing. On the other end it has a stainless steel Swagelock fitting which connects to the sample exhaust tube of the gas chromatograph. In order to maintain these

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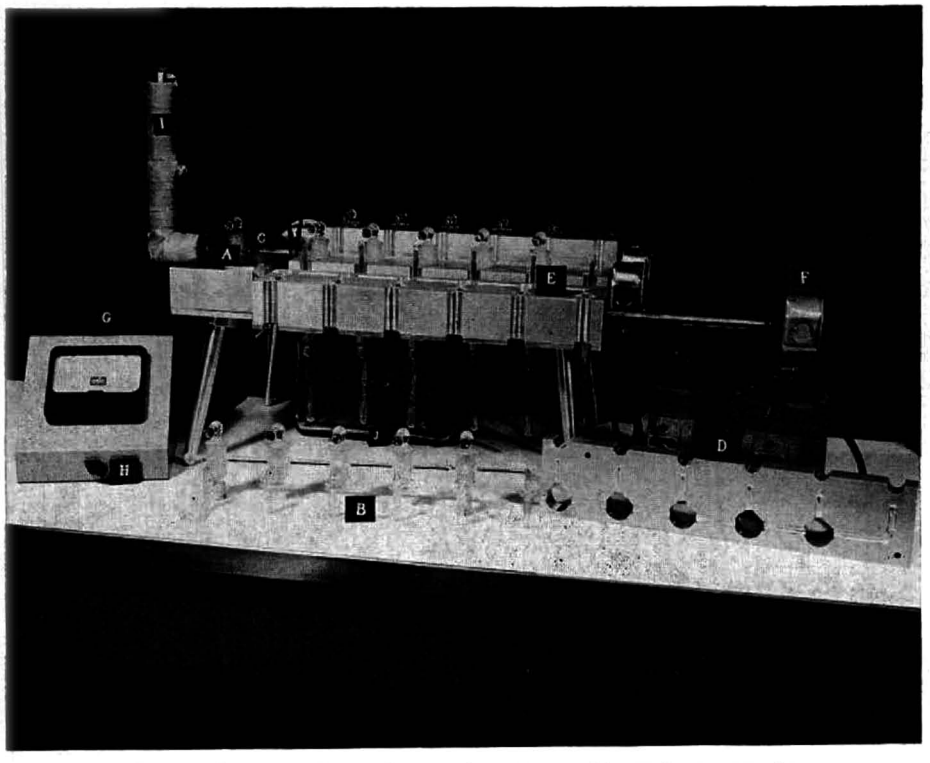

Fig. 1. Fraction collector for gas chromatographic peak compounds.

connecting tubings at a required constant temperature, the capillary glass tubing is imbedded in the center of an aluminum rod (1 in. diameter), which is split into two halves with channels engraved in each of them (I). The aluminum rod is wrapped with a Briskeat heating tape ($^1/_2$ in. wide, 4 ft. long). A thermal couple is inserted into the rod at a position close to the borosilicate glass capillary tubing. This thermocouple is connected to the pyrometer through one position of the rotary switch (H). The stainless steel tubing is inserted into an aluminum tubing ($^1/_4$ in. O.D., $^1/_8$ in. I.D.) which is wrapped with a 10 in. piece of Briskeat heating tape.

Construction of the cold traps

Each of the peak compounds is collected in a cold trap connected to one of the exit ports of the manifolds through a S/T 10/30 borosilicate glass joint. The traps used are the same as that described by CHANG *et al.*[1] except that borosilicate glass capillary tubing (3 mm O.D.), bent into U shape, and restrictions drawn out at every 2 cm, is used to replace the polyethylene capillary tubing.

Performance of the fraction collector

The present fraction collector can be used to trap 18 peak compounds eluted from a gas chromatograph without loss of resolution. A mixture of acetone and isoamyl alcohol, was chromatographed with a Carbowax 1000 column at 130°, and a mixture

of caproic, caprylic and capric methyl esters was chromatographed with a DEGS column at 175°, both with a flow rate of 70 ml/min. Each of the peak compounds from six runs of chromatograph was accumulatively collected in one trap. The recoveries for the five compounds were 95, 100, 94, 90 and 100 % respectively. Each of the peak compounds thus collected was rechromatographed. Their chromatograms indicated that they were as pure as when they were collected with individual traps directly connected to the sample exhaust tube of the gas chromatograph.

The collector does not create measurable back pressure to the chromatographic column even when the flow rate is as high as 150 ml/min. When the flow rate is 60 ml/min, the time lag for the carrier gas to reach the trap farthest from the detector is $^1/_3$ sec. For compounds of a higher boiling point which have a tendency to form "fog", the temperature of the coolant in bath (J) for the cold traps should not be lower than necessary for condensing the compound.

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The effect of ultraviolet irradiation on chlorpromazine*

I. Aerobic condition

Introduction

The picture of the metabolic end-products of chlorpromazine in psychotic patients has become clearer in recent years following a number of reports on the isolation and identification of the metabolites. Glucuronidation was found to be the major mechanism of the metabolism of this drug. A number of studies[1–4] concerning the excretion of non-polar metabolites, namely, unchanged chlorpromazine (CP) and its sulfoxides (CPO), have been reported. Chlorpromazine sulfoxide was found to be 1–18 % and unchanged drug was reported to be less than 1 % of the administered dose[2]. The ratio of the unchanged drug to its sulfoxide was approximately 1:16. The CPO was identified to be a mixture of chlorpromazine sulfoxide and two demethylated products, namely Nor_1-CPO and Nor_2-CPO in which Nor_2-CPO predominates. The same findings have been reported by FISHMAN AND GOLDENBERG[5]. In the report[6] on a quantitative analysis of four groups of urinary metabolites in psychotic patients, chlorpromazine glucuronides (CPGL) were found to be the major metabolites. An average of 44.6 % of CPGL was found in urine followed by CPO (7.7 %), $CPOH_1$**

* This study was supported by the grant MY-5785, National Institute of Mental Health, U.S. Public Health Service. The grant was administered by Friends of Psychiatric Research, Inc.

** $CPOH_1$ = Hydroxyl chlorpromazine (urinary metabolite, R_F 0.57, reacted blue with 50 % H_2SO_4, purple with 5 % $FeCl_3$, I.R.: 2.70–2.85 μ)[6].

(4.8 %) and CP (1 %). The chlorpromazine glucuronides were further fractionated into 9 fractions and their physical properties were described[7].

In the quantitative analysis of urinary chlorpromazine metabolites it was observed that when samples were exposed to room light over a period of 72 h, a new compound (R_F 0.89) appeared on the chromatogram. It is speculated that a small amount of ultraviolet (U.V.) emission produced by the fluorescent lamps resulted in the oxidation of some of the metabolites.

A nitrite and dinitrophenylhydrazine derivative of a substance from the U.V.-irradiated CP solution were reported[8,9]. However, it appears that the compounds produced by the U.V.-oxidation are mixtures and not a single compound. Photosensitization[10,11] and photo-allergy were reported after the administration of chlorpromazine. It is speculated that the photo-oxidation products might be responsible for these conditions. Therefore, it will be of interest to pursue the photo-oxidation study of chlorpromazine.

Experimental

An aqueous solution of chlorpromazine·HCl was placed in a porcelain evaporating dish and irradiated with U.V.-light (Mineralight R-51, 70 W) for a period of 24, 48 and 72 h. After the irradiation, changes in the color as well as pH (from 5.9 to 1.72) of the solution were noted. The solution was applied in a 1 cm band on 30 × 35 cm Whatman 3MM paper. The chromatogram was developed in ascending manner in a neutral solvent system, *n*-butanol–ethanol–water (5:2:2), overnight. The dried chromatogram was sprayed with 50 % sulfuric acid. Twelve compounds with R_F values ranging from 0.10 to 0.92, and color reactions ranging from pink–blue–purple were observed (Table I). Several compounds reacted blue to purple with 10% ferric chloride solution. Differences in the intensity of fluorescence among the chromatograms of these compounds were observed.

The developed unsprayed chromatograms were cut and separated into 12 fractions using a strip of acid-sprayed chromatogram as a guide. The accumulated paper strips of each fraction were extracted with 50 % ethanol. The ethanol extract of each fraction was evaporated in a Buchler flash-evaporator below 50°. The residue of each fraction was rechromatographed twice as described above. Picrates were prepared from the purified fraction by adding an equal quantity of saturated picric acid in ethanol into the sample solution (in ethanol). The precipitate was collected on a filter paper and recrystallized from methanol. The melting point, R_F value, color reactions with 50 % sulfuric acid and 10 % ferric chloride reagent, ultraviolet absorption peaks (in a Bausch & Lomb "Spectronic 505") and infrared spectra (in a Perkin-Elmer Model 237) of each fraction were obtained.

Results and discussion

Table I shows the physical properties of the 12 compounds isolated from the mixture of ultraviolet irradiated solution of chlorpromazine·HCl. The unchanged chlorpromazine was recovered from the top of chromatograms. The fraction 1 was identified to be chlorpromazine-N-oxide (Table I) from its color reactions, mixed melting point of the picrate with an authentic specimen (no depression, 174–177°) and R_F on cochromatograms (0.92). The electrophoretic pattern of this fraction was found to be the same with that of reference chlorpromazine-N-oxide. The fraction 2 showed a purple

TABLE I

ULTRAVIOLET IRRADIATED PRODUCTS OF CHLORPROMAZINE·HCl

Fractions	*Reference material*[a]	*Color on paper*	*Color under U.V.-lamp*	*Color reactions*		*R_F (pure fraction)*	*M. p. (°C)*[b] *(picrate)*	*U.V.-absorption peak (mμ)*
				50% H_2SO_4	*10% $FeCl_3$*			
1		No color	Violet	Pink	Pink	0.92	173–175	258, 308
	CPNO	No color	Violet	Pink	Pink	0.92	175–177	257, 308
2		No color	Yellow	Purple	Brownish-purple	0.84	120–122	266, 340
3		No color	Reddish-purple	Red	NR[c]	0.79	207–210	240, 278, 300, 342
	CPO	No color	Reddish-purple	Red	NR	0.80	208–210	240, 278, 300, 343
4		No color	Grey	Orange	NR	0.76		262, 283, 310, 362
5		Yellow	Light blue	Purple	Brownish-purple	0.71	76–80 (base)	262, 324
6		Grey	Sky blue	Lavender[d]	Bluish-green	0.63	127–132	260, 321
7		Green	Blue	Blue	Yellowish-brown	0.56	153–158	226, 260
8		Red	Pinkish-purple	Bluish-green	NR	0.49	78–80	240, 272, 333
9		Yellow	Light blue	Bluish-purple	Purple	0.42	92–93	260, 310
10		Tan	Yellowish-brown	Purple	Purple	0.33	140–143	258, 310
11		No color	Purple	Purple	NR	0.30	120–122	259
12		Light brown	Orange	Purple	NR	0.08	152–156	258

[a] CPNO = chlorpromazine-N-oxide; CPO = chlorpromazine sulfoxide.
[b] Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.
[c] NR = no color reaction.
[d] Color changed to blue in the presence of excess 50% H_2SO_4.

color reaction with ferric chloride reagent, suggesting a ring oxidation in this compound, I.R.: 2.8 μ (hydroxyl). A urinary chlorpromazine metabolite ($CPOH_2$)* from patients was found to have similar physical properties with this compound[6], except ultraviolet absorption peaks. The fraction 3 was identified to be chlorpromazine sulfoxide from its color reaction, R_F value, melting point of the picrate, and ultraviolet and infrared absorption spectra. Mixed melting point of the picrate with an authentic specimen did not show depression (208–212°). The electrophoretic pattern of the fraction 3 was identical with that of reference chlorpromazine sulfoxide. The nature of the other fractions is currently under investigation. About 20–25% of the starting material was recovered from the 24 h irradiated solution. As the duration of irradiation time was increased to 48–72 h, the intensity of chromatograms of the fractions 6, 7, 8, 9 and 10 increased while that of the fractions 3, 4 and 5 decreased. At the end of 72 h, only a trace of the starting material was found on chromatograms. Ninhydrin test and nitroprusside test were negative in all fractions, indicating that no demethylated derivative was formed under these conditions. This experiment was also carried out under anaerobic conditions. The results of this experiment will be reported later.

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* $CPOH_2$ = Hydroxyl chlorpromazine (urinary metabolite, R_F 0.83, reacted purple with 50% H_2SO_4, brownish-purple with 10% $FeCl_3$, I.R.: 2.8 μ).

A spot drier for paper chromatograms*

The repeated application of samples to paper chromatograms is a tedious and time-consuming operation, especially when numerous samples are to be examined. Techniques in which infrared lamps or hot air driers are used to reduce the drying time of the sample create an uncomfortable situation for the worker. The spot drier described in this note is easy to build and will reduce spotting time to about one-quarter of that required by other methods.

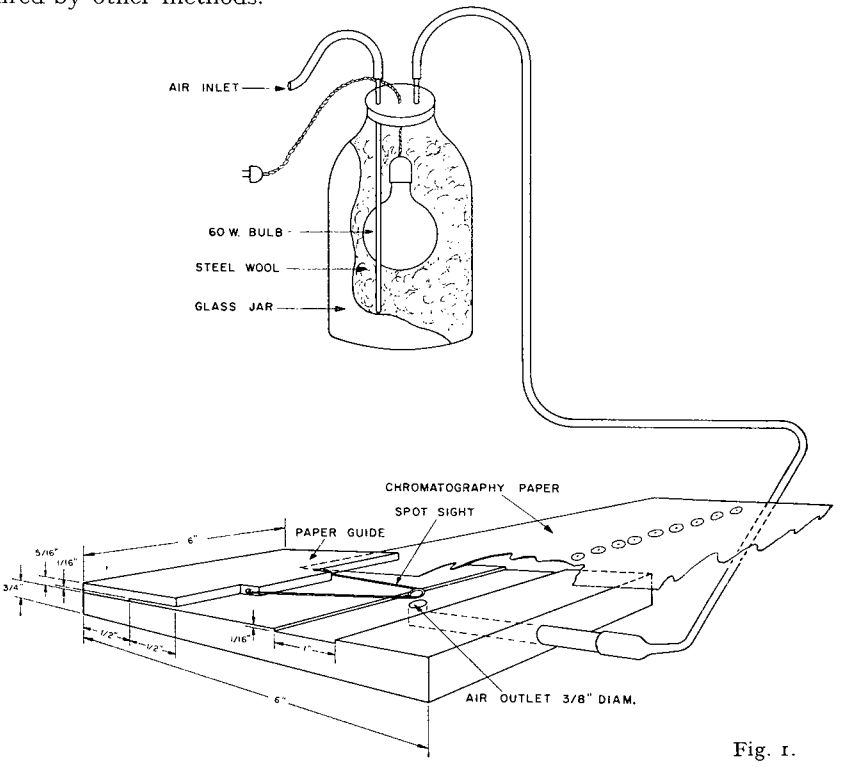

Fig. 1.

The drier is made of lucite plastic and its construction is self-explanatory from the figure shown. The distance from the air outlet to the inside edge of the paper guide (spotting distance from the edge of the paper) is not given and can be varied to suit the needs of the researcher. Since the chromatogram paper covers most of the drier when in use, a wire sight locates the spot on the paper immediately over the warm air outlet. Nitrogen can be used instead of air when spotting compounds which are sensitive to oxidation. A 60 W bulb fitted inside a glass jar loosely filled with steel wool makes a convenient air heater.

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The use of acetate sheets as records for chromatographic patterns

Taxonomists are often faced with marking and analysing chromatographic patterns of large samples of taxa. If sheets, 46 × 57 cm are used, the recording of the position of compounds, their fluorescence and color under U.V. light and their color changes with different chromogenic sprays is often cumbersome, if done on separate sheets.

The recording upon the original chromatogram is not advisable, since these can repeatedly be exposed to different vapor phases for color change, and can then be subjected to a chromogenic spray to find further color reactions. If, in such a case, the position of compounds on the original chromatogram has been marked, the previously drawn lines may suggest compounds that are not in fact visible.

My work of recording chromatographic patterns was greatly facilitated by superimposing a thin sheet of clear acetate (Grumbacher Tuffilm 196–150) upon the original chromatogram. With wax pencil or china marker the exact outline of the compounds can be recorded on the acetate and their coloration noted. This can also be done in a vapor chamber or under U.V. light. The original can again be subjected to NH_3 or other vapors, U.V. light and finally be sprayed, without being cluttered with notations, and without shadow spots produced by finger marks. In two-dimensional chromatography the exact position and relation of the compounds to each other in the pattern to be recorded is greatly facilitated. Since the application point and front line are also noted on the acetate sheet, several of these can be superimposed and deviating patterns immediately recognized. The acetate is inexpensive, resistant to many vapors, easily stored and can be used as a permanent record.

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"Crossing" paper electrophoresis for the detection of complexes of enzymes with their inhibitors

The inhibition of lysozyme activity occurs in the presence of high molecular weight acidic polymers. It was found that the following substances effectively inhibited lysozyme activity: heparin[1–3], ribo-nucleic acid, deoxyribonucleic acid, hyaluronic acid, pneumococcus polysaccharide, Vi antigen, glutamyl polypeptide[4]. The inhibition was of the competitive type. The lysozyme–trypsine complex was isolated[5] and the precipitation and aggregate formation of deoxyribonucleic acid with lysozyme was examined[6].

A similar type of inhibition can be observed with ribonucleic acid, which is the inhibitor of deoxyribonuclease[7,8]. The reaction between these inhibitors belonging to the group of macromolecular acid compounds and lysozyme or deoxyribonuclease has an electrostatic character. In our previous work, we have demonstrated with the help of "crossing" electrophoresis that inhibitors of macromolecular acidic polymer nature

inhibit the activity of ribonuclease by forming stable enzyme–inhibitor complexes[9]. The principle of "crossing" electrophoresis is that by applying two substances as two lines that form an angle, the substances can be made to cross each other during paper electrophoresis. If the substances possess chemical affinity, then an addition product is formed at the crossing point[10].

The aim of this work was the investigation of complexes of lysozyme with its inhibitors and a complex of deoxyribonuclease with ribonucleic acid by "crossing" paper electrophoresis.

Materials and methods

The substances employed were: (a) lysozyme (Mann Research Laboratories Inc., New York), (b) crystalline deoxyribonuclease (Mann Research Laboratories Inc., New York), (c) yeast ribonucleic acid (BDH, England), (d) deoxyribonucleic acid (Light, England) and (e) heparin (Polfa, Poland).

The substances to be tested were applied to the filter paper as lines drawn obliquely to each other. The paper used was Whatman No. 1, 12 × 40 cm or 18 × 40 cm. Electrophoresis was carried out for 20 to 60 min, at 700 V with acetate buffer, pH 4.0, and ionic strength 0.05, 0.1 or 0.25. Lysozyme and deoxyribonuclease were stained with bromphenol blue[11], ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) with Victoria Blue B[12] and heparin with toluidine[13] or Victoria Blue. In addition the nucleic acids were examined under a U.V. lamp.

Results

The enzymes migrate at pH 4.0 towards the cathode, while the inhibitors examined migrate towards the anode. Fig. 1 shows an electrophoregram on which lysozyme has been obliquely applied to the lines of RNA, DNA or heparin. During the electrophoresis as a result of the formation of an enzyme–inhibitor complex, the direction of the

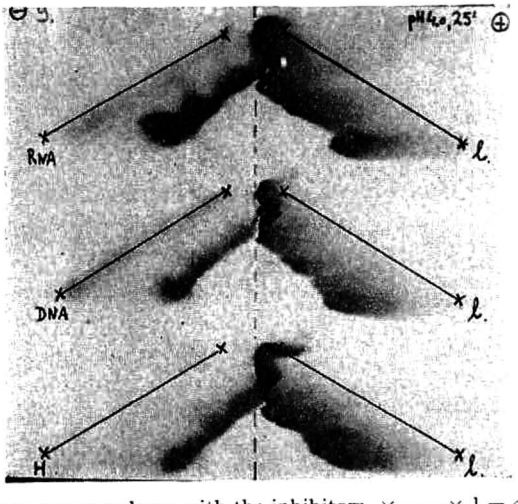

Fig. 1. Detection of lysozyme complexes with the inhibitors. ×——× l = 20 µl/8 cm of 2% lysozyme; ×——× RNA = 20 µl/8 cm of 10% ribonucleic acid; ×——× DNA = 20 µl/8 cm of deoxyribonucleic acid; ×——× H = 20 µl/8 cm of 2.5% heparin; — — — — = the middle of the paper strip. Stained with bromophenol blue and Victoria Blue.

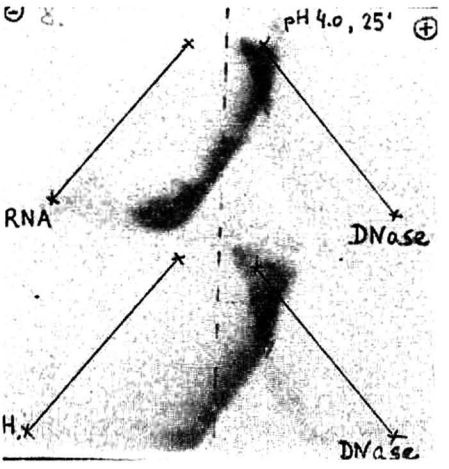

Fig. 2. Detection of deoxyribonuclease complexes with ribonucleic acid and heparin. ×——× DNase = 15 μl/6 cm of 2% deoxyribonuclease; ×——× RNA = 15 μl/6 cm of 10% ribonucleic acid; ×——× H = 15 μl/6 cm of 2.5% heparin; — — — — = the middle of the paper strip. Stained with bromophenol blue and Victoria Blue.

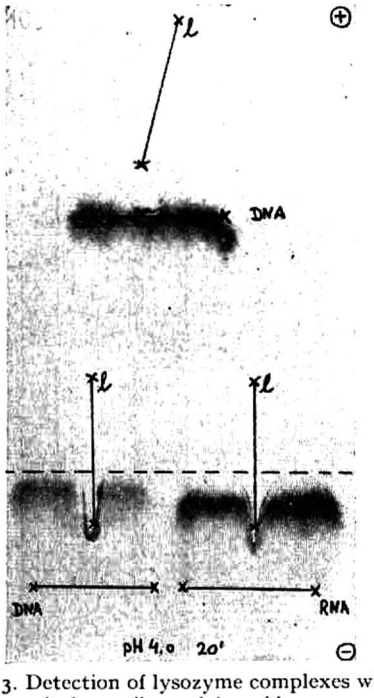

Fig. 3. Detection of lysozyme complexes with ribo- and deoxyribonucleic acids. ×——× l = 12.5 μl/5 cm of 2% lysozyme; ×——× RNA = 10 μl/4 cm of 10% ribonucleic acid; ×——× DNA = 10 μl/4 cm of 10% deoxyribonucleic acid; — — — — = the middle of the paper strip. Stained with Victoria Blue.

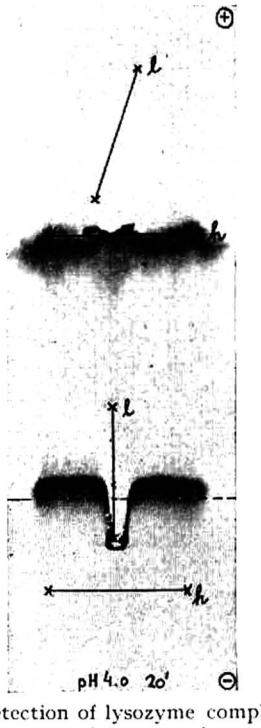

Fig. 4. Detection of lysozyme complex with heparin. ×——× l = 12.5 μl/5 cm of 2% lysozyme; ×——× h = 12.5 μl/5 cm of 2.5% heparin; — — — — = the middle of the paper strip. Stained with toluidine blue.

lines changes at the crossing point. These complexes can be seen as dark-staining bands in the places where lysozyme has come in contact with its inhibitors.

In the experiments with deoxyribonuclease and RNA or heparin a distinct change of direction of the substances is visible at the crossing point (Fig. 2).

A different picture of the complexes is obtained when lysozyme is applied perpendicularly or at an angle to the inhibitors (Figs. 3 and 4). Application nearer to the anode results in the formation of a visible complex where the enzyme and inhibitor meet. Application of the substances in the cathode field, however, in addition to the complex formation, leads to considerable distortion of the RNA, DNA or heparin lines.

The formation of complexes and bending of the lines is caused by a reaction of an electrostatic nature between the enzyme, of which the pH is in the alkaline range, and the macro-acidic polymers. Evidence of this is the complete crossing without any mutual interaction of two macro-acidic polymers.

The influence of the time of electrophoresis and the ionic strength of the buffer was also examined. Neither prolonging of the time even to 60 minutes nor change in the ionic strength had any effect on the stability of the complex, once formed. It should be remembered that the complexes obtained are sometimes visible even before the staining of the electrophoregrams. After the strips are dried, a yellowish band corresponding to the complex can be seen at the crossing point of the two substances. This can also be seen under ultraviolet light.

The results of the above experiments indicate that in inhibition of the competitive type between polyanions and the substrate of the enzyme, stable enzyme–inhibitor complexes can be obtained by means of "crossing" electrophoresis.

This method also enabled us to obtain positive results in experiments on the action of antibiotics[14].

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Rapid electrophoresis of RNA nucleotides in a dilute agar gel paper medium*

Much interest has been shown in the electrophoretic separation and subsequent quantitation of nucleotides in biological systems[1–4]. An attempt was made by GORDON AND REICHARD[5], to separate nucleotides in concentrated agar gel. However, this medium was found to be unsuitable[6] and most of the subsequent investigations used paper as the anticonvection medium. Several existing zone electrophoresis techniques appear to have been used successfully[7–9]. The EDSTRÖM procedure, which used alkali treated copper silk fibers as the anticonvection medium, required an application of 1800–6000V to the fiber[10]. Acid soluble nucleotides were separated by KLOUWEN using high voltage paper electrophoresis[11]. Others have used equally arduous techniques[12]. A need still exists for a simple system which can yield rapid well resolved separation of nucleotides and which possesses both quantitative and qualitative characteristics. In the present study agar gel overlaying vegetable parchment paper was the medium in which the electrophoresis took place[13,14]. Yeast ribonucleic acid nucleotides were used as a model for investigating the following:

1. The rapid electrophoretic separation of nucleotides in an open unequilibrated system.

2. The elution of the resolved and dried nucleotides for their subsequent qualitative elucidation by differential spectrophotometry.

3. The quantitative determination of separated nucleotides by ultraviolet scanning.

Materials and methods

Electrophoresis was carried out in agar gel overlaying vegetable parchment paper on a $3^1/_4$ in. $\times$ 4 in. lantern slide as previously described[13,14]. The gel was prepared by dissolving 300 mg of Ionagar No. 2 in acetate buffer of pH 3.1 and approximate ionic strength of 0.045. The buffer was made by diluting a stock buffer 1:4 where the stock was prepared by titrating 0.18 moles of sodium acetate to pH 3.1 with glacial acetic acid. 10 mg of each nucleotide were dissolved in 1 ml of distilled water, or shaken to form a saturated solution for those nucleotides which did not dissolve completely. Small strips of filter paper were impregnated with the nucleotides, blotted free of excess material by touching them to filter paper and then placed on the agar gel parallel to the 4 in. axis about 1 in. from the cathode. Application of 250 V for 20–30 min caused a rapid movement of the nucleotides toward the anode. The distance each moved was observed by holding a short wave U.V. light over the plate. In the case of mixtures, the electrophoresis was discontinued when clear separation of the components was observed, and this always occurred during the 20–30 min period. When separation was completed, the gel was quickly dried with an infrared lamp. The spots were penciled after ultraviolet visualization, cut out and eluted with 3 ml of 0.05 *N* NaOH. These solutions were then scanned across the ultraviolet spectrum with an automatic recording spectrophotometer.

Results and discussion

Fig. 1 shows an experiment which delineates the electrophoretic separation of RNA

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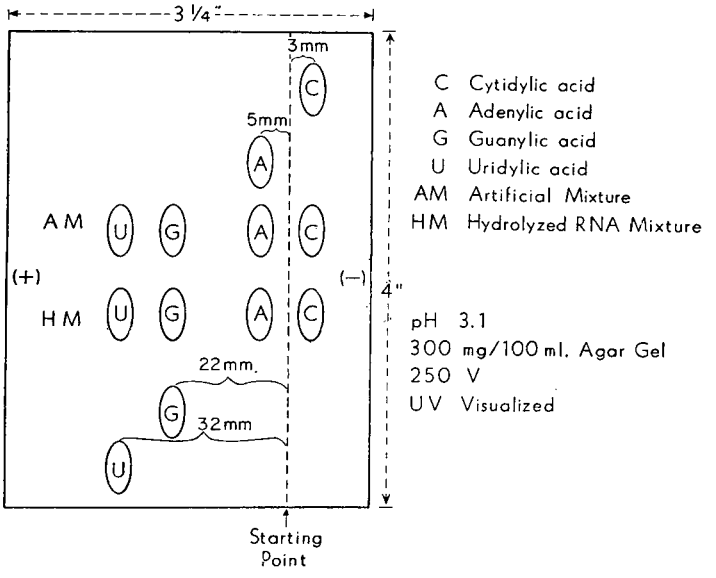

Fig. 1. Schematic representation of mobilities and separations of RNA nucleotides.

nucleotides in the pure state from artificially controlled mixtures and from natural mixtures obtained by mild hydrolysis of yeast ribonucleic acid. The upper and lower single components moved with exactly the same mobility as when they were present in the mixtures shown in the two center separations in the electropherograms. The ratios of the mobilities U:U, G:U, A:U and C:U were 1.00, 0.67, 0.15 and 0.08 respectively, as uridylic acid moved approximately 32 mm, guanylic acid 22 mm, adenylic acid 5 mm and cytidylic acid 3 mm. These ratios were the same for the

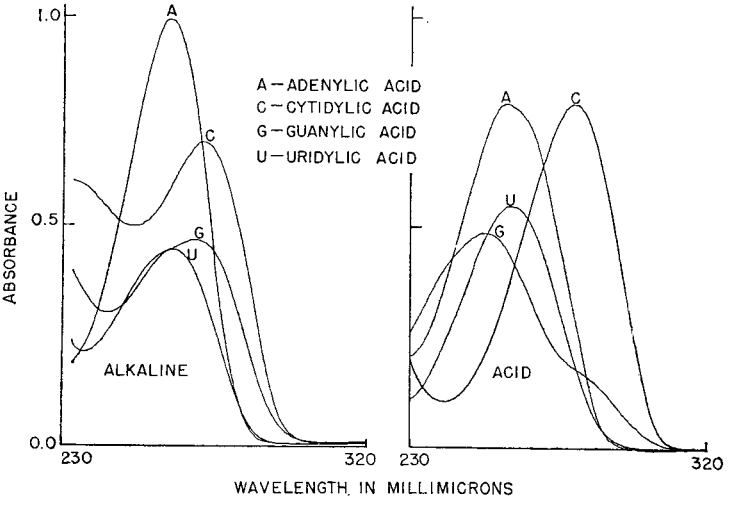

Fig. 2. Spectra of RNA nucleotides dissolved in alkali and acid.

artificially contrived mixtures, the hydrolysate as well as the individually moved components. This experiment showed a lack of influence on the mobility of any one nucleotide because of the presence of the other three nucleotides. When purified agarose was substituted for Ionagar No. 2, the endosmotic decrease caused the slowest nucleotide to remain at the starting line with equal diffusion to both sides of the line. This was not as clear cut a result as when the nucleotide moved behind the line and away from the filter paper site of application.

Fig. 2 shows the ultraviolet spectra obtained for the nucleotides when one of two

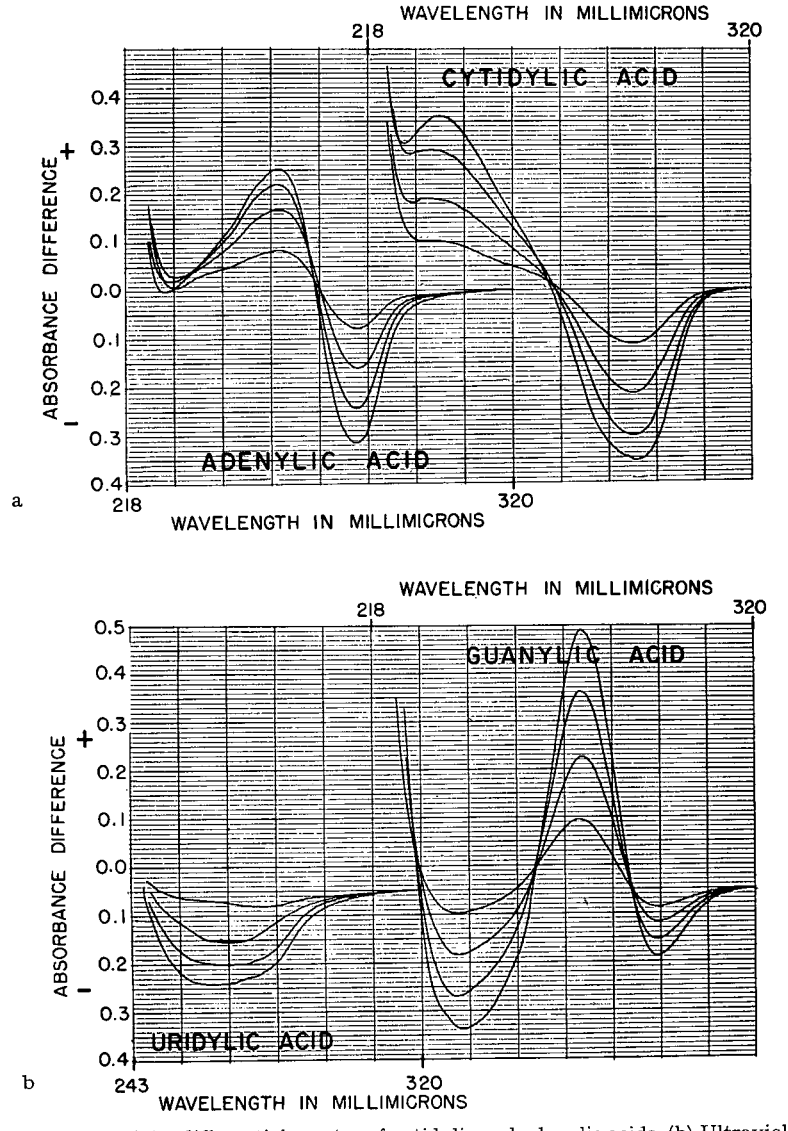

Fig. 3. (a) Ultraviolet differential spectra of cytidylic and adenylic acids. (b) Ultraviolet differential spectra of guanylic and uridylic acids.

aliquots of the same nucleotide was acidified with hydrochloric acid and the other alkalinized with sodium hydroxide. Samples prepared in this manner could be scanned simultaneously through the ultraviolet range by placement of one aliquot in the reference compartment and a second aliquot in the sample compartment of a double beam spectrophotometer. Fig. 3a and 3b show the distinct qualitative differences one can obtain with this automatic differential spectroscopy technique[15]. Ordinary ultraviolet readings at a fixed wavelength thus serve as a quantitative measure of the nucleotide being considered, while relative electrophoretic mobility and differential spectrophotometry can serve as a double checking qualitative system. The isosbestic points which are different for the several nucleotides can be seen as those wavelengths at which the differential spectra are isoabsorptive and cross the zero absorbance lines of Fig. 3a and 3b. These points may also be useful in the identification process. There is some acetate left in the parchment paper from which the elution of the resolved electropherogram of nucleotides was carried out. This did not appear to distort the spectra when compared to solution in the NaOH alone, although the peaks may shift several millimicrons. However, scanning of the entire spectra with an automatic recording instrument avoided any error due to bathochromic or hypsochromic shifting. Hyperchromic and hypochromic effects with small pH differences seem to be negligible.

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A rapid paper chromatographic method for separation and identification of soybean sapogenols*

The biological effects of soybean saponins have been studied in this laboratory**. In order to differentiate between the various soybean saponins, a simple and rapid method was required for the separation and identification of the sapogenols of which these saponins are composed.

The aglucone moiety of soybean saponins is known to consist of four sapogenols, designated A, B, C, and D, respectively (OCHIAI *et al.*[1] and MEYER *et al.*[2]). The procedures described by these authors are, however, intended for a preparative separation of the sapogenols and have been found to be too lengthy for analytical purposes. DIECKERT *et al.*[3], using glass-paper chromatography for establishing the relationship between groundnut sapogenols and soybean sapogenols, achieved a satisfactory separation between the soybean sapogenols only when the paper was impregnated with silicic acid.

Soybean saponins were prepared from ether-extracted soybean meal by a method developed in this laboratory[4]. The saponins were subjected to acid hydrolysis in ethanolic hydrochloric acid, as described by DJERASSI *et al.*[5]. The resulting sapogenols were precipitated by adding water to the hydrolysate. The precipitate was dissolved in a small amount of chloroform and chromatographed on Whatman No. 1 and No. 3MM papers by ascending and descending techniques. Several solvent systems were examined and it was found that the water-free system of petrol ether–chloroform–acetic acid, recommended by SANNIÉ *et al.*[6] for the separation of steroid sapogenols, provided an excellent medium also for the separation of the triterpenoid soybean sapogenols. Preliminary experiments showed that the most suitable ratio of the solvents is petrol ether–chloroform–acetic acid (100 : 10 : 2.5) and that better resolution can be achieved with Whatman No. 3MM than with Whatman No. 1 paper. After the completion of the chromatographic run the papers were dried in a hood and then developed with a saturated solution of $SbCl_3$ in chloroform, as described by COULSON[7]. Although in some chromatograms five distinct spots could be identified, in most cases the differentiation was difficult because of considerable "tailing" of the spots.

This major obstacle was overcome by introducing the horizontal, circular chromatography technique. Whatman No. 3MM papers, 20 cm × 20 cm, were spotted with 5 λ samples 1.5 cm from the center and 2 cm apart, and a paper wick was introduced into the center of the paper. The latter was put above an open petri dish (15 cm diameter) leaning on the rim with the wick immersed in the solvent (25 ml) and then covered with the other part of the dish. When the solvent reached the rim of the petri dish, the paper was taken out and treated as described above. As can be seen in Fig. 1, the tailing effect was completely eliminated and satisfactory separation into five distinct zones could be achieved. The R_F values of the hydrolysate constituents were compared to those of the chemically defined soybean sapogenols A, B, C and D***.

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*** Kindly donated by Prof. O. JEGER of The Federal Institute of Technology, Zürich.

When a mixture of sapogenols C and D was chromatographed some overlapping was observed even when the minimal amount of sapogenol (4–5 μg/spot) necessary to produce a distinct colour reaction with $SbCl_3$ was used. This difficulty was overcome by substituting hexane (b.p. 68–70°) for petrol ether in the solvent system. As shown

Fig. 1. Circular paper chromatography of soybean-sapogenols A, B, C, D and of the acid hydrolysate of soybean saponins. Paper: Whatman No. 3MM. Solvent: hexane–chloroform–acetic acid (100:10:2.5). Developing reagent: $SbCl_3$ in chloroform.

in Fig. 1, the latter modification of the solvent brought about a very clear separation between the different sapogenols. It should be pointed out that this separation was obtainable only in the range of 5–20 μg/spot. The chromatogram of the soybean saponin hydrolysate contains an additional zone (R_F 0.76), which stains green with $SbCl_3$, but is not detectable when the paper is dipped in a mixture of acetic anhydride–sulfuric acid (1:1). The chemical nature of this compound has not yet been established.

The R_F values of chromatograms performed in the two solvent mixtures are summarized in Table I. It is obvious from Table I that in the solvent which contains hexane, the difference between the R_F values of sapogenols C and D is greater, whereas the difference between the R_F values of sapogenols A and B does not change appreciably. Very good agreement was found between the R_F values of the components of the hydrolysate, and those of sapogenols A, B, C and D.

The use of circular paper chromatography enables separation between different sapogenols after a very short distance of migration of the solvent (6 cm), which is completed within 10 minutes. The high volatility of the solvent makes possible complete drying in 5 min; including the staining process, the chromatography of the soybean sapogenols can be accomplished in less than one hour.

TABLE I

R_F VALUES OF SOYBEAN SAPOGENOLS AND OF ACID HYDROLYSATES OF SOYBEAN SAPONINS

Compound	*R_F values*		*Colour with $SbCl_3$*
	Petrol ether–chloroform–acetic acid	*Hexane–chloroform–acetic acid*	
Soya-sapogenol A	0.32	0.27	brown
Soya-sapogenol B	0.50	0.47	violet
Soya-sapogenol C	0.71	0.86	violet
Soya-sapogenol D	0.67	0.73	violet
Zone I hydrolysate	0.32	0.26	brown
Zone II hydrolysate	0.50	0.47	violet
Zone III hydrolysate	0.65	0.73	violet
Zone IV hydrolysate	0.67	0.76	green
Zone V hydrolysate	0.70	0.85	violet

Since soybean sapogenols are frequently found in the saponins of other legumes[3,8,9], it is hoped that the described procedure may also be applicable in their analyses.

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The detection of amides on paper chromatograms

No convenient test appears to have been described for the detection of amides on paper chromatograms. In the case of an amide being ninhydrin-negative the only method of detection is the general test for N–H-containing compounds based on the formation of N-chloro-derivatives[1–3]. SOLOWAY AND LIPSCHITZ[4] described a colorimetric test for amides and nitriles, based on their reaction with hydroxylamine to give hydroxamic acids and amidoximes respectively, followed by the formation of coloured ferric complexes. It was found that this test could be modified for use as a spray reagent for the detection of amides on paper chromatograms. The method is as follows.

Reagents. Saturated solution of hydroxylamine hydrochloride in methanol and ferric chloride hexahydrate (1 %) in ethanol.

Procedure. The paper was sprayed with hydroxylamine reagent with simultaneous drying of the reagent on the paper using a hot air drier. In order to apply a thick even coating of the reagent the paper was covered twice in this fashion. It was then hung in an oven at 100° for 20 min. Finally the paper was sprayed very lightly with ferric chloride reagent, when a positive test was the immediate production of a red colour stable over several hours.

SOLOWAY AND LIPSCHITZ[4] recommended the use of a solvent with a high boiling point, propylene glycol, since the reaction of amides with hydroxylamine proceeded better at elevated temperatures. In the adaptation to paper chromatograms it was found that the non-volatile solvent led to extensive diffusion of the spots with greatly lowered sensitivity. Furthermore the reaction proceeded well when the reactants were in the solid state. Hydroxylamine was therefore applied to the paper in a more volatile solvent which was evaporated as rapidly as possible after application.

As little as 50 μg of asparagine, glutamine, nicotinamide and isobutyramide could be detected after one-dimensional chromatography using this procedure; 30 mg amide was used in the colorimetric procedure[4]. In view of the wide applicability of the colorimetric test to amides, the procedure described here should also have wide applicability for non-volatile amides. For amides with melting points in the region of 100° or less, the reaction can be carried out at a lower temperature with heating for correspondingly longer periods.

The ureides allantoin and citrulline gave negative tests, while urea reacted anomalously giving a blue colour which faded rapidly. Several organic acids and amino acids gave weak positive reactions, so that the test can only be employed when standard markers are also chromatographed. The cyanoglucosides linamarin and lotaustralin (which contain a nitrile group) also reacted positively, but required longer heating (30 min) for optimum colour production.

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Separation and identification of pyridoxal and pyridoxal-5-phosphate by paper chromatography

SILIPRANDI, SILIPRANDI AND LIS studied the electrophoretic separation of vitamin B_6 compounds[1] and data on the paper chromatography of these compounds has been given by other workers[2–5]. On the whole, however, the paper chromatographic separation of these compounds has, so far, been little investigated, possibly because the methods for locating them on the paper are either insensitive or complicated. The classical method is biological and depends on their ability to promote growth of certain micro-organisms[2]. Another detection method depends on reaction with the phosphate group in pyridoxal-5-phosphate and pyridoxamin-phosphate. All vitamin B_6 compounds can be developed by diazotization as well. These methods cannot be used for the demonstration of less than μg amounts of these agents. When present on the paper in high concentrations (5–10 μg) all B_6 compounds give a spontaneous fluorescence in the U.V. region after chromatography in certain solvent systems.

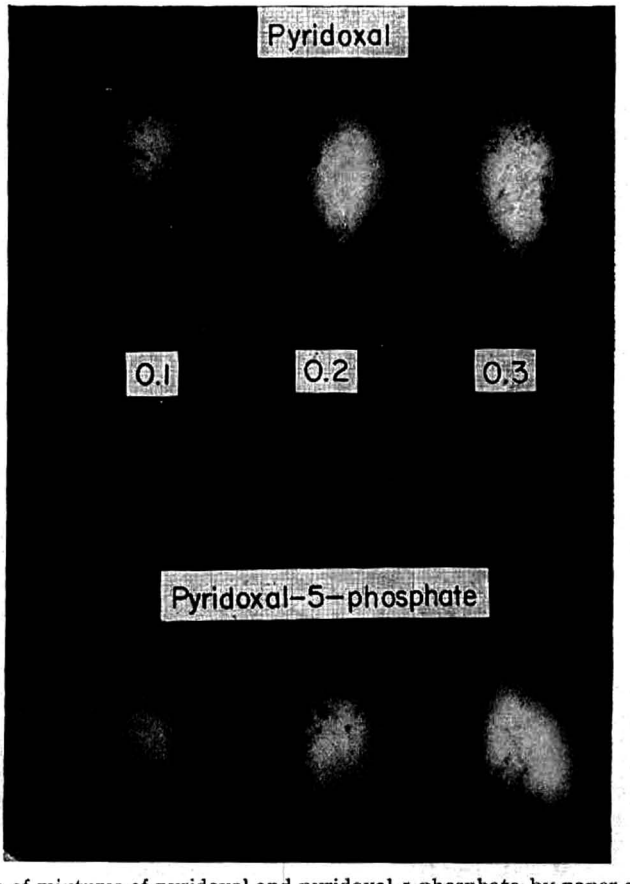

Fig. 1. Separation of mixtures of pyridoxal and pyridoxal-5-phosphate by paper chromatography in *n*-butanol saturated with water for 8 h. The amount of each compound is given in μg. The photograph was taken in U.V. light using Gevaert Scopix G film, with an alkaline solution of picric acid serving as a yellow filter, the exposure time was 40 sec.

TABLE I

R_F VALUES AND FLUORESCENCE REACTIONS OF VITAMIN B_6 COMPOUNDS

*Compounds**	*R_F values in solvents***:*					*Comments*
	I	*II*	*III*	*IV*	*V*	
Pyridoxine	0.48	0.55	0.42	0.75	0.41	weak fluorescence after chromatography in solvents II, III and V.
Pyridoxamine	0.09	0.10	0.30	0.42	0.27	blueish fluorescence after chromatography in solvents II and V.
Pyridoxal	0.78 0.42	0.58	0.39	0.76	0.40	strong greenish fluorescence after chromatography and subsequent treatment with semicarbazide.
Pyridoxic acid	0.61	0.50	0.65	0.73	0.74	blueish fluorescence after chromatography in solvents II, IV and V.
Pyridoxal-5-phosphate	0.70	0.13	0.02	0.63	0	strong greenish fluorescence after chromatography and subsequent treatment with semicarbazide.

* Pyridoxine hydrochloride and pyridoxamine dihydrochloride were furnished by Hoffmann-La Roche, Basel. Pyridoxal-5-phosphate and 4-pyridoxic acid were obtained from L. Light & Co., England. Pyridoxal hydrochloride was delivered by Sigma, U.S.A.

** Solvents: I = *n*-Butanol saturated with *N* hydrochloric acid. II = *n*-Butanol saturated with water. III = *n*-Butanol saturated with *N* ammonia. IV = Methanol–butanol–benzene–water (2:1:1:1). V = Isopropanol–ammonia–water (20:1:2). Chromatography was performed descending in solvent I, ascending in all the other solvents.

The intensity and colour of the fluorescence varies markedly with the solvent system used as seen in Table I.

It was observed in this laboratory that the reaction of semicarbazide with pyridoxal and pyridoxal-5-phosphate resulted in products which gave intense greenish fluorescence under test tube conditions and also on a paper chromatogram. The former reaction has been developed into a sensitive analytical method for the measurement of very small amounts of pyridoxal-5-phosphate. The details of this method will be given elsewhere.

The compounds were applied on filter papers (Whatman No. 1) in amounts ranging from 0.05–1 μg in the case of pyridoxal and pyridoxal-5-phosphate and 1–5 μg of other vitamin B_6 metabolites. Chromatography was performed in different solvents in the dark for approximately 16 h at room temperature. The paper chromatograms were dried at about 65°. When sprayed with a solution of 0.1 *M* semicarbazide in a mixture of 0.1 *M* tris buffer, pH 9, and ethanol (2:3), the spots containing pyridoxal and pyridoxal-5-phosphate emitted an intense greenish fluorescence in U.V. light (Sterisol UV-lamp, Original Hanau, applied with a filter absorbing visible light, Jena Farb- und Filter-glas, UG 1). The fluorescence was found to be stable for weeks. Less than 0.1 μg of each compound could be detected. The sensitivity of the reaction is illustrated in Fig. 1. The R_F values and the details of the spontaneous fluorescence reactions in different solvents are given in Table I.

The routine procedure when viewing the paper chromatograms was as follows:

(1) Observation of spontaneous U.V. fluorescence.

(2) Semicarbazide treatment and registration of spots containing pyridoxal and pyridoxal-5-phosphate.

(3) Diazotization by the Pauly reagent.

The semicarbazide reaction seems to be a simple and sensitive method for the identification of pyridoxal and pyridoxal-5-phosphate on paper chromatograms. Adaptations of this technique for the study of biological material is in progress.

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Eine papierchromatographische Trennung der isomeren Dijodbenzole und ihr aktivierungsanalytischer Nachweis

Nach der Uranspaltung in Gegenwart organischer Verbindungen wird ein Teil des Spaltjods in homöopolarer Bindung vorgefunden. So liegen beispielsweise bei der Uranspaltung in Gegenwart von Benzol mehr als 50 % des in die organische Phase übergetretenen Radiojod in Form von Jodbenzol vor[1]. Darüber hinaus war früher schon gezeigt worden, dass bei einer speziellen Auswahl der Fängerverbindung sogar eine Positionsmarkierung mit den Spaltfragmenten möglich ist[2,3].

Um nun besonders im Falle der Jod-Markierung mittels Kernspaltung die Möglichkeiten einer Positionsmarkierung näher untersuchen zu können, musste eine Trennung von Jodbenzol und den isomeren *o*-, *p*-, und *m*-Dijodbenzolen durchgeführt werden.

Gaschromatographisch ist diese Trennung mit einer Golay-Kapillarsäule bei 150° ohne besondere Schwierigkeiten möglich. Die Reaktionsprodukte der Spaltmarkierung auf diese Weise zu analysieren schien uns jedoch nicht ratsam. HERR und Mitarbeiter haben nämlich unter den Bedingungen der Gaschromatographie einen raschen Isotopenaustausch gefunden[4]. Da im Gemisch der organischen Reaktionsprodukte der Spaltmarkierung elementares und wahrscheinlich auch ionogenes Radiojod in trägerfreier Form enthalten ist, war hier ebenfalls mit einem Isotopenaustausch zu rechnen. Die Folge wäre ein Fehler bei der Ausbeutebestimmung der markierten Verbindungen gewesen.

Aus diesem Grunde wurde von uns eine bei Normalbedingungen verlaufende, einfache, papierchromatographische Trennung der drei Isomeren und Jodbenzol ausgearbeitet. Da die Verbindungen ausgesprochen lipophiler Natur sind arbeiteten wir nach den bekannten Regeln mit Umkehrung der Phasen und verwendeten dazu vollacetyliertes Papier. Als Laufmittel erwies sich auch hier die schon von WIELAND UND KRACHT[5] zur Trennung isomerer, mehrkerniger Aromaten angewandte Mischung von Methanol, Aceton in Wasser (4:4:1) als sehr gut geeignet.

Der Mikronachweis der Verbindungen gelang dagegen nicht mit bekannten chemischen Methoden. Auch das sonst sehr empfindliche Verfahren mit Cer(IV)-Sulfat und arseniger Säure[6], mit dem in Vergleichsversuchen andere Jodverbindungen wie Dijodthyrosin oder NH_4J noch in Mengen von 10^{-8} g sichtbar gemacht werden konnten, versagte bei den Dijodbenzolen, vermutlich wegen einer zu geringen Dissoziation der Moleküle. Wir wendeten deshalb zum Nachweis die Neutronenaktivierungsanalyse an. Aber auch hierbei gab es anfangs Schwierigkeiten. Chromatographiepapier enthält nämlich, auch wenn es vorher mit reinen Lösungsmitteln gewaschen worden ist, immer noch einen so hohen Chlor- und Natriumgehalt, dass neben der Aktivität dieser Elemente die Jodaktivität nicht direkt gemessen werden kann[7]. Auch in den Halbwertszeiten sind ^{38}Cl und ^{128}J so wenig unterschiedlich, dass hiermit keine Unterscheidung möglich ist, (^{38}Cl: 37 Min.; ^{128}J: 25 Min.). Wir analysierten deshalb die neutronenaktivierten Chromatogramme mit einem Einkanal-Impulshöhenanalysator der selektiv auf die 0.45 MeV-γ-Strahlung des ^{128}J eingestellt war.

(Die γ-Energien von ^{38}Cl betragen 2.15 und 1.6 MeV, von ^{24}Na 1.38 und 2.75 MeV.)

Fig. 1 zeigt einen unter diesen Bedingungen erhaltenen Messstreifen. Die so ermittelten R_F-Werte betragen für:

m-Dijodbenzol:	0.39;
p-Dijodbenzol:	0.32;
o-Dijodbenzol:	0.20;
Monojodbenzol	0.26.

Experimentelle Ausführung

Je 20 μg der isomeren Dijodbenzole werden in Form einer 1%igen methanolischen Lösung auf vollacetyliertes Chromatographiepapier (Fa. Schleicher und Schüll, Acetylgehalt 40–45%) aufgetragen und mit einem Gemisch von Methanol–Aceton–

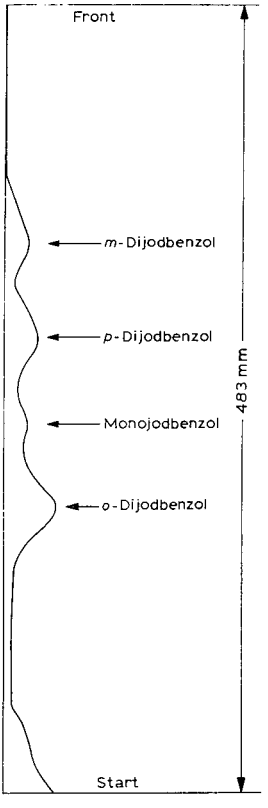

Fig. 1. Papierchromatographische Trennung der isomeren Dijodbenzole, absteigend, auf vollacetyliertem Papier. Lösungsmittelgemisch Methanol–Aceton–Wasser (4:4:1). Aufgegebene Substanzmengen je 20 μg. Laufzeit 2 Std. 15 Min.

Wasser (4:4:1) als Fliessmittel absteigend entwickelt. Nach 2.25 Std. ist die Lösungsmittelfront ca. 50 cm gewandert und die Trennung kann beendet werden. Nach Markieren der Lösungsmittelfront und einem kurzen, unvollständigen Trocknen mit Warmluft wird das feuchte Papier in der Laufrichtung in Streifen geschnitten, die man

sofort in Polyäthylenhüllen einschweisst. Auf diese Weise lässt sich ein allmähliches Verflüchtigen der organischen Mikromengen verhindern und das Chromatogramm ist ausserdem gegen Verunreinigungen geschützt, die durch die Neutronenaktivierung mit einer zu hohen Radioaktivität den Jodnachweis stören könnten. Die eingeschweissten Streifen werden dann zusammengerollt und 10 Min. lang mit Neutronen aktiviert. Der thermische Neutronenfluss betrug bei uns $5 \cdot 10^{12}$n/cm^{2}sec. Unmittelbar nach der Aktivierung — die Halbwertszeit des ^{128}J beträgt nur 25 Min. — wird das Chromatogramm ausgemessen. Vorher klebt man noch eine Standardaktivität ausserhalb der Laufstrecke der Verbindungen auf den Chromatographiestreifen, um einen Bezugspunkt für die Lokalisierung der gesuchten Aktivitätsmaxima der Jodflecken zu haben. Am einfachsten verwendet man dazu einen dünnen, jodgetränkten Filterpapierstreifen, der zusammen mit dem Chromatogramm aktiviert worden ist.

Der so vorbereitete Streifen wird dann in eine neue Polyäthylen-Schutzhülle eingelegt und an das Papier des Aktivitätsschreibers angeheftet. Mit der Vorschubgeschwindigkeit der Schreibers (bei uns war es 1 cm/Min.) bewegt sich dann der Chromatographiestreifen vor einer Bleiblende mit schlitzförmiger Öffnung vorbei, hinter der sich ein Szintillationsdetektor zum Nachweis der γ-Strahlung befindet. Der Detektor ist an einen Einkanal-Impulshöhenanalysator (Fa. Telefunken) angeschlossen, der auf die 0.45 MeV-γ-Linie des ^{128}J eingestellt ist. Dadurch erfolgt eine weitgehend selektive Aktivitätsmessung des Radiojod. Der Zählratenmesser des Impulshöhenanalysators ist mit dem Schreiber verbunden, der den Vorschub des Chromatographiestreifens steuert. Auf diese Weise wird die Verteilung der Jodaktivität auf dem Chromatogramm im Massstab 1:1 auf dem Schreiberpapier wiedergegeben und die Lage der Jodverbindungen kann durch einen Streckenvergleich sofort angegeben werden.

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The chromatographic separation of diastereoisomeric dipeptides

Diastereoisomeric dipeptides and their derivatives have variable physical properties and therefore can be sometimes separated by crystallisation[1–4], countercurrent distribution[5], ion-exchange[6], or gas chromatography[7]. Some authors report a difference of the paper chromatographic R_F values for diastereoisomeric peptides[2,8].

The present work reports a satisfactory separation of some diastereoisomeric dipeptides by ascending paper chromatography on Whatman paper No. 1.

The simplest dipeptide containing two asymmetric carbon atoms can exist in four isomeric modifications: L-L, D-D, L-D, D-L. Several dipeptides of known configuration were obtained from L- and/or D-amino acids (see Table I). The values of R_F for the L-L peptides were identical with those observed for the D-D compounds (since L-L is the mirror image of D-D) and differed markedly from those of the L-D and D-L isomers.

The best separation of diastereoisomers was obtained with the following basic solvent systems:

TABLE I

THE R_F VALUES OF DIASTEREOISOMERS OF DIPEPTIDES IN RELATION TO THEIR CONFIGURATION

Solvents: S_1 = Ethyl acetate–pyridine–acetic acid–water (5:5:1:3).
S_2 = Pyridine–water (4:1).
Paper: Whatman No. 1 (ascending), length 30 cm.
Detection: Ninhydrin.

Dipeptides	*Configuration of component amino acids*									
	R_F *in* S_1					R_F *in* S_2				
	DL-DL	L-L	D-D	L-D	D-L	DL-DL	L-L	D-D	L-D	D-L
Ala-Ala	do not separate					0.44 0.37	0.44			
Ala-But	0.53		0.53			0.48		0.48		
	0.47				0.47	0.43				0.43
Ala-Leu	0.79	0.79				0.60	0.60			
	0.73					0.54				
Ala-Phe	0.69	0.69				0.72	0.71			
	0.62				0.62	0.55				0.56
Ala-Val	0.60	0.60				0.73	0.73			
	0.54				0.54	0.50				0.51
But-Ala	0.58					0.68				
	0.51				0.50	0.51				0.51
But-But	0.68					0.73				
	0.59				0.59	0.51				0.51
Glu-Asp	0.29	0.28								
	0.23									
Glu-Glu	0.41	0.40								
	0.34									
Phe-Ala	0.70	0.70				0.73				
	0.58			0.58		0.60			0.60	
Phe-Val	0.80	0.80				0.83	0.82			
	0.70					0.69				
Val-Leu	0.80	0.80				0.83	0.82			
	0.70					0.71				
Val-Phe	0.86	0.86				0.80	0.80			
	0.78					0.71				
Val-Val	0.83	0.83				0.80	0.80			
	0.72					0.71				

S_1: Pyridine–ethyl acetate–acetic acid–water (5:5:1:3)

S_2: Pyridine–water (4:1)

The differences of the R_F coefficients (ΔR_F) in these systems had the values 0.05–0.23.

The dipeptides synthesised from two racemic amino acids (a mixture of four diastereoisomers: L–L, L-D, D-D, D-L) were separated in these systems and gave two non-overlapping spots which corresponded to two racemates (L-L/D-D and L-D/D-L). The upper spot of the mixture of these racemates had the R_F value corresponding to the L-L and D-D isomers, the lower spot to the L-D and D-L isomers.

Relative amounts of diastereoisomers can be quantitatively determined according to BOISSONNAS[9] with an accuracy of 2%.

There is a regular dependence of the R_F value of the dipeptides upon the relative configuration of the amino acids in all experiments:

$$R_F \text{ (L-L/D-D)} > R_F \text{ (L-D/D-L)}$$

Similar regularities are observed in the paper chromatography of hydroxy-amino acids with two asymmetric centres; *erythro*-isomers of phenylserine[10] or threonine[11] travel more quickly than the *threo*-isomers.

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Paper partition chromatography of mixtures of chloride, chlorite, chlorate and perchlorate

Chloride ion and several inorganic species containing chlorine and oxygen are capable of coexistence in the pH range 3–11, but also undergo interconversions, either by interaction with each other or with other inorganic or organic materials present in the aqueous solution[1–5]. The complexity of such reactions has made facile identification of the various species highly desirable. Detection of chlorine dioxide is no problem, since it is highly volatile and absorbs in the near ultraviolet[6]. A method for identifying the remaining (anionic) species was sought, however. Separation of chlorate from chlorite on paper chromatograms had been achieved by SERVIGNE[7] and by LAUB[8], but neither achieved a good separation of chloride; LAUB was unable to keep hypochlorite ion stable in the solvent system used by him, and in this regard we were also unsuccessful. The separation of chloride from chlorite achieved in the present work is of particular significance, since the heavy metal salts of both these ions have similar solubilities, making precipitation tests with compounds of these metals uncertain at best. At the same time, it has also been possible to improve the detection sprays used by the previous authors, both with regard to ease of handling and to sensitivity.

Procedure

Strips of Whatman No. 1 filter paper measuring 5 × 43 cm were spotted 4 cm from one end with various mixtures made from 1 % solutions of sodium chloride, sodium chlorite, sodium perchlorate and potassium chlorate and permitted to dry. At least 10 μg of each anion was applied. Each strip was then developed for about 16 h* in a "Chromatobox" (Model A-801, obtainable from the Emil Greiner Company, 20 N. Moore St., New York 13, N.Y.) with a 15:2:2:2 mixture of 2-propanol, water, pyridine and concentrated ammonium hydroxide, and dried at room temperature. The portion of the strip up to 11 cm from the starting line was sprayed with 0.2 N aqueous silver nitrate while the remainder of the strip was shielded from the spray; after drying, the strip was exposed for 2 min to an ultraviolet lamp, which caused a dark purple spot, due to chloride ion, to appear at R_F 0.25 $\pm$ 0.02 (center of spot). Tannish streaks due to hydroxides of sodium and potassium appeared at R_F 0.09–0.12; the presence of these hydroxides was evidently due to the separation of the salts originally applied into fast-moving anions and slow-moving cations, the latter acquiring hydroxyl from the solvent as their counter ion. The region from 11 cm to 24 cm was sprayed with a fresh mixture of equal parts of 3 M aqueous hydrochloric acid–acetone (1:1) and 5 % ethanolic diphenylamine; this revealed chlorite immediately as a blue spot, R_F 0.36 $\pm$ 0.02, and chlorate, after drying 2–3 min, as a green spot of R_F 0.54 $\pm$ 0.02. The remainder of the strip was sprayed with saturated aqueous sodium acetate, dried, and oversprayed with 0.2 % aqueous methylene blue to locate perchlorate as a violet spot of R_F 0.71 $\pm$ 0.02 against a light blue background. There was no difficulty in detecting as little as 10 μg of each anion.

Experiments of the type described above showed that hypochlorite ion, stable for a short time as a dried spot on filter paper, decomposes on exposure to the solvent

* Good chromatograms can be obtained in even 4 h, in which case the R_F values are 0.25, 0.34, 0.50 and 0.65 for the four ions.

mixture. Without chromatographing, the dried spot could be visualized as a brown spot by spraying with iodide buffered with carbonate–bicarbonate; under these conditions the other oxy-chloro anions are unreactive.

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Use of 2-(o-hydroxyphenyl)-benzoxazole in detection of phosphate esters and other ferric-complexing compounds on paper chromatograms

Spots of phosphate esters on paper chromatograms are usually detected by the method of HANES AND ISHERWOOD[1] or one of its modifications, involving hydrolysis to inorganic phosphate, reaction with molybdate and reduction to molybdenum blue. This is sensitive to about 1 nanomole (0.03 μg) of P but is relatively slow and inconvenient to use. WADE AND MORGAN[2] introduced a detection based on the masking of the color reaction between ferric ions and sulfosalicylic acid. This method is less specific and is sensitive only to 30 nanomoles of P, but it is fast and does not destroy the compounds being detected. We have found that the fluorescent compound, 2-(*o*-hydroxyphenyl)-benzoxazole, which reacts with many metallic ions[3], can be used instead of sulfosalicylic acid. The benzoxazole fluorescence reaction requires only very low reagent concentrations, it is somewhat more sensitive, and works well over a wide pH range (while the sulfosalicylic color requires a pH near 2).

Reagent PD-1A is a 0.54 % solution of $FeCl_3 \cdot 6\ H_2O$ in 80 % (v/v) ethanol and is stable for months. Before use, a 1:50 dilution in acetone (reagent PD-1B, approximately 0.0004 *M* ferric ion) is prepared. It is applied to the paper with a pipet, in quantity just sufficient to saturate it. The paper is allowed to dry for a few minutes, and then reagent PD-1C, a 0.05 % (0.0024 *M*) solution of 2-(*o*-hydroxyphenyl)-benzoxazole and 2 % (v/v) dimethyl sulfoxide in 2-butanone is applied with a pipet. This kind of application is more convenient than spraying or dipping and gives a more uniform deposit of reagents. When the paper is examined in long-wave ultraviolet light, spots of ferric-complexing compounds fluoresce yellow (if the paper is acidic) or

blue (if alkaline) on a dark background. The effect of the dimethyl sulfoxide seems to be to intensify weak spots by preventing adsorption of minute amounts of the benzoxazole on the paper (which quenches the fluorescence); it can be replaced by other slightly volatile solvents such as ethylene glycol but not by formamide. Sensitivity varies for various phosphate esters but is of the order of 10 nanomoles of P. If the concentration of benzoxazole is halved or that of ferric ion is doubled the fluorescence of both background and spots is reduced but there is no striking change in sensitivity.

The reaction also detects many other ferric-complexing compounds (such as citric or malic acids) in the 10–100 nanomole range. Heavy metals (such as copper, nickel and cobalt) and substances that absorb long-wave ultraviolet light strongly or quench the benzoxazole fluorescence (such as dinitrophenyl derivatives of amino acids) may show as black spots on the dark blue background. The lack of specificity makes the reaction useless with complex mixtures, and we have applied it primarily to characterize spots which have been through at least one purification step[4]. It is also useful in identifying traces of phosphate esters labeled with ^{14}C or ^{32}P; larger amounts of known phosphate esters are added and spots detected with the benzoxazole reagent, and if radioactivity coincides with one of the known spots in several chromatographic or ionophoretic separations the radioactive material is tentatively identified.

The very low reagent concentration in the benzoxazole reaction facilitates recovery of spots from paper. They can be cut out, washed with acetone to remove reagents, extracted in water and run in other solvent systems or subjected to microcolorimetric analysis for phosphate. It is also possible to apply other reagents, such as ninhydrin, to ferric–benzoxazole-treated chromatograms or ionograms without serious interference.

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Comportement de peptides de l'acide *meso*-α,α'-diaminopimélique au cours de leur chromatographie sur papier*

RHULAND *et al.*[1] avaient constaté que l'acide α,α'-diaminopimélique, après chromatographie sur papier dans un mélange méthanol–pyridine–acide chlorhydrique 10*N*–eau (80:10:2.5:17.5, v/v) se différencie des autres acides aminés de deux manières: d'une part, il donne, avec la ninhydrine, une coloration vert-olive qui vire avec le

* 4ème Communication sur les peptides de l'acide α,α'-diaminopimélique; 3ème communication, voir réf. 4. Ce texte a fait l'objet d'une Communication à la Réunion du Groupe d'Étude de la Chromatographie de la Société Chimique de France, le 31 mai 1963.

temps en jaune et, d'autre part, il se dédouble en deux taches distinctes, l'une correspondant à la forme DD et l'autre à son antipode LL, la forme *meso* ayant le même R_F que la forme DD.

On peut se demander si l'on retrouve chez les peptides de l'acide α,α'-diaminopimélique cette même particularité de l'acide libre au cours de leur chromatographie sur papier.

L'obtention des divers peptides de l'acide *meso*-α,α'-diaminopimélique (*meso*-DAP) soit par synthèse chimique[2], soit par l'action de peptidases sur des peptides synthétiques[3,4] nous a donné l'occasion d'étudier leur comportement chromatographique. Dans le Tableau I nous avons résumé les constatations concernant les colorations obtenues avec la ninhydrine pour un certain nombre de ces peptides. D'après ces résultats, l'apparition d'une coloration verte est caractéristique (i) de la présence sous forme non substituée des deux fonctions α-amine du DAP et (ii) de la présence d'au moins une fonction carboxyle libre, la seconde pouvant être substituée.

L'intérêt pratique de ces constatations est évident pour la caractérisation des peptides du *meso*-DAP que l'on obtient par hydrolyse acide partielle d'une chaîne peptidique provenant par exemple de la paroi bactérienne d'*Escherichia coli* et de *Mycobacterium tuberculosis*[5–7]: en effet, il suffit d'effectuer une chromatographie de ces peptides dans le mélange de RHULAND *et al.*[1] pour déceler, après coloration à la ninhydrine, la présence éventuelle, parmi eux, de peptides ayant en position N-terminale le *meso*-DAP, étant entendu que les deux fonctions amines sont libres et une des fonctions carboxyles impliquée en liaison peptidique.

Dans le Tableau II nous avons indiqué les valeurs des R_F après chromato-

TABLEAU I

COLORATION À LA NINHYDRINE APRÉS DÉVELOPPEMENT DANS LE MÉLANGE: MÉTHANOL–PYRIDINE–HCl 10*N*–EAU (80:10:2.5:17.5)

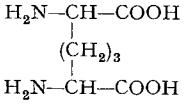
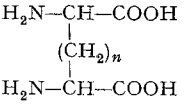
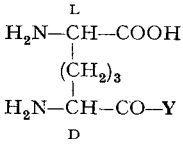
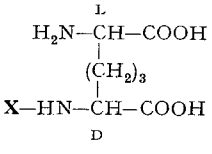
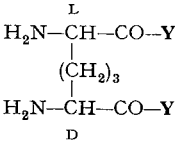
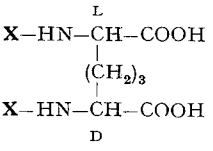
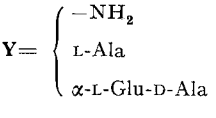
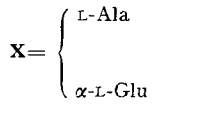
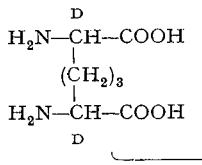
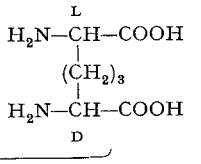
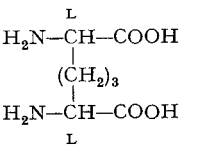
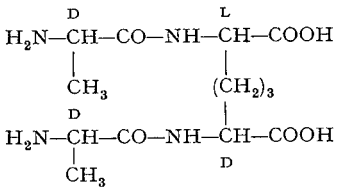
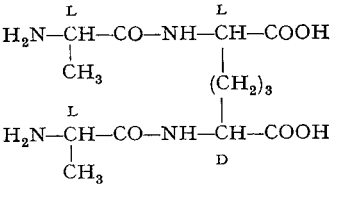
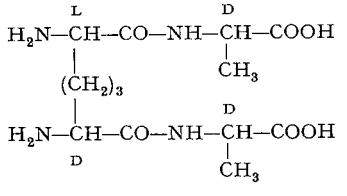
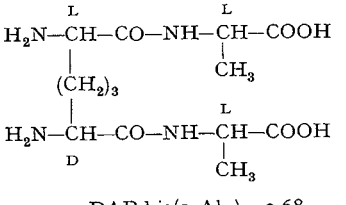
	vert-olive → jaune
	violette
	vert-olive ↓ jaune
	violette
	violette
	violette
	

TABLEAU II

VALEURS DES R_F DANS LE MÉLANGE RHULAND *et al.* (WHATMAN NO. 1)

		
DD-DAP et *meso*-DAP=0.24		LL-DAP=0.30
		
bis-(D-Ala)-*meso*-DAP=0.60		bis-(L-Ala)-*meso*-DAP=0.60
		
meso-DAP-bis-(D-Ala)=0.58		*meso*-DAP-bis(L-Ala)=0.68

graphie dans le mélange précédent de quatre peptides du *meso*-DAP et de la D- et L-alanine, deux à deux énantiomorphes; à titre de comparaison sont donnés aussi les R_F des trois formes du DAP libre. On constate que les deux peptides énantiomorphes ayant les deux fonctions amines du DAP libres migrent avec des vitesses différentes et que le peptide *meso*-DAP-bis-(L-alanine) possède un R_F plus grand que son antipode *meso*-DAP-bis-(D-alanine). Cette différence de R_F est comparable à celle que l'on observe entre les formes DD et LL du DAP libre.

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EVANGHÉLOS BRICAS
CLAUDE NICOT

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Reçu le 4 juin 1963

Chromatographic separation of amino acid isomers

Several naturally occurring amino acids possess two asymmetric centres and can exist in diastereomeric forms. We have succeeded in separating the *threo* and *erythro* isomers of a number of these compounds by paper chromatography. The solvent systems used also discriminated between the straight-chain and branched-chain forms of some aliphatic amino acids.

The most generally useful solvent investigated was the upper phase of *tert.*-amyl alcohol–acetic acid–water (20:1:20). Table I gives the conditions used for the separation of several groups of isomers together with their $R_{Leucine}$ values.

This method provided a poor alternative to paper electrophoresis[1] for resolving *threo* and *erythro* β-methyl aspartic acid, but was more successful in separating the geometrical forms of β-hydroxy aspartic acid. It also distinguished between the corresponding diastereoisomers of γ-methyl glutamic acid, which were separated by ion exchange chromatography on Dowex 1 X 8, acetate form. A column of this resin,

TABLE I

SEPARATION OF AMINO ACID ISOMERS ON PAPER USING THE UPPER PHASE OF FRESHLY PREPARED *tert.*-AMYL ALCOHOL–ACETIC ACID–WATER (20:1:20)

	$R_{Leucine}$ at 20°	*Time in days taken for separation of each group of isomers on Whatman 3 MM paper**
β-Methyl-L-aspartic acid (*threo* form)***	0.20	
β-Methyl-DL-aspartic acid (*erythro* form)***	0.23	20
β-Hydroxy-DL-aspartic acid (*threo* form)***	0.03	
β-Hydroxy-DL-aspartic acid (*erythro* form)***	0.06	12
γ-Methyl-DL-glutamic acid (natural form)	0.41	
Allo-γ-methyl-DL-glutamic acid	0.48	4
L-Isoleucine (*erythro* form)***	0.88	
D-Alloisoleucine (*threo* form)***	0.81	4
Cis-α-(methylcyclopropyl)glycine	0.56	
Trans-α-(methylcyclopropyl)glycine	0.67	3
4-Hydroxy-L-proline (*erythro* form)***	0.23	
Allo-4-hydroxy-D-proline (*threo* form)***	0.19	4
DL-Valine	0.49	
DL-Norvaline	0.66	1
L-Isoleucine	0.88	
L-Leucine	1.00	2
L-Norleucine	1.14	
Homoisoleucine** (2-Amino-4-methyl hexanoic acid)	1.39	
Homoleucine** (2-Amino-5-methyl hexanoic acid)	1.50	2
Homonorleucine** (2-Amino-heptanoic acid)	1.59	

* Samples (10 γ) of the compounds were applied to the origin of 44 cm long descending chromatograms as streaks 2 cm × 0.3 cm. The amino acids were finally revealed with ninhydrin.

** These identifications are only tentative. The compounds were obtained by catalytic hydrogenation of hypoglycin A[9].

*** The relationship between the two asymmetric centres, where this is known, is expressed by the use of the terms *threo* and *erythro* as proposed by VICKERY[10].

65 cm long and 1.2 cm in diameter almost completely resolved 750 mg of synthetic γ-methyl glutamic acid when eluted with 0.2 *N* acetic acid. The second isomer eluted from the column co-chromatographed with natural γ-methyl glutamic acid present in tulip leaf and peanut seedling extracts.

Separation of isoleucine and alloisoleucine, previously achieved with ion exchange resins[2], was also obtained by paper chromatography in *tert.*-amyl alcohol–acetic acid–water.

Our solvent clearly distinguished between the two geometrical forms of the ring compound α-(methylcyclopropyl)glycine[3], but was less successful when applied to the isomers of certain proline derivatives. It adequately resolved a mixture of 4-hydroxyproline and 4-allohydroxyproline but probably did this no more satisfactorily than the solvents described by FRIEDBERG[4] and BEYERMAN[5]. It did not distinguish at all between the *threo* and *erythro* forms of 4-methylproline. These latter isomers were, however, separated by paper chromatography, using water-saturated butan-1-ol in the presence of 3 % (w/v) ammonia, a system first described by CONSDEN *et al.*[6]. An acceptable result was obtained in 5 days under the conditions given in Table I (see footnote*), providing the solvent was equilibrated at 2–4° and the chromatogram was run at the same temperature. *Cis*-4-methylproline (the *threo* form) ran at $R_{Leucine}$ 0.61 and its diastereoisomer at $R_{Leucine}$ 0.57.

The original amyl alcohol-based system readily distinguished between the isomers of some aliphatic amino acids and has been used routinely in this laboratory to resolve valine and norvaline as well as leucine, isoleucine and norleucine. It may be superior to the other solvents already recommended for this purpose[7,8] partly because chromatograms, after treatment with ninhydrin, show relatively compact spots on a clean background. Finally, it satisfactorily separated the three higher homologues of these leucine isomers. Only a mixture of the relevant compounds was available and identifications given in the last section of Table I are tentative, being based on chromatographic position alone.

We are grateful to Dr. H. J. SALLACH, Dr. H. A. BARKER, Prof. G. W. KENNER and Prof. C. H. HASSALL for kindly providing us with authentic samples of some of the amino acid isomers used in this investigation.

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Received July 4th, 1963

Ion exchange chromatography of acidic amino acids by using a weakly basic anion exchange resin

A chromatographic procedure for the quantitative separation of acidic amino acids such as α-aminoadipic, glutamic, aspartic acids, and isovalthine[1,2] has been described. On an analytical scale, a 0.9 × 15 cm column of Amberlite CG 45 Type II in the acetate form was used. Acetic acid of the following concentration, 0.05, 0.15, 0.25 and 2.0 *M*, was employed as eluent, and a satisfactory resolving power and quantitative recoveries were obtained by this method.

Preparation of column

Amberlite CG 45 Type II, freed of fine particles, was converted to the acetate form after washing with 2 *N* sodium hydroxide and water, and was packed into 0.9 × 15 cm column. The column was equilibrated with 100 ml of 0.05 *M* acetic acid before use.

Fractionation

An authentic sample mixture containing about 2 micromoles of each amino acid dissolved in 3 ml of 0.05 *M* acetic acid was placed on the top of the column and washed in two 0.5 ml portions of the same acetic acid. Then the amino acids were eluted stepwise with 0.05, 0.15, 0.25 and 2.0 *M* acetic acid. Each ml of the eluate was collected with an automatic fraction collector. A flow rate up to 20 ml per hour could be used without difficulty. The neutral and the basic amino acids passed through the column. α-Aminoadipic acid and glutamic acid were eluted with 0.15 *M* acetic acid, aspartic acid was eluted with 0.25 *M*, and isovalthine with 2.0 *M* acetic acid (Fig. 1). Even

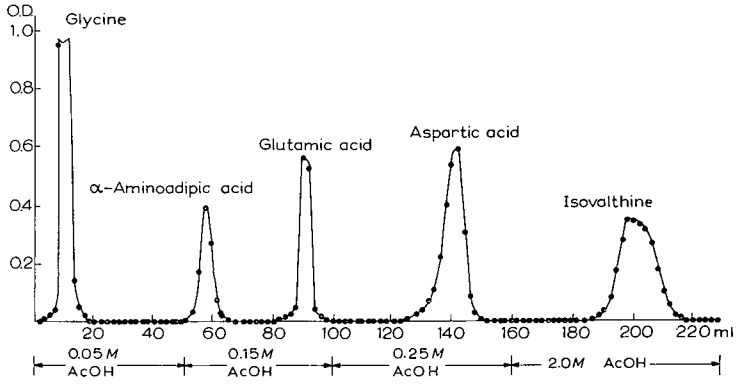

Fig. 1. Separation of the acidic amino acids on a 0.9 × 15 cm column of Amberlite CG 45 Type II. The column was operated in the acetate form at 20° with the sequence of acetic acid concentrations as indicated. Flow rate was 8–10 ml/h.

numbered fractions were used for the photometric determination by the modified ninhydrin method of MOORE AND STEIN[3]. Sufficient colour development could never be attained unless the fractions eluted with 2.0 *M* acetic acid were neutralized before the addition of ninhydrin reagent. Odd numbered fractions were dried under reduced pressure and used for identification by paper chromatography. Each fraction of

acidic amino acids was found to contain essentially one compound. The recoveries of α-aminoadipic, glutamic, aspartic acids, and isovalthine were 103, 98, 97, and 101%, respectively. In order to analyze acidic amino acids in urine, it is necessary to desalt the urine sample by appropriate procedures. In the analysis of isovalthine in urine it is desirable to concentrate the isovalthine fraction by the procedure described above, except that a column with larger cross section is used first, because the concentration of isovalthine in urine is very low.

Gradient elution

The separation of acidic amino acids by gradient elution chromatography was possible with the same column and a mixing chamber of 235 ml. The column was equilibrated with 0.05 *M* acetic acid. After the neutral amino acids had been eluted with 25 ml of 0.05 *M* acetic acid, a gradual increase in the concentration of eluent was effected with 2.0 *M* acetic acid. Glutamic and aspartic acids were well resolved by this procedure. When the concentration of eluent was increased by 7.0 *M* acetic acid, isovalthine was eluted as a separate peak from aspartic acid.

Effect of temperature, sodium chloride and urea

Changes of the temperature of the column in the range from 5 to 35°, and the presence in 3 to 5 ml solution of sodium chloride and urea in physiological concentration, caused little effect on the elution curve.

Another eluent

When formic acid was employed as eluent in a stepwise procedure, α-aminoadipic acid was eluted with 0.05 *M* acid, glutamic acid with 0.15 *M* acid, and aspartic acid with 0.25 *M* acid, respectively.

Preparative scale

5 g of casein hydrolyzate (CASAMINO ACID. DIFCO Lab. Inc.) was dissolved in 0.05 *M* acetic acid and applied on a 6.5 × 13 cm column of Amberlite CG 45 Type II, equilibrated with 0.05 *M* acetic acid, and analyzed. The basic and the neutral amino acids were eluted with 1300 ml of 0.05 *M* acetic acid, and successive elutions with 2000 ml of 0.15 *M* acetic acid and 2000 ml of 0.25 *M* acetic acid yielded glutamic and aspartic acids, respectively. The yields were 682 and 218 mg.

In the case of human urine small portions of two unknown ninhydrin positive substances which resisted hydrolysis partially overlapped with isovalthine in the chromatograms obtained by the stepwise or the gradient elution procedures. Further studies on these substances are necessary.

The author wishes to acknowledge the technical assistance of MISS NORIE SUGINO of his laboratory in the course of these experiments.

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Book Reviews

Chromatographie Symposium II—1962 (Proceedings) organised by the "Société Belge des Sciences Pharmaceutiques", 14 and 15 September 1962 (published by the Société Belge des Sciences Pharmaceutiques, Bruxelles, 1963), price B.F. 150.

Four conferences were held at this symposium of which two (by R. PARIS and by J. E. COURTOIS AND F. PERCHERON) are given in full in this volume, while only summaries are given of the lectures on "Polyamidchromatographie" by L. HÖRHAMMER and "Gas liquid chromatography" by A. T. JAMES AND E. A. PIPER.

The thirty communications to the symposium are mostly printed in full. There is of course a strong bias towards clinical and pharmaceutical problems and paper chromatography and thin-layer chromatography dominate over gas-liquid and column methods. However, there are also some papers on inorganic separations and some on theoretical questions. One paper on teaching experiments in inorganic paper chromatography has been published previously and this fact is not mentioned.

These proceedings should be bought by larger reference libraries and considered as a type of irregular issue of a scientific journal, its value lying mainly in the original papers.

M. LEDERER (Rome)

The Nth volume

Volume 10 of *Protides of the Biological Fluids*, edited by H. PEETERS (Elsevier, Amsterdam, 1963), price 90/-; *Gas Chromatography Abstracts 1962*, edited by C. E. H. KNAPMAN AND D. AMBROSE (Butterworths, London, 1963), price 42/-.

It is rather pointless to discuss at length yearly volumes which do not vary essentially from one year to the next, being published by the same publishers and edited by the same editor.

Volume 10 of the *Protides* is a good cross-section of the original work published on the separation of proteins and its clinical applications. The general trend in this field of research seems to be the combination of various techniques rather than the development of new methods.

Gas Chromatography 1962 contains 763 abstracts, which is slightly less than the preceding volumes. The reviewer would like to suggest that a cumulative index would be very helpful in one of the forthcoming volumes, which incidentally will not be published by Butterworths but by the Institute of Petroleum.

Characteristic Frequencies of Chemical Groups in the Infra-red, by M. ST. C. FLETT, Elsevier Publishing Co., Amsterdam, 1963, ix + 98 pages, price Dfl. 12.50, DM. 14.–, £ 1.5.0.

This short monograph is not in the same class as the formally analogous, but much more comprehensive (and hence generally more valuable) books by L. J. BELLAMY (*The Infrared Spectra of Complex Molecules*) or K. NAKAMOTO (*Infrared Spectra of Inorganic and Coordination Compounds*). Nevertheless it should find use by and be of occasional value to some organic chemists engaged primarily in synthesis and prone to rely (often too heavily) upon infrared spectra for identification and even structure determination.

The book is divided into four sections. 1. Correlation Charts (8 pp.). There are seven given, each representing a distinct spectral range within the range from 3600 to 500 cm^{-1}. Most laboratories using infrared will have large, more detailed and easier to read charts available. 2. Characteristic frequencies of chemical classes. Characteristic frequencies of the commoner classes (74 in all) of organic compound, in alphabetical order (from Acetylene Derivatives to Uretidiones) are briefly discussed. Data of this kind may be useful when enough is known about the chemistry of an unknown species for possible structures to be suggested. The main virtue of the book lies in its convenient, albeit brief, presentation of the characteristic frequency data. Approximate peak intensities are given as well as frequent references (183 in all) to the original and recent literature on which the band assignments are based. 3. Some sources of infrared spectra (2 pp.). Thirteen are cited, each with comment. 4. Bibliography on the interpretation of the infrared spectra of organic materials ($1^1/_2$ pp.). Twelve references are cited with comment. There follows $3^1/_2$ page index, by compounds and groups.

In this book the author offers convenience and conciseness in place of thoroughness and understanding. However, for those for whom the book is primarily intended, often the former are much preferred and indeed more valuable.

J. SELBIN (Baton Rouge)

Zone Electrophoresis in Blocks and Columns, by H. BLOEMENDAL, Elsevier Publishing Co., Amsterdam, 1963, viii + 219 pages, price Dfl. 20.—.

The following techniques are described in this book: block electrophoresis, gel electrophoresis, continuous electrophoresis, column electrophoresis and column electrophoresis in density gradients.

For each technique the author describes the methods employed in detail and discusses the various packing materials etc. Though applications are only briefly mentioned, the biochemical applications are given the main emphasis. All topics of this volume with the exception of electrophoresis in a density gradient have been reviewed in the *Journal of Chromatography* either by BLOEMENDAL or others; however, each chapter has been brought up to date and enriched with supplementary illustrations and material.

The volume will be found useful as a companion to the various treatises on paper electrophoresis which usually do not discuss block and column electrophoresis adequately.

Announcements

Colloquium on Protides of the Biological Fluids

The XIIth Annual Colloquium on Protides of the Biological Fluids organised by the Sint-Jan's Hospital, Bruges, Belgium, will be held on 30th April and 1st, 2nd and 3rd May 1964.

PROGRAMME

Topics:

I Phylogeny of proteins
II Tissue proteins
III Glyco- and mucoproteins, especially urinary proteins
IV Methods.

Round Table Discussions:

I Tissue proteins
II Methods
III Proteins of diagnostic significance.

For application and communications, please apply to:

Laboratory, St. Jan's Hospital,
Bruges (Belgium)

Anschluss des Deutschen Arbeitskreises für Spektroskopie an die Fachgruppe "Analytische Chemie" der Gesellschaft Deutscher Chemiker

In Fortsetzung des von Prof. W. Seith im Jahre 1948 gegründeten Deutschen Zentralausschusses für Spektrochemie und angewandte Spektroskopie wurde am 24. April 1963 in Erlangen der "Deutsche Arbeitskreis für Spektroskopie" neu gegründet. Der in Erlangen gewählte vorläufige Vorstand, bestehend aus den Herren:

Prof. Dr. G. Scheibe, München
Prof. Dr. J. Fischer, Frankfurt
Prof. Dr. H. Kaiser, Dortmund
Prof. Dr. W. Lochte-Holtgreven, Kiel
Prof. Dr. R. Mecke, Freiburg

hat auftragsgemäss mit der Gesellschaft Deutscher Chemiker verhandelt und am 28. Oktober 1963 in Frankfurt (Main) beschlossen das Angebot der Gesellschaft

Deutscher Chemiker anzunehmen, ein Arbeitskreis im Rahmen der GDCh-Fachgruppe "Analytische Chemie" zu werden.

Für die Zusammenarbeit wurden Arbeitsrichtlinien festgelegt.

Der endgültige Vorstand wird auf der nächsten Mitgliederversammlung, die für Herbst 1964 vorgesehen ist, gewählt.

Nähere Auskunft erteilt die GDCh-Geschäftsstelle, 6000 Frankfurt/Main, Postfach 9075.

THE SOCIETY FOR ANALYTICAL CHEMISTRY

MIDLANDS SECTION

Elwell Award, 1964

The object of this award is to encourage young Midland scientists in the profession of analytical chemistry.

Entries are invited for the annual competition for the Elwell Award from any scientist, including those engaged in full-time post-graduate studies under the age of 30, working or residing in the area covered by the Midlands Section of the Society for Analytical Chemistry, *i.e.* South of, but including, Stoke-on-Trent and North of, but excluding, Carmarthen.

The Award, consisting of a silver trophy, will be retained for one year by the candidate deemed by a panel of referees to have submitted the best paper dealing with some aspect of analytical chemistry. In addition, the successful candidate will receive a gift of scientific books of his own choice to the value of 10 guineas; the runner-up will receive scientific books to the value of 3 guineas.

Papers submitted should describe, in a form suitable for presentation at a local Section Meeting, work in which he or she has been actively associated, though not necessarily entirely responsible.

The basic requirement is that the contribution should advance, even in a small way, existing knowledge of analytical chemistry.

Selected papers will be presented at a Meeting of the Section during September, 1964, and the name of the successful candidate will be announced on the same evening. The closing date for entries is 8th June, 1964, and the latest date for submission of papers is the 29th June, 1964.

Entry forms and other particulars may be obtained from: M. L. RICHARDSON, A.R.I.C., A.C.T. (Birm.), Honorary Secretary, Midlands Section of the Society for Analytical Chemistry, c/o John & E. Sturge Ltd., Lifford Chemical Works, Lifford Lane, Kings Norton, Birmingham, 30, England.

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REDUCED PLATE HEIGHT EQUATION: A COMMON LINK BETWEEN CHROMATOGRAPHIC METHODS

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It has long been recognized that a critical requirement for chromatographic separation is the development of zones whose "width" is no greater in magnitude than the distance separating them[1]. Since the plate height is conventionally used to express zone width or zone spreading it is particularly important to understand the physical basis of this parameter and the lower limits to which it can be restrained.

While the first step in improving the resolving power of a method is in understanding the ultimate potential of the system, one hopes to avoid the necessity of treating each variation in technique as a new and separate problem. Since nearly all chromatographic techniques are based on the same molecular physics, it should be possible to relate the different methods to a single plate-height equation. This "universal" equation would then serve to demonstrate the ultimate capabilities of all systems at one and the same time.

In an earlier paper[2] it was shown that inert (non-sorbed) zones in gas and liquid chromatography could be treated by the same equation providing reduced variables were used. The present development is an extension of this concept, along with its practical application, to working chromatographic systems.

The theoretical equation for the plate height of a chromatographic system may be written in the form[3]:

$$H = \frac{2\gamma D_m}{v} + \frac{2\gamma_s D_s (1 - R)}{v \quad R} + Cv + \sum_i \frac{1}{1/2\lambda_i d_p + D_m/\omega_i d_p^2 v} \qquad (1)$$

where v is the average velocity of the mobile phase, γ and γ_s are the longitudinal diffusion parameters for the mobile and stationary phases, respectively, and D_m and D_s the corresponding diffusion coefficients for these phases, R is the retention factor ($\cong R_F$), d_p is the mean particle diameter, ω_i is one of the mass transfer parameters for the mobile phase and λ_i is the corresponding eddy diffusion parameter. The coefficient C, of great importance, is a mass transfer term representing the finite rate of adsorption–desorption in adsorption chromatography and the finite rate of diffusion in the stationary liquid of a partion column. This parameter, representing the total "rate" effects of the stationary phase, has been studied in detail[3], but the numerous forms assumed by C in different circumstances precludes its more specific elaboration here.

Dimensionless variables of the following nature[2] serve to reduce equation (1) to a more universal form:

$$h = H/d_p, \quad v = d_p v/D_m \tag{2}$$

where h is the "reduced plate height" and v the "reduced flow velocity". In addition the two dimensionless parameters:

$$\Omega = CD_m/d_p^2, \qquad \beta_s = (1 - R)\gamma_s D_s/R\gamma D_m \tag{3}$$

are useful. With these changes eqn. (1) gives the following dependence of h upon v:

$$h = \frac{2\gamma}{v} + \frac{2\gamma\beta_s}{v} + \Omega v + \sum \frac{1}{\frac{1}{2}\lambda_i + 1/\omega_i v} \tag{4}$$

The first and the last terms of this equation, representing the mobile phase effects, depend only on the structural parameters γ, the λ_i's and ω_i's (in addition to the reduced velocity). These terms do not depend on the specific properties of the solute and the mobile phase, such as the diffusivity D_m, and they are independent of the mean particle diameter d_p. Since the structural factors are nearly the same for any random packing of particles, the first and last terms of the equation have essentially the same dependence on reduced velocity v irregardless of the nature of the chromatographic system. (This rule is violated when the column diameter to particle diameter ratio becomes excessive and trans-column mass transfer becomes significant[4].). Thus the h — v curve due to mobile phase effects alone shows an irreducible minimum below which the plate height cannot be taken by *any* manipulation of column parameters. An approximation to this minimal curve (using approximate structural parameters for packed media) is shown by the lowest line in Fig. 1. Even at optimum

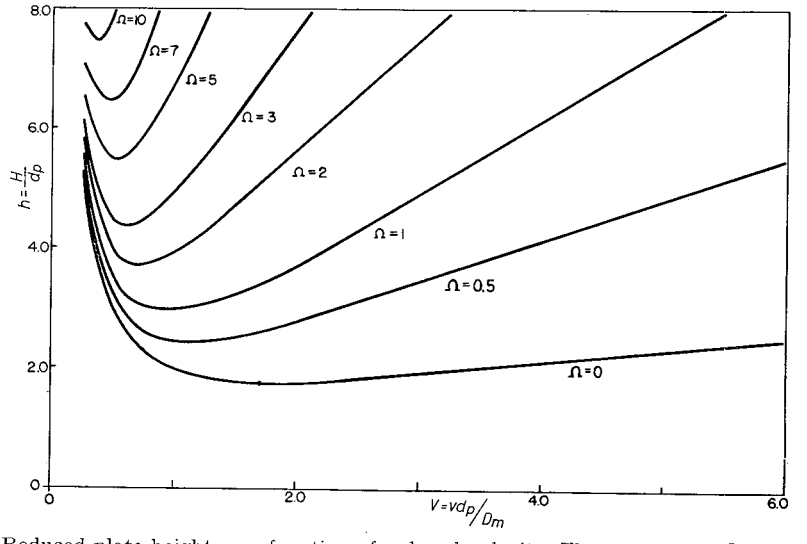

Fig. 1. Reduced plate height as a function of reduced velocity. The curve with $\Omega = 0$ is caused by mobile phase effects only. The other curves show the increase in plate height as the sorption–desorption processes become unduly slow.

velocity the value of h cannot be reduced much below 2, *i.e.*, the plate height H cannot be pushed much below 2 particle diameters.

While the mobile phase contribution to plate height in a packed column is determined within rather inflexible limits, the same is not true for the stationary phase contribution given by the middle two terms of eqn. (4). The $\Omega\nu$ term, particularly, may vary over wide limits. Excessive values of Ω may destroy the resolution of an otherwise excellent column. This is shown in Fig. 1 where the $\Omega\nu$ term is added to the "minimal curve" for different values of Ω. (The β_s term, negligible in many columns, will be assumed as zero in these plots.). The effectiveness of separation, indicated by the smallness of h, is decreased as Ω increases. The plots show that resolving power is seriously hindered for Ω values greater than unity. We will thus regard unity as an upper desirable limit to Ω, and briefly indicate how this limit may be achieved in practical columns.

The parameter C, which has the dimensions of time, is a measure of the speed of the sorption–desorption process in or on the stationary phase. Since the Ω parameter has been limited to a value of unity or less the maximum C value, from equation (3), is:

$$C \leq d_p^2/D_m \tag{5}$$

Two general rules can be formulated from the above expression. They are:

(1) The requirement for rapid equilibration (small C) increases with the square of the particle diameter. If C already contributes a significant part of the plate height there is little use in reducing the particle diameter further. For those systems in which C is naturally small (some forms of adsorption chromatography) a reduction in d_p may be very helpful.

(2) The speed of equilibration must increase as the mobile phase diffusion coefficient increases. Thus gas chromatography, with D_m values about 10^5 times larger than in liquid chromatography, makes much more stringent demands on the equilibration processes. Likewise a hydrogen or helium carrier gas demands more of the system than a nitrogen carrier, but the effect is not so large in this case.

An application of rule (2) serves as a useful example. It is known that gas–liquid partition chromatography operates effectively when the stationary liquid is accumulated in pores about one micron in diameter[5]. Therefore liquid-partition chromatography should operate successfully with the partitioning liquid in units as large as $\sqrt{10^5 \times 1\ \mu} \cong 300\ \mu$ in diameter, if necessary. Since particle diameters are usually less than this, it can be concluded that equilibration in the stationary liquid phase, when there are no side effects due to adsorption or other influences, is rapid enough not to hinder chromatographic performance. This conclusion applies, of course, only when the liquid system is operated in the same range of ν values as the gas system. This example does show, however, that empirical results from one chromatographic system may have an important bearing on the performance and limitations of many different techniques.

The curves shown in Fig. 1 demonstrate that the optimum performance of an efficient column occurs at a reduced velocity of between 1 and 2. If the minimum occurs at a point much less than this the system is in need of redesigning. Quite often the velocity will be increased beyond the optimum in order to increase the speed of analysis. This is particularly true in liquid chromatography where the optimum ve-

locity, obtained from equation 2 with $\nu = 1$, is about 10^{-4} cm/sec (the optimum velocity in gas chromatography is about 10 cm/sec). It is especially important in such high-speed chromatography that C be small, perhaps even less than the limit suggested by equation (5).

In summary we may say that the reduced plate-height expression of equation (4) contains a group of structural factors (γ, λ_i, ω_i) which are essentially universal constants. It also contains two parameters, β_s and Ω, characteristic of the stationary phase. The desirability of the stationary phase must be judged in terms of the properties of the mobile phase. This comparison is already made once we have reached the stage of equation (4), and relatively simple criteria thus exist for the effectiveness of the stationary phase, *i.e.*, β_s and Ω should be less than unity. These criteria are not quantitatively rigid because of the varying requirements of the experimentalist; indeed they could not be quantitatively exact because the structural factors are not precisely the same for any two columns. In addition some difficulty might be encountered in determining the molecular diffusion coefficients, and approximate estimates might be required. Nonetheless equation (4) comes very close to forming a practical and quantitative bridge between the different forms of chromatography, and the universal plots of Fig. 1 demonstrate the common goal sought in the resolving power of any systems.

ACKNOWLEDGEMENT

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SUMMARY

In order to correlate the behavior and performance obtained from the many diverse forms of chromatography, a universal plate-height equation is proposed. This equation is based on the fundamental processes which are characteristic of all methods. A plot of reduced plate height versus reduced flow velocity should yield a set of universal curves, approximated by the equation, which will be applicable to any system under any operating conditions (temperature, etc.). This concept shows the possibility for relating experimental results obtained on different systems and thus transferring experimental knowledge from one system to another.

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DUAL COLUMN–DIFFERENTIAL FLAME IONIZATION DETECTOR SYSTEM AND ITS APPLICATION WITH PACKED AND GOLAY TYPE COLUMNS*

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In gas–liquid partition-chromatography, bleeding of the liquid phase is usually unavoidable. Every liquid phase has a certain vapor pressure at a given temperature and because the gas in the column is constantly moving, the column effluent always contains a certain amount of this vapor.

Since gas chromatographic detectors usually respond to any substance other than the inert gas (and certainly to any organic material), substrate bleeding will result in a detector background signal. However, if column temperature and carrier gas flow rate are unchanged during analysis (*isothermal operation*), the rate of bleeding is constant. Thus, the background signal—due to substrate bleeding—is also kept constant and can therefore electrically be compensated.

The situation is different when the column temperature is constantly raised (*programmed temperature operation*). Since the vapor pressure of any substance increases exponentially with temperature, the rate of bleeding is not constant any more but will increase during the program. This will result in a continuous (and exponential) base line drift with both thermal conductivity and ionization detectors. The sample component peaks will be superimposed over this drifting base line making peak identification and the quantitative evaluation of the chromatogram practically impossible.

THE INFLUENCE OF LIQUID PHASE BLEEDING

In order to demonstrate the effects of substrate bleeding on background current, a column was prepared with squalane liquid phase. The column dimensions were 3 ft. × 0.085 in. I.D. (0.125 in. O.D.) and it consisted of 15 wt.-% squalane on Chromosorb W, 80–100 mesh. The column was placed in a gas chromatograph equipped with a flame ionization detector and the carrier gas (helium) flow was kept constant during the whole investigation at 15.5 ml/min (uncorrected value, at column outlet). The recorder base line was adjusted to zero at room temperature, and then the temperature of the column was raised in steps of 25° from 50° up to 175° and the background current from the detector was recorded after the base line reached equilibrium. The results are given in Table I and plotted in Fig. 1. This graphical presentation results

* Condensed text of the papers presented at the 13th and 14th Pittsburgh Conferences on Analytical Chemistry and Applied Spectroscopy, Pittsburgh, Pa., March 1962–1963.

TABLE I

BACKGROUND SIGNAL OF SQUALANE COLUMN AT DIFFERENT TEMPERATURES WITH 15.5 ml/min* CARRIER GAS (He) FLOW

Column temperature (°C)	*Background signal (recorder deflection) ($\times 10^{-12}$ A)*
50	0.14
75	0.29
100	0.94
125	12.5
150	93.0
175	810.0

* Uncorrected value measured at column outlet.

in a curve similar to the actual base line drift during programmed temperature operation.

The ordinate (*i.e.* the background) of Fig. 1 is expressed in two different ways: the scale on the left gives the background signal in ampères while the scale on the right indicates full scale deflection at different attenuations on a 5 mV recorder giving the approximate magnitude of base line drift during programmed temperature operation with the given flow rate. For example, the background signal at 150° was $93 \cdot 10^{-12}$ A. With the system used, $2.5 \cdot 10^{-12}$ A signal corresponds to full scale deflection at attenuation $\times$ 1; thus, full scale deflection with *e.g.* attenuation $\times$ 500 corresponds to $1250 \cdot 10^{-12}$ A. This means that the programming of this particular column from room temperature up to 150° with the given flow rate, using attenuation $\times$ 500 would result in 7.4% base line drift. However, with attenuation $\times$ 50, the same programming would result in a 74% base line drift since with this attenuation, full scale recorder deflection is equal to $125 \cdot 10^{-12}$ A.

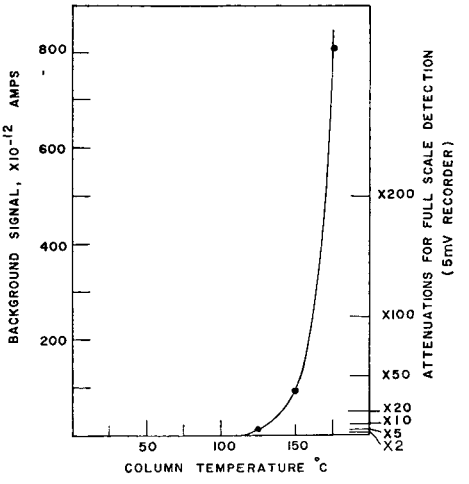

Fig. 1. Background signal of a packed column with 15 wt.-% squalane on Chromosorb W, 80–100 mesh. Column dimensions: 3 ft. $\times$ 0.085 in. I.D. Carrier gas (He) flow rate: 15.5 ml/min.

This short calculation means that the base line drift during programmed temperature operation is less significant when low detector sensitivities are used; at high sensitivities, however, it makes the analysis practically impossible.

Another conclusion can be made when the measured values are compared with the vapor pressure data of squalane. According to the literature, usually the temperature corresponding to 0.1 mm Hg vapor pressure is considered as the maximum operating temperature of a gas chromatographic column[1]. Fig. 2 gives the vapor

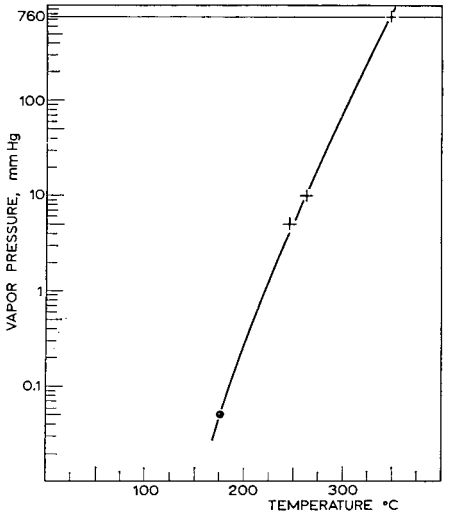

Fig. 2. Vapor pressure curve of squalane. Data marked with + are from Ref. 2 while the value corresponding to 0.05 mm Hg is from Ref. 3.

pressure curve of squalane[2,3]. According to this, a vapor pressure of 0.1 mm Hg corresponds to about 185°. However, it is clear from Fig. 1 that a squalane column could not be programmed higher than about 125–130° if the high sensitivity of the flame ionization detector is utilized. Thus, maximum temperature values reported in the literature are only valid with thermal conductivity detectors, under isothermal conditions.

DUAL COLUMN OPERATION

In 1961, EMERY AND KOERNER[4,5] introduced a new approach for the elimination of base line drift when the column temperature is programmed. In their system, a second column parallel to the analytical column is bleeding at identical rate into the reference side of the thermal conductivity detector. Since the output of such detector bridge is a measure only of the differences in thermal conductivity in the two chambers, it will be zero except when sample components enter the sensing cell. Thus, the base line drift due to substrate bleeding can be eliminated.

The only limitation of their system is that since it is utilizing a thermal conductivity detector, its sensitivity is restricted by the detector itself and some of the advantages of base line compensation are lost although otherwise, it can be used with practically any type of liquid phase[6]. Therefore, TERANISHI *et al.*[7] substituted *two*

identical flame ionization detectors (FID) for the katharometer feeding their output into a differential amplifier.

An alternate approach is the use of a *differential* flame ionization detector with a single output feeding one amplifier. In such system, differential amplifiers and complicated controls are avoided: the single output from a differential FID eliminates the problems associated with matching high megohm input resistors and designing complex zero suppression circuitry.

In this paper, such a system is briefly described and its application is illustrated.

APPARATUS

Fig. 3 gives the simplified electrical circuitry of the differential detector. Two flames housed within a single chamber are oppositely polarized by two 300 V batteries; the other terminal of each battery is connected to amplifier ground. A platinum screen acting as a common collecting electrode for each flame is connected to the input of an electrometer amplifier. Since both flames are oppositely polarized and commonly connected to the electrometer amplifier, the circuit is equivalent to a differential ampèremeter. The current appearing at the input of the electrometer is the difference

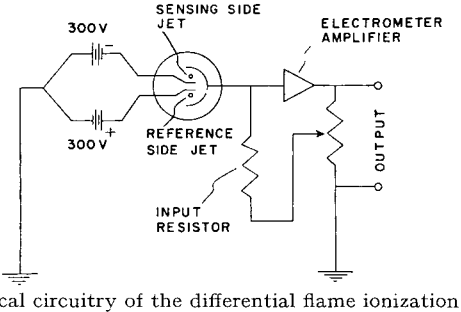

Fig. 3. Simplified electrical circuitry of the differential flame ionization detector system.

between the signals generated in the sensing and reference flames. Whereas signals from the sensing flame tend to decrease the current at the electrometer amplifier, signals from the reference flame tend to increase this current. The net result is a measure only of the difference on the two generated signals. The differential voltage developed across the input resistor is amplified by the electrometer amplifier and recorded on a suitable recorder connected to the output. The amplifier used is a negative feedback amplifier which is capable of driving both galvanometric and potentiometric recorders.

Fig. 4 shows a more detailed view of the detector. The jets are made of stainless steel tubes of 0.020 in. I.D. and are connected directly to the detector base with internal Swagelok fittings. Platinum wires running vertically in close proximity to the flames serve as the polarizing electrodes; these electrodes pass insulated through the detector base. The common collecting electrode is constructed of fine mesh platinum screening. An ignitor coil is placed between the jets for ignition of the hydrogen flames. Filtered air is purged into the detector through a sintered stainless steel disc to insure smooth air flow within the chamber. The combustion products and excess air escape through vents in the top of the detector cover.

The detector construction described shows no electrical communication between the two flames; therefore, shielding or separate detector chambers are not needed. Due to the symmetrical relationship of the electrode pairs, both flames exhibit equal sensitivities after initial optimization of gas flows. The presence of grounded jets and ignitor does not affect detector response. The detector is insensitive to gross temperature changes and may be programmed up to 400°.

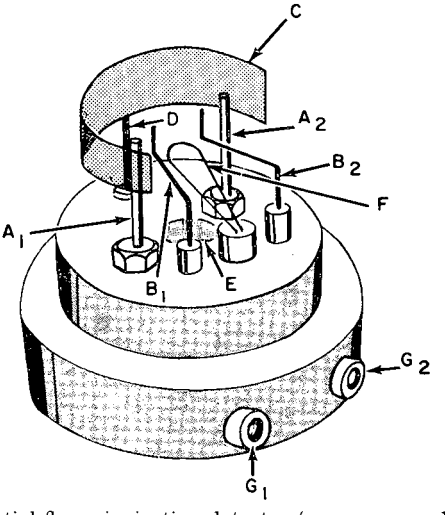

Fig. 4. View of the differential flame ionization detector (cover removed). (A_1, A_2) jets; (B_1, B_2) polarization electrodes; (C) common collecting electrode; (D) connection to electrometer input; (E) air filter disk; (F) ignitor; (G_1, G_2) column connections.

The symmetrical construction of the detector allows each flame to be used separately (but not simultaneously) as a single flame ionization detector. In this case, the hydrogen flow through the unused jet is shut off.

The described differential FID is incorporated into a gas chromatographic system* shown schematically in Fig. 5. Individual proportional flow controllers with needle valve adjustment are used to independently balance column flows. The system has dual injection ports housed within a single block which can be heated up to 500°. The columns are enclosed in a high velocity circulated air bath oven compartment capable of operation up to 400°. The effluents of each column proceed to effluent splitters which allow a specified percentage of the flow to enter the detector, the remainder of which vents to atmosphere for sample collection, auxiliary detection (*e.g.* additional hot wire or electron capture detector, etc.) or discard. The vents may also be plugged to allow full column flow to the detector. The detector jets, as described previously, are housed within a single chamber and supplied with externally regulated hydrogen gas. A needle valve and snubber is installed in each hydrogen line for establishing the desired flow rates. Externally regulated filtered air provides the necessary oxygen for the flame and purges the combustion products from the detector chamber.

* The system described is the Model 800 gas chromatograph of the Perkin-Elmer Corporation, Norwalk, Conn. All analyses reported in this paper were performed with this instrument.

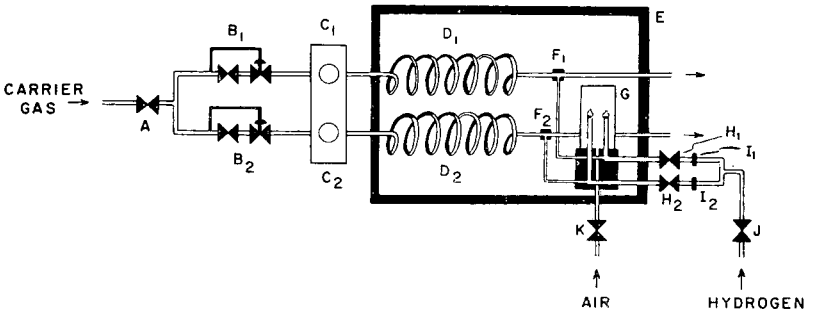

Fig. 5. Flow scheme of a dual column–differential FID gas chromatograph. (A) carrier gas pressure regulator; (B_1, B_2) flow controllers with needle valve adjustment; (C_1, C_2) sample introduction ports; (D_1) analytical and (D_2) reference columns; (E) column oven compartment; (F_1, F_2) column effluent splitters; (G) differential flame ionization detector; (H_1, H_2) needle valves; (I_1, I_2) snubbers; (J) hydrogen pressure regulator; (K) air pressure regulator.

The dual injection ports permit special applications of the system. In this way, both columns can be used alternately for separation. It is also possible to install two *different* columns into the instrument and use them alternately, operating the differential FID as a single detector. Two consecutive analyses on different columns can be carried out under identical conditions, greatly simplifying *e.g.* relative retention time or retention index measurements.

COMPENSATION OF LIQUID PHASE BLEEDING

The compensation of liquid phase bleeding is carried out by the adjustment of the reference carrier gas flow. The columns are stabilized at the initial temperature of the program and the base line is electrically zeroed. Now, the oven is programmed up to the maximum temperature; as a result, a base line drift will be observed which will level off at the end of the program. The flow through the reference column is now adjusted to rezero the recorder base line. After this procedure, the instrument is cooled down and can be used for actual analysis.

Fig. 6 illustrates base line compensation of a 6 ft. × 0.085 in. I.D. packed column with SE-30 liquid phase. The column was programmed from 75 to 355° at 20°/min and then cooled to 75°. The chromatogram shows the actual base line with single column operation and also, base line compensation with dual columns.

APPLICATIONS

Dual column–differential FID systems can be used in practically all applications where programmed temperature operation over a wide range is necessary. The following examples illustrate some typical applications of the system described.

Figs. 7 and 8 demonstrate the analysis of a high boiling sample containing trace components. Two 6 ft. × 0.085 in. I.D. columns containing Apiezon L on hexamethyldisilazane (HMDS) treated Chromosorb W, 80–100 mesh were used in both single and dual column mode. The samples consisted of high boiling aromatics. Peaks 4 and 5 corresponded to approximately $6 \cdot 10^{-9}$ g each. Since attenuation × 5 was used, the minimum detectable limit for this sample with 1 μl injection is about 1 p.p.m. in the

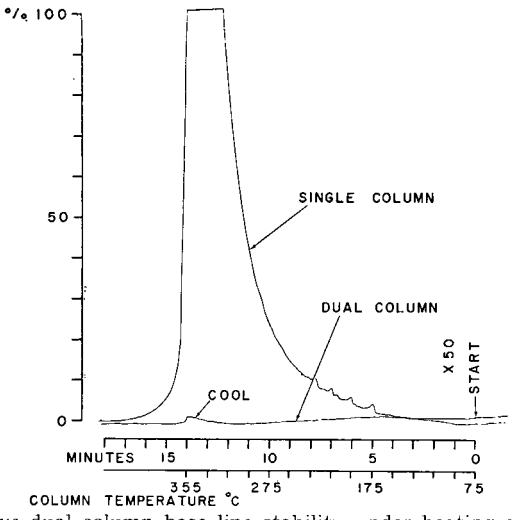

Fig. 6. Single *versus* dual column base line stability under heating and cooling. Each column 6 ft. × 0.085 in. I.D. containing 1.5 wt.-% SE-30 silicone gum rubber on Chromosorb W, 80–100 mesh. 5 mV recorder with attenuation × 50; full scale response corresponds to $1.25 \cdot 10^{-10}$ A.

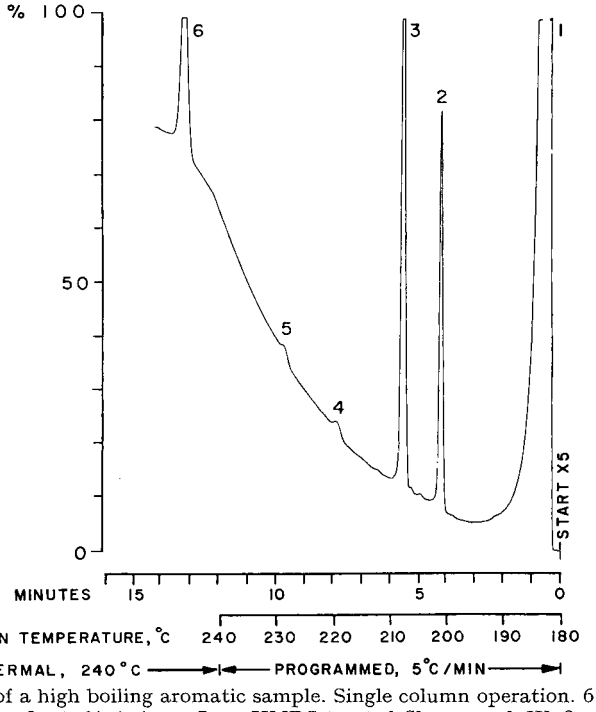

Fig. 7. Analysis of a high boiling aromatic sample. Single column operation. 6 ft. × 0.085 in. I.D. column containing 8 wt.-% Apiezon L on HMDS-treated Chromosorb W, 80–100 mesh. Injection block: 260°. Sample: 1 μl solution. Peaks: 1 = benzene (b.p. 80°) (solvent); 2 = biphenyl (b.p. 255°); 3 = 2,3-dimethylnaphthalene (b.p. 268°); 4 = fluorene (b.p. 298°); 5 = anthracene (b.p. 354°). Peaks 4 and 5 correspond to approximately $6 \cdot 10^{-9}$ g each. 5 mV recorder with attenuation × 5; full scale response corresponds to $1.25 \cdot 10^{-11}$ A.

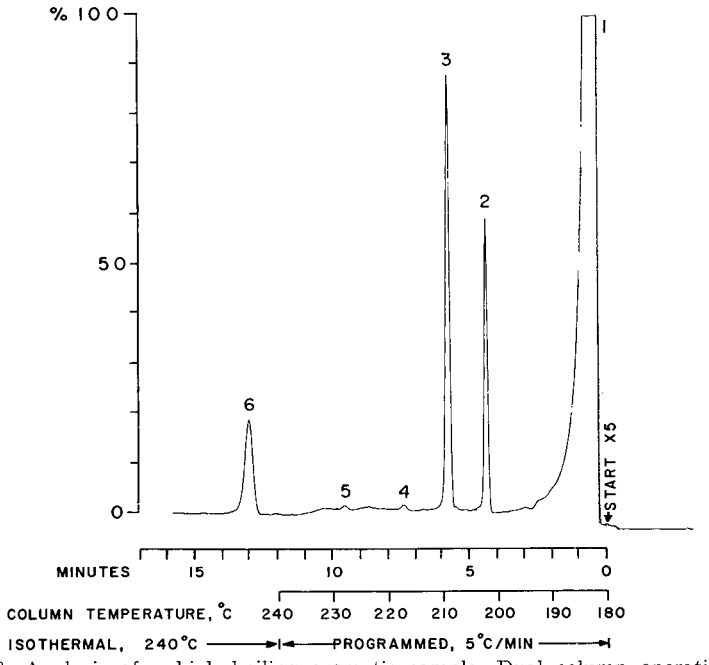

Fig. 8. Analysis of a high boiling aromatic sample. Dual column operation. Each column, conditions, sample and peaks as given in Fig. 7.

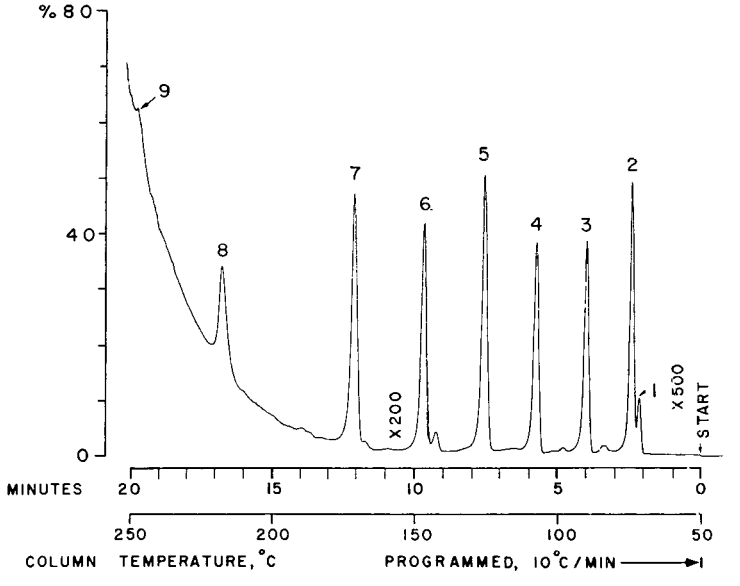

Fig. 9. Analysis of halogenated hydrocarbons. Single column operation. 6 ft. × 0.085 in. I.D. column containing 10 wt.-% DC-550 phenylsilicone oil on Chromosorb W, 80–100 mesh. Sample: 0.02 μl mixture. Peaks: 1 = chloroform (b.p. 61°); 2 = 1-chlorobutane (b.p. 78°), 3 = 1-chloropentane (b.p. 108°); 4 = 1-chlorohexane (b.p. 132°); 5 = 1-chloroheptane (b.p. 159°); 6 = *o*-dichlorobenzene (b.p. 180°); 7 = hexachlorobutadiene (b.p. 215°); 8 = 1-iodo-3-nitrobenzene (b.p. 280°); 9 = hexachlorobenzene (b.p. 326°). 5 mV recorder with given attenuations; with attenuation × 200, full scale response corresponds to $5 \cdot 10^{-10}$ A.

sample. However, if higher sample volumes were used or the flow rate of the carrier gas increased, concentration in the p.p.b. range could also be detected.

Figs. 9 and 10 compare the results of the analysis of a wide range halogenated hydrocarbon mixture. Using a single column, it is difficult to judge whether the base line disturbance at 20 min is a peak. With dual column operation, this peak—corresponding to a small amount of hexachlorobenzene (b.p. 326°)—can clearly be

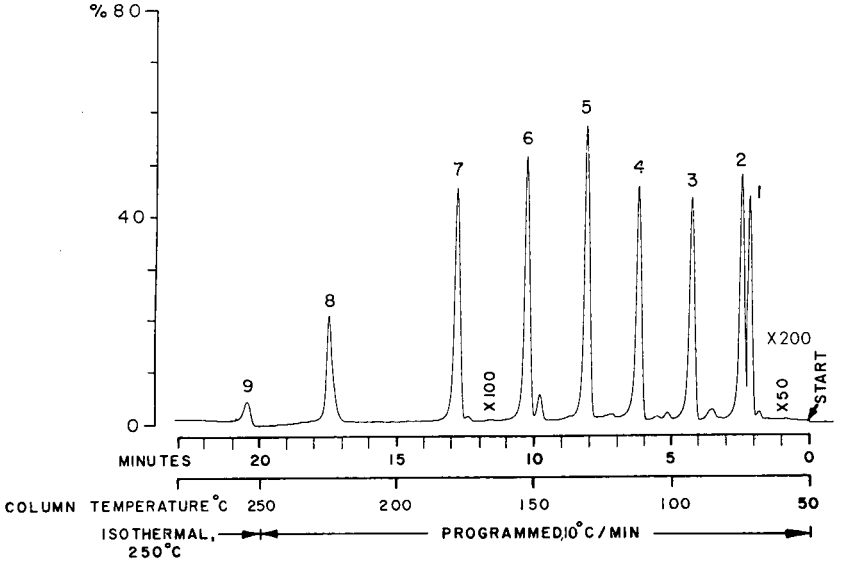

Fig. 10. Analysis of halogenated hydrocarbons. Dual column operation. Each column, conditions, sample and peaks as given in Fig. 9.

distinguished. For this analysis, the DC-550 phenylsilicone oil was heated 25° higher than its maximum recommended temperature[8].

A recent publication[9] showed some interesting comparative chromatograms on the analysis of higher fatty acid methyl esters. Under isothermal conditions, at 180°, erucic acid methyl ester emerged in about 67 min; using temperature programming and dual column operation, this time could be reduced to 23 min without significant loss in the resolution of the stearate–oleate pair.

The dual column analysis of a mixture of high boiling condensed ring aromatics is shown in Fig. 11. The Apiezon L columns were relatively new and except for the usual preconditioning at 200° for a few hours, no special conditioning took place. The columns were heated higher than the recommended maximum operating temperature, without any observable base line drift.

Fig. 12 is a typical example for the analysis of another high boiling sample. In this case, Versamid 900, a long chain linear polyamid (a product of the Chemical Division of the General Mills Corporation, Hensakee, Ill.) was used as liquid phase and programmed up to 275°.

Dual column operation is interesting not only at high temperatures but can also be applied at any temperature if the relatively high bleed rate of the column makes it necessary. Fig. 13, for example, shows the analysis of two beer head space gas samples.

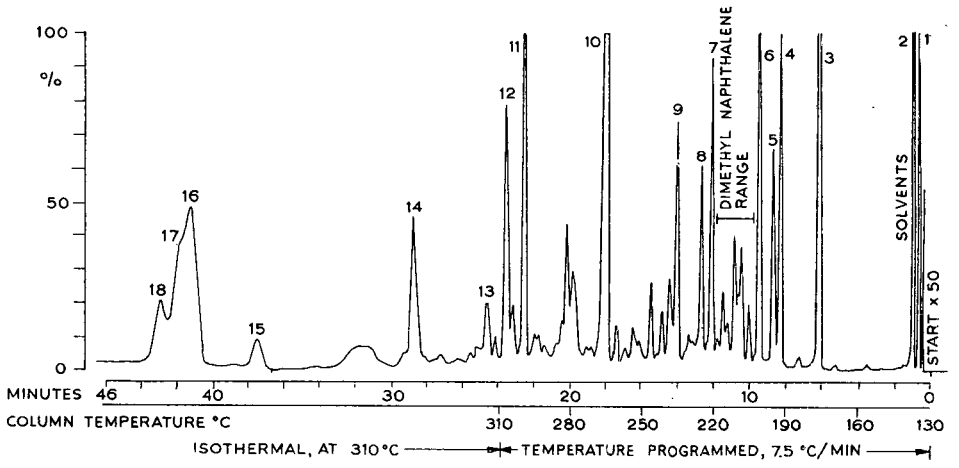

Fig. 11. Analysis of a neutral creosote oil with added polycyclic substances. Dual column operation. Two 6 ft. × 0.085 in. I.D. columns containing 8 wt.-% Apiezon L on HMDS-treated Chromosorb W, 80–100 mesh. Injection block: 350°. Sample: 0.2 μl of a benzene–toluene solution. Peaks: 1,2 = solvents; 3 = naphthalene (b.p. 218°); 4 = 2-methylnaphthalene (b.p. 245°); 5 = 1-methylnaphthalene (b.p. 240–243°); 6 = biphenyl (b.p. 254–255°); 7 = acenaphthene (b.p. 277°); 8 = diphenylene oxide (b.p. 288°); 9 = fluorene (b.p. 295–298°); 10 = phenanthrene (b.p. 340°) + anthracene (b.p. 354°); 11 = fluoranthene (b.p. 384°); 12 = pyrene (b.p. 394°); 13 = 1,2-benzofluorene (b.p. ~ 407°); 14 = chrysene (b.p. 448°); 15 = perylene (b.p. ~ 460°); 16 = 1,2-benzpyrene (b.p. 493°); 17 = 3,4-benzpyrene; 18 = 1,2-benzoperylene. 5 mV recorder with attenuation × 50; full scale response corresponds to $1.25 \cdot 10^{-10}$ A.

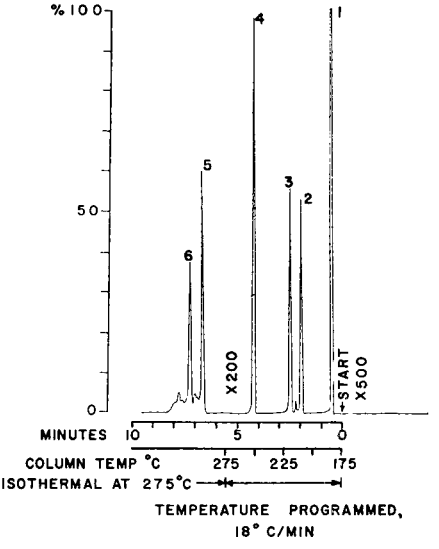

Fig. 12. Analysis of a phthalate mixture. Dual column operation. Two 6 ft. × 0.085 in. I.D. columns containing 2 wt.-% Versamid 900 on HMDS-treated Chromosorb W, 80–100 mesh. Injection block: 350°. Sample: 0.5 μl solution in acetone. Peaks: 1 = acetone (solvent); 2 = dimethyl phthalate (b.p. 282°); 3 = diethyl phthalate (b.p. 296°); 4 = dibutyl phthalate (b.p. 340°); 5 = dioctyl phthalate (b.p. 384°); 6 = dinonyl phthalate (b.p. 420–440°). 5 mV recorder with given attenuations; with attenuation × 200, full scale response corresponds to $5 \cdot 10^{-10}$ A.

Although Carbowax type columns can usually be heated isothermally higher than 120°, they have a relatively high bleed rate at the sensitivities used when programming and therefore, they would show a significant base line drift under single column condition. These two chromatograms also illustrate the reproducibility of dual column operation.

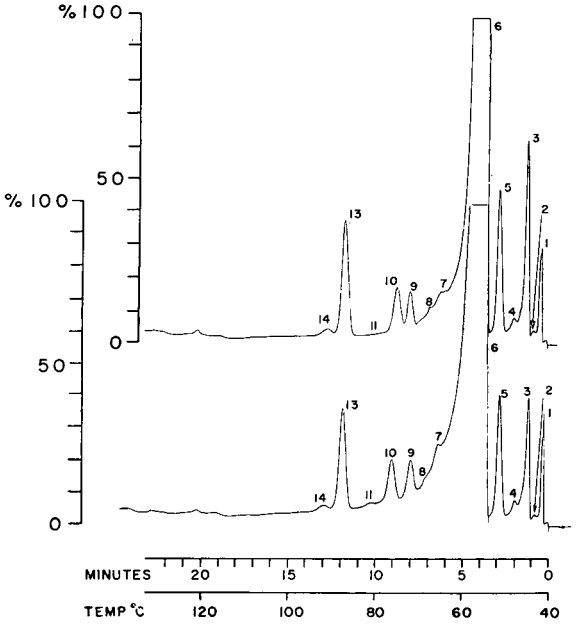

Fig. 13. Analysis of beer head space gas samples. Dual column operation. Two 6 ft. × 0.085 in. I.D. columns containing 8 wt.-% Carbowax 1540 on Chromosorb W, 80–100 mesh. Sample: 3 ml gas. Identified peaks: 1 = acetone; 3 = acetaldehyde; 5 = ethyl acetate; 6 = ethanol; 9 = isobutanol; 13 = isoamyl alcohols. 5 mV recorder with attenuation × 5; full scale response corresponds to $1.25 \cdot 10^{-11}$ A.

CAPILLARY (GOLAY) COLUMNS

Dual column systems can easily be adopted for use with open tubular (Golay) columns. When working with these columns of "capillary" dimensions (0.010 in. I.D.), the flow rates are usually less than 5 ml/min. Such low flows prevent the use of standard flow controllers. It was found, however, that base line compensation is possible using independent pressure regulation on each column. A previous paper[10] showed that under proper conditions, the change in the carrier gas flow during programming does not influence the quantitative analysis if constant pressure drop is maintained through the column, when working with FID.

The modification of the standard system for operation with capillary columns is shown in Fig. 14. The reference capillary column is connected directly to an independent source of regulated carrier gas. A linear stream splitter is installed between one injection port and the analytical capillary column. The outlet of this split system is connected to the second injection port normally used for the reference column. Split ratios are easily obtained by inserting various gauge calibrated needles into the septum of the second injection port.

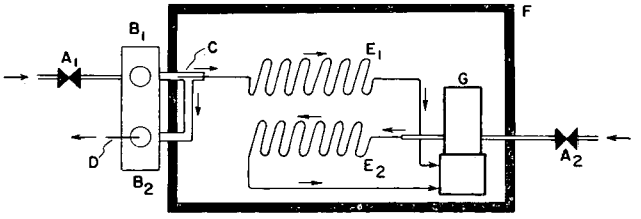

Fig. 14. Flow scheme of the dual column–differential FID gas chromatograph for capillary columns. (A_1, A_2) carrier gas pressure regulators in the analytical and reference gas lines; (B_1) sample introduction port; (B_2) injection block in the former reference line; (C) linear stream splitter; (D) interchangeable restrictor at vent; (E_1, E_2) analytical and reference columns; (F) column oven compartment; (G) differential FID, the construction of which is identical to that shown in Fig. 5.

The technique of dual column compensation with capillary columns is identical to that described previously for packed columns except for the use of pressure regulation in place of flow controllers. With Golay columns of larger diameters, standard flow regulators can again be utilized.

Figs. 15 and 16 illustrate the difference between uncompensated single column and compensated dual columns when analyzing commercial gasoline. The columns used were 150 ft. × 0.010 in. I.D. and coated with squalane.

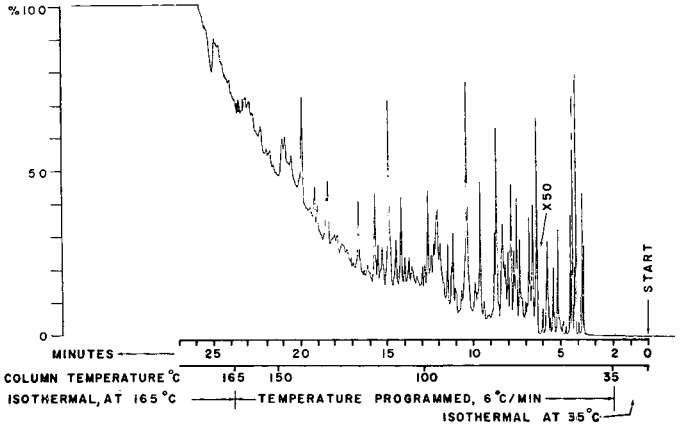

Fig. 15. Analysis of a commercial gasoline sample. Single column operation. 150 ft. × 0.010 in. I.D. capillary column coated with squalane as liquid phase. Injection block: 240°. Sample: 1 μl, split ratio about 1:500. 5 mV recorder with given attenuations; with attenuation × 50, full scale response corresponds to $1.25 \cdot 10^{-10}$ A.

QUANTITATIVE ASPECTS

In order to demonstrate the quantitative accuracy of a dual column–differential flame ionization detector system, a mixture of fatty acid methyl esters with known concentration was selected. The sample was a standard of the National Institutes of Health, obtained from the Applied Science Laboratories, University Park, Pa. The conditions were as follows:

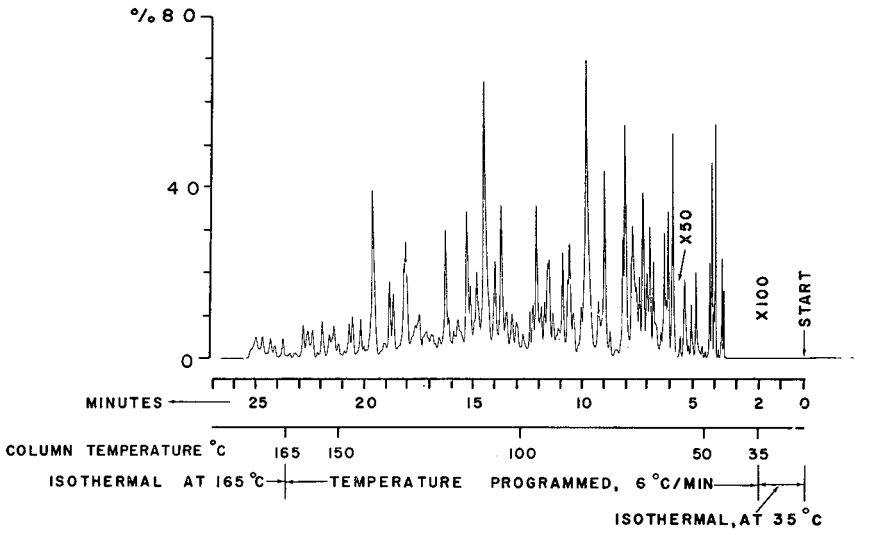

Fig. 16. Analysis of a commercial gasoline sample. Dual column operation. Each column, conditions and sample as given in Fig. 15.

Column: 6 ft. × 0.085 in. I.D. packed columns containing 8 wt.-% butanediol succinate on Chromosorb W, 80–100 mesh.

Injection block temperature: 300°.

Column temperature: (a) Isothermal at 180°.
(b) Programmed 150–210° at 2°/min.
(c) Programmed 150–210° at 4°/min.

Sample volume: 0.2 μl.

Gas inlet pressures and flow rates: (a) Carrier gas (He): 50 p.s.i.g., 30 ml/min.
(b) Air: 40 p.s.i.g., 500 ml/min.
(c) Hydrogen: 12 p.s.i.g., 33 ml/min.

Table II summarizes the analytical results. The isothermal and programmed results show a deviation of less than 0.5 %. The table also compares the analytical results with the original composition of the sample; these results confirm again that—as

TABLE II

QUANTITATIVE ACCURACY OF A DUAL COLUMN–DIFFERENTIAL FLAME IONIZATION DETECTOR SYSTEM

	Composition of sample (wt.-%)	*Isothermal at 180°*		*Programmed 150–210° at 2°/min*		*Programmed 150–210° at 4°/min*	
		Peak area (%)	*Deviation (%)*	*Peak area (%)*	*Deviation (%)*	*Peak area (%)*	*Deviation (%)*
Methyl myristate	11.83	12.08	+0.25	11.83	—	12.02	+0.19
Methyl palmitate	23.62	24.06	+0.44	23.74	+0.12	24.00	+0.38
Methyl palmitoleate	6.84	6.39	−0.45	6.60	−0.24	6.53	−0.31
Methyl stearate	13.09	12.58	−0.51	13.09	—	12.44	−0.65
Methyl oleate	44.62	44.89	+0.27	44.74	+0.12	45.01	+0.39

shown in a previous paper[11]—in the analysis of fatty acid methyl esters with flame ionization detection, the relative peak area values are very close to the actual concentration by weight of the sample when analyzing methyl esters with relatively high carbon number.

ACKNOWLEDGEMENTS

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SUMMARY

The influence of liquid phase bleeding on the analytical results and the possibilities for its compensation in programmed temperature for chromatography were discussed. A new differential FID was described; the dual column system incorporating this detector allows the use of both packed and open tubular (Golay) columns. The application of the system to a few problems was shown and its quantitative accuracy was demonstrated.

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SEPARATION AND DETERMINATION OF SEVERAL CHLORINATED PHENOXY-ACIDS BY GAS–LIQUID CHROMATOGRAPHY

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Various chemical methods of analysis have been developed for chlorinated phenoxy-acids, such as 2,4-dichlorophenoxyacetic acid (2,4-D), involving the cleavage of the phenyl ether linkage with pyridine and subsequent colorimetric determination of the chlorinated phenol[1]. Recently 2,4-D and related compounds have been determined quantitatively by gas-liquid chromatography combined with a microcoulometric detector[2–5]. Although considerable work has been done on the quantitative determination of microamounts of single phenoxy-acids, no attempt was made to determine closely related phenoxy-acids from a mixture with sufficient sensitivity and specificity. For this reason two methods have now been developed for the simultaneous determination of three closely related phenoxy-acids combining gas–liquid chromatography of the methyl esters with spectrophotometry or radioactive counting technique.

EXPERIMENTAL

Gas–liquid chromatography

The phenoxy-acids were separated by gas–liquid chromatography as their respective methyl esters. To a 1-ml solution in benzene containing 10 mg each of 2,4-D, 2,4,5-T, and PCPA*** was added 1 ml of diazomethane solution in diethyl ether prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wis.) as described previously[6,7]. Carbon-14 methyl esters of the phenoxy-acids were prepared from ^{14}C-Diazald (New England Nuclear Corp., Boston 18, Mass.). Aliquots of freshly prepared ester solutions were chromatographed on an Aerograph Model A-90C instrument, using a 6-ft. long, $^1/_4$ in. O.D. copper tubing, packed with 20% (w/w) Dow-11 high-vacuum silicone grease on 30/60-mesh Chromosorb P at a column temperature of 210° and a He gas flow of 60 ml per min. Fractions were manually collected at the predetermined retention times of the respective methyl esters using a 1.0 × 14.0 cm glass tube fitted with a 7/15 S/T outer joint and containing a small wad of glass wool saturated with the appropriate solvent as specified below[8].

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* Parts of this paper were taken from the M.S. thesis of DAVID L. GUTNICK, University of California, Davis, Calif., 1963.

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*** Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; 2,4,5-T = 2,4,5-trichlorophenoxyacetic acid; PCPA = 4-chlorophenoxyacetic acid.

Spectrophotometric analysis

This method is based on the qualitative test for esters as the red Fe (III) complex of the hydroxamates[9].

(*a*) *Preparation of reagents.* About 3 g of reagent grade hydroxylamine hydrochloride (Eastman Chemicals Co.) was placed in a test tube, and 15 ml of absolute ethanol added. The solution was allowed to boil on a steam bath for 10 min. About 6.8 g of KOH was dissolved in 20 ml absolute ethanol in a 100-ml Erlenmeyer flask and boiled on a steam bath for 10 min.

(*b*) *Colorimetric determination.* One hundred μg of the methyl esters was dissolved in 1 ml of absolute ethanol in a test tube. Gentle heating was applied to bring the material into solution. Four-tenths milliliter of the alcoholic hydroxylamine hydrochloride solution was pipetted into the test tube followed by about 0.9 ml of the KOH solution or until the pH was greater than 10. The mixture was boiled on a steam bath for 1 min with stirring, and 2.5 ml of 1 *N* HCl was added until the pH was less than 1. The reaction mixture was cooled in an ice bath, and 1 ml of 1% (w/v) aqueous ferric chloride added. The test tube was shaken for 10 sec, the solution was transferred to a 10-cm path length spectrophotometric cell, and the absorbance at 500 mμ was measured with a Beckman DU spectrophotometer using a reagent blank as reference. The total volume of the final solution was 5.8 ml.

(*c*) *Preparation of the standard curve.* A stock solution of each of the methyl esters was prepared by adding 50 mg of each of the methyl esters to a 50-ml volumetric flask and diluting to volume with absolute ethanol. Aliquots of 100, 50, 30 and 20 μl (100, 50, 30 and 20 μg, respectively) were pipetted into test tubes and the volume of each was made up to 1 ml with absolute ethanol. The colorimetric reactions were carried out as above, and the absorbancies were measured at 500 mμ immediately and plotted against micrograms PCPA, 2,4-D, and 2,4,5-T.

(*d*) *Gas–liquid chromatography followed by colorimetric analysis.* In order to determine the percentage recovery of the methyl esters of the three phenoxy acids from the gas chromatographic column, 100 μg of each of the methyl esters was injected into the gas chromatograph and collected manually over glass wool saturated with absolute ethanol. The condensed material was eluted with 1 ml of absolute ethanol into test tubes and the colorimetric analysis was performed as above. The absorbance at 500 mμ was compared with the absorbance for 100 μg from the standard curve and the percentage recovery calculated.

Analysis of radioactive derivative

An alternate method of analysis was the separation of ^{14}C-methyl esters of the phenoxy-acids by gas–liquid chromatography and the radioassay of the collected fractions by liquid-scintillation spectrometry.

(*a*) *Preparation of stock solution of ^{14}C-methyl esters.* 1 ml containing 1 mg of each of the phenoxy-acids was pipetted into a graduated centrifuge tube and 1 ml of ethereal diazomethane-^{14}C was added. A freshly prepared solution of ^{14}C-diazomethane had a specific activity of about $1.75 \cdot 10^5$ d.p.m. per ml. The solution was gently swirled for 5 min at room temperature and evaporated under an air stream to a volume of 1 ml. The ^{14}C-methyl esters thus prepared were stored in the refrigerator until further use.

(*b*) *Counting technique.* A Tri-Carb semi-automatic liquid scintillation spectrometer (Model 314-E, Packard Instruments, Inc.) was used to count the radioactive

samples. A non-aqueous counting solution was prepared by dissolving 4 g 2,5-diphenyloxazole (PPO, Eastman Chemicals Co.) and 50 mg 2,2-phenylene-bis-(5-phenyloxazole) (POPOP, Arapahoe Chemical Corp.) in 1 l redistilled toluene. The solutions were counted in 20 ml potassium-free counting vials fitted with disposable screw caps. The spectrometer was operated at a tap setting of 2–425 (825 V).

(c) *Calibration curve.* Aliquots of the ^{14}C-methyl esters (5-30 μg) were separated by gas–liquid chromatography, and each ester was collected for a period of 3 min, 1 min before and after its respective retention time. The collector contained glass wool soaked in liquid phosphor counting solution (see above). The condensed methyl esters were washed from the collector into a counting vial with 10 ml additional liquid phosphor solution, and the total volume was made up to about 15 ml with additional phosphor solution. One ml of benzene alone was treated in the same manner as the ester solution and served as a blank. For quantities of esters that were too low to give sufficient response by thermal conductivity (5, 10 μg respectively) 100 μg of the non-radioactive compounds were mixed with the radioactive material as internal standards. Calibration curves were prepared by plotting counts per minute against μg phenoxy-acid. A calibration curve was prepared for each new batch of diazomethane.

RESULTS AND DISCUSSION

Gas–liquid chromatography

The separation of the methyl esters of PCPA, 2,4-D, and 2,4,5-T is shown in Fig. 1. The retention times under the stated conditions (see EXPERIMENTAL and caption under Fig. 1)

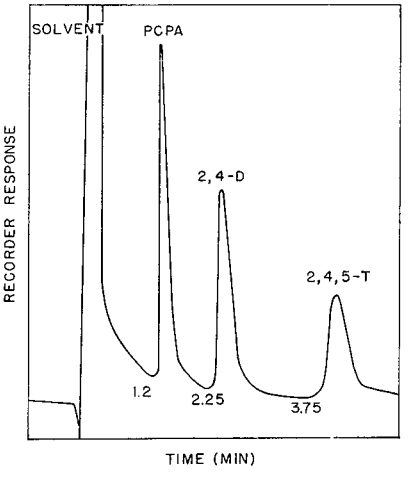

Fig. 1. Gas chromatography of methyl esters of PCPA, 2,4-D and 2,4,5-T on a Dow 11 high vacuum silicone column (20% on 30–60 mesh chromosorb) at 210°.

were 1.25, 2.25, and 3.75 min for PCPA, 2,4-D, and 2,4,5-T, respectively. The response of the thermal conductivity detector (hot-wire katharometer) of the Aerograph 90C instrument was directly proportional to the amount of esters chromatographed over the range from 25 to 100 μg. The esters of 2,4-D and PCPA had about the same thermal response, while the detectability for 2,4,5-T was reduced by about 30%.

Yield of esterification

The yield of esterification was determined by microcoulometric analysis[10], and the results are found in Table I. The percent recovery of the esterification process was determined for four different samples of diazomethane and the results represent aver-

TABLE I

YIELD OF ESTERIFICATION AS DETERMINED BY MICROCOULOMETRIC METHODS[10, *]

Compound	*Peak area (sq. in.)*	*Wt. of ester recovered (μg**)*	*Wt. of acid (μg)*	*% Recovery*
2,4,5-T	1.70	3.06	2.89	96.3
2,4-D	1.29	3.03	2.84	94.7
PCPA	0.81	3.20	2.96	98.7

* Average of two independent experiments in which 3 μg-quantities were esterified.
** Micrograms chlorinated phenoxy acid =

$$\frac{\left(\begin{matrix}\text{peak}\\ \text{area}\end{matrix}\right) \times \left(\begin{matrix}\text{recorder}\\ \text{sensitivity}\end{matrix}\right) \times 35.5\,\frac{\text{g}}{\text{equiv.}} \times 60\,\frac{\text{sec}}{\text{min}} \times 10^{6}\,\frac{\mu\text{g}}{\text{g}} \times 10^{-3}\,\frac{\text{V}}{\text{mV}} \times 10^{2}}{\left(\begin{matrix}\text{recorder input}\\ \text{resistance, } \Omega\end{matrix}\right) \times (\%\ \text{chlorine in pesticide}) \times 96{,}500\,\frac{\text{C}}{\text{equiv.}}}$$

Recorder sensitivity = 1.09 mV./in.
Recorder input resistance = 64 Ω.

ages of these four separate analyses. As may be seen, 3 μg of each ester was recovered almost quantitatively but lower yields might have been caused by volatilization of the methyl esters during evaporation.

VORBECK *et al.*[11] have reported on a comparison of methylation techniques. In addition to diazomethane, they employed methanol–hydrochloric acid with microsublimation, methanol– hydrochloric acid on ion exchange resin, and methanol–boron-trifluoride. Diazomethanolysis was found to be the most efficient technique in the preparation of the methyl esters of low molecular weight fatty acids, and for this reason it was chosen as the methylating agent in these studies.

The use of microcoulometry in determining the yield of esterification had the advantage of providing a true electrochemical measurement of the amount of chloride in the sample. The quantitative application of thermal conductivity is by necessity a relative process. However, the combination of gas–liquid chromatography and microcoulometry[10] permits the effluent fractions to be combusted to hydrochloric acid which in turn is titrated with Ag^+ ions.

Stability of esters during gas–liquid chromatography

Fig. 2 illustrates infrared spectra of 2,4-D methyl ester before and after gas–liquid chromatography. The primary bands in the infrared spectra are: aliphatic C–H = 3.4 μ, C = O ester = 5.7 μ, C_6H_5–O–C = 8.26 and 9.25 μ. Although it is difficult to deduce the exact structure from the infrared data, it would seem apparent from the data that

no structural changes in the molecule took place as a result of gas–liquid chromatography. Similar results were observed with the methyl esters of PCPA and 2,4,5-T. Extreme care was taken to omit contamination such as moisture, which might have interfered with the spectra.

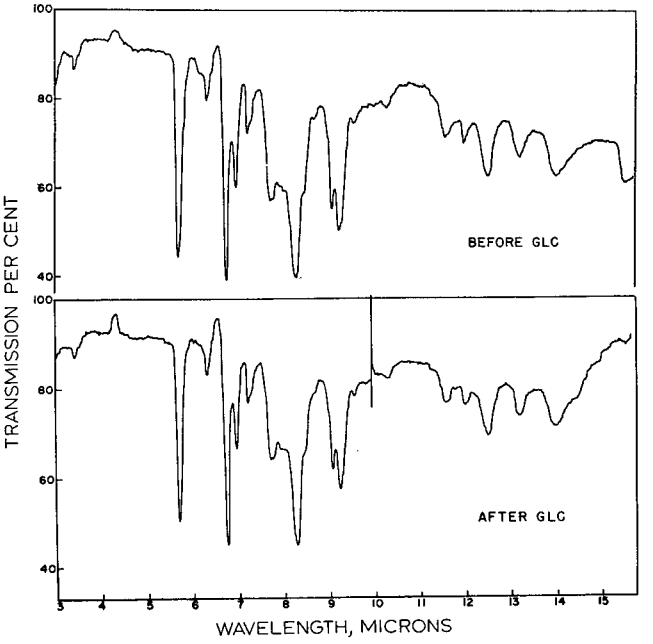

Fig. 2. Infra-red absorption spectra of 2,4-D methyl ester before and after gas–liquid chromatography.

Colorimetric analysis

The technique represents a modification of a qualitative determination of esters[9]. It was necessary, however, to use 10-cm path length cells for the spectrophotometric analysis owing to the low absorbance of the color. The minimum quantity, when

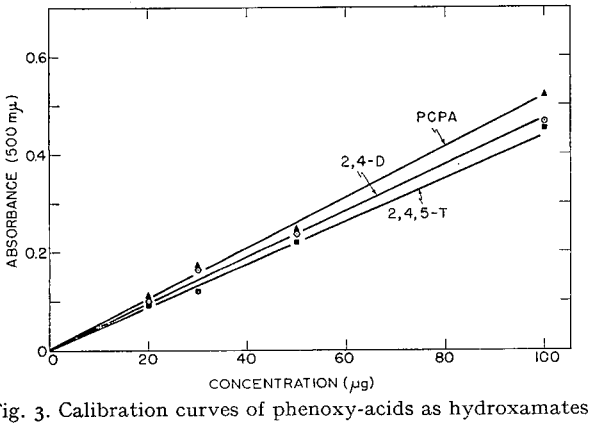

Fig. 3. Calibration curves of phenoxy-acids as hydroxamates.

analyzed in a 1-cm cell was about 200 μg of phenoxy-acid, while with a 10-cm long optical path, the least detectable amount was 20 μg.

The pH requirements for the stability of the color as determined by HESTRIN[12] were followed, but the color remained stable for only 4 min before degrading rapidly, making this method less desirable than the radiotracer technique discussed below.

The linear relationship between absorbance of the color complex and the quantity of phenoxy-acid analyzed is illustrated in Fig. 3. Within the 20 to 100 μg range, the standard curve obeys Beer's Law. The sensitivity of this method compares with that of the gas chromatography–thermal conductivity analysis. Since the direct colorimetric technique is non-specific, it was examined in conjunction with gas chromatography as a method of separating the phenoxy-acids from other compounds containing carboxyl groups prior to the colorimetric analysis.

The data in Table II show the percentage recovery of 100 μg of each of the acids from a mixture of PCPA, 2,4,-D, and 2,4,5-T. Recovery of each component by itself

TABLE II

RECOVERY OF PCPA, 2,4-D, AND 2,4,5-T FROM A MIXTURE OF THE THREE BY COMBINATION GAS–LIQUID CHROMATOGRAPHY AND COLORIMETRY*

Compound	*Absorbance*		*Quantity recovered (μg)*	
	1	*2*	*1*	*2*
PCPA	0.463	0.497	89.2	97.6
2,4-D	0.455	0.429	93.2	88.0
2,4,5-T	0.427	0.389	95.2	86.4
Average % recovery = 91.6% ± 4.5%.				

* A mixture of each of the three esters (100 μg of each) was injected into the gas chromatographic column and the fractions were collected at the respective retention times. The colorimetric technique was then applied to the analysis of each sample.

and from a mixture was over 90%. In all cases, the color was measured in the spectrophotometer within 4 min after development. The precision of the method as calculated by the standard deviation was ± 3.1%.

Radioactive analysis

Calibration curve. Data illustrating the linearity of the radioisotope assay are presented in Fig. 4. It was found that the background varies both with the type of compound and with the quantity of acid analyzed. A small amount of radioactivity remained on the column after each methyl ester was collected. Before each compound was chromatographed, therefore, the collector was placed on the exit port for 5 min, the effluent stream was collected, eluted from the collector, and a background count was taken. This background count ranging from 100–300 c.p.m. was subtracted from the total count. The standard deviation of each net count represented the error in the counting instrument and is the square root of the count[13]. It may be seen from Fig. 4 that with the radioactive-derivative method an ultimate sensitivity of 0.5 μg or less of each ester may be achieved; 5 μg of the esters of the three phenoxy-acids studied gave counts ranging from 1957 to 2683 c.p.m. at 57% counting efficiency of the liquid scintillation spectrometer.

Mixtures of the three ^{14}C-methyl esters were chromatographed and each respective fraction was collected and counted. It was found that quantitative recoveries could be made as shown in Table III, and the same standard curves plotted as for single com-

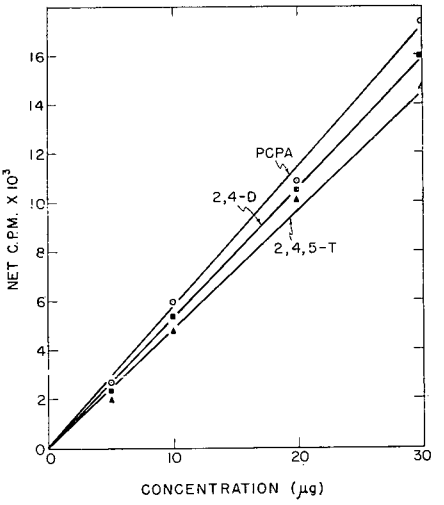

Fig. 4. Calibration curve for determination of phenoxy-acids by combination of gas–liquid chromatography and radioisotope technique.

ponents (Fig. 4). Overall comparison of the analytical techniques described led to the conclusion that the radioisotope analysis was more sensitive than either the colorimetric or thermal conductivity methods.

TABLE III

RECOVERY OF DIFFERENT QUANTITIES OF PHENOXY-ACIDS FROM A MIXTURE BY RADIOISOTOPE TECHNIQUE

	Fraction	% *Recovery*	% *Standard deviation*	*Number of determinations*
1	(PCPA)	91.6	8.9	15
2	(2,4-D)	95.3	6.2	16
3	(2,4,5-T)	96.7	6.8	16

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SUMMARY

Two procedures for the quantitative determination of micro-amounts of 2,4-dichloro-, 2,4,5-trichloro-, and 4-chlorophenoxyacetic acids are described. The first procedure is

based on the separation of the methyl esters by gas–liquid chromatography and the colorimetric determination of collected fractions as the hydroxymate–Fe (III) complex. The second procedure involves the formation of the ^{14}C-methyl esters, their separation by gas–liquid chromatography and the radioassay of collected fractions by liquid-scintillation spectrometry.

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SÉPARATION DES AMINES PAR CHROMATOGRAPHIE GAZ–LIQUIDE EN UTILISANT LE TEFLON COMME SUPPORT

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INTRODUCTION

L'analyse des amines par chromatographie gaz–liquide a fait l'objet de nombreuses publications dont les premières remontent aux origines de la méthode, qui fut appliquée à cette séparation par JAMES ET MARTIN[1,2] dès 1952. Les difficultés rencontrées alors dans la séparation de mélanges d'amines ou d'amino-alcools et de composés analogues ont été difficilement surmontées et les méthodes d'analyses de tels mélanges sont loin d'être aussi bonnes que celles dont on dispose pour quantité d'autres dérivés fonctionnels. Ces difficultés proviennent des traînées importantes causées par l'adsorption sur le support que présentent les pics élués des colonnes faites à partir des supports classiques (célite, chromosorb). La forte adsorption des amines est due à leur grande polarité et au caractère basique de l'azote qui peut conduire à la formation de liaisons hydrogène remarquablement fortes. C'est ainsi qu'on observe dans certains cas la disparition totale du pic lorsque la quantité de produit pur injectée est réduite au-dessous d'une certaine valeur[3]. Les volumes de rétention observés ne sont plus alors caractéristiques car ils varient beaucoup avec la masse injectée. De nombreux auteurs ont décrit des traitements variés du support utilisé qui, avec des variantes de détail, consistent essentiellement à traiter le chromosorb W par la potasse alcoolique[4–6] ou la célite par la soude alcoolique[1,3,7,8], comme l'avaient fait JAMES ET MARTIN. La concentration de la solution à utiliser ne semble pas critique, l'essentiel est de ne pas se contenter d'un lavage mais d'introduire dans le support une quantité de base supérieure à celle nécessaire pour assurer la neutralisation complète des sites actifs[5]. On a également préconisé l'emploi comme support du produit obtenu par extraction à l'éther de pétrole de la lessive "Tide" et traitement à la potasse alcoolique[9]. L'emploi de supports ainsi traités permet d'utiliser des phases stationnaires peu polaires telles que l'huile de paraffine et l'huile de silicone[2] mais, le plus souvent, il est nécessaire, pour obtenir de bons résultats, d'utiliser des mélanges de phase polaire et apolaire[1,6,10], tels que l'huile de paraffine et le 5-éthylnonanol-2[1]: ceci n'est pas très favorable à l'obtention de résultats bien reproductibles; il semble préférable d'employer des phases polaires sur lesquelles les traînées sont réduites[6,11], la triéthanolamine par exemple. Le traitement du support par silanisation semble donner de bons résultats dans certains cas[10,12], mais ce traitement est très délicat et il ne semble pas avoir été appliqué au cas des amines aliphatiques. On peut enfin combattre les phénomènes

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d'adsorption en utilisant un gaz vecteur contenant de la vapeur d'eau, de l'ammoniac ou une amine légère[10]. Ce procédé est d'une application délicate et ne semble pas pouvoir être généralisé.

La poudre à mouler de téflon n'a été que très peu utilisée jusqu'à présent en raison des grandes difficultés que présente la fabrication de colonnes suffisamment efficaces. Dans une étude antérieure[13], nous avons décrit le mode de fabrication de colonnes utilisant ce support et donnant des efficacités environ moitié de celles des colonnes classiques pour des composés non polaires, mais bien supérieures dans le cas des amines en particulier. L'avantage du téflon, qui est presque chimiquement inactif et n'adsorbe que très peu, est alors pleinement utilisable.

MÉTHODE D'IDENTIFICATION

Les mélanges d'amines à analyser sont souvent très complexes: on y trouve des amines primaires, secondaires et tertiaires, parfois des diamines ou de l'ammoniac, souvent des composés oxygénés (amino-alcools) et de l'eau.

Il convient donc d'abord de disposer de colonnes assez efficaces pour résoudre de tels mélanges. Il faudra ensuite identifier les composés correspondants et, pour cela, caractériser d'abord leur fonction. Nous avons utilisé dans ce but les indices de rétention de KOVATS[14,15]: l'incrément d'indice ou différence entre les indices de rétention mesurés sur une phase polaire et sur une phase apolaire est caractéristique, dans certaines limites, d'un type de dérivé fonctionnel. La comparaison entre l'indice mesuré pour un corps inconnu et ceux déterminés pour les dérivés du même type permet alors l'identification.

Nous avons préparé dans ce but deux colonnes de polarités différentes, l'une apolaire avec de l'Apiézon L, l'autre polaire avec un Polyglycol 1500*. La polarité de cette phase est relativement grande: 0.76 dans l'échelle arbitrairement choisie par CHOVIN ET LEBBE[16] où le squalane a une polarité nulle et le β,β'-oxydipropionitrile une polarité égale à 1. Cette polarité est due au grand nombre de groupes hydroxyles libres, susceptibles de former des liaisons hydrogène avec les solutés basiques, en particulier avec les amines.

Interprétation théorique des résultats

L'indice de rétention est donné par la formule de KOVATS[3]:

$$I = 200 \frac{\log V_N(\mathrm{X}) - \log V_N(n\mathrm{P}_z)}{\log V_N(n\mathrm{P}_{z+2}) - \log V_N(n\mathrm{P}_z)} + 100\,z$$

où $V_N(\mathrm{X})$ désigne le volume de rétention totalement corrigé du composé X, $n\mathrm{P}_z$ et $n\mathrm{P}_{z+2}$ désignant les paraffines normales paires éluées juste avant et après le composé X.

Les propriétés intéressantes des indices de rétention résultent directement de la formule:

(i) Ils varient peu avec la température. Si cette variation est en toute rigueur représentée par une fonction homographique[17], dans un domaine de température limité cette variation est à peu près linéaire.

* Polyglycol 60-U, K-35, Naphtachimie, 203, Fbg. Saint-Honoré, Paris (8ème).

(ii) L'indice de rétention ne dépend théoriquement pas de la quantité de phase stationnaire contenue dans la colonne, contrairement aux volumes de rétention absolus qui lui sont proportionnels. Cette conclusion doit être nuancée lorsque l'on utilise des solutés polaires et un support classique. L'adsorption sur le support joue parfois un rôle important dans la rétention de tels solutés malgré que l'on obtienne des pics à peu près symétriques. Un support inerte comme le téflon est tout particulièrement intéressant à ce point de vue.

(iii) La comparaison entre les indices de rétention d'un même corps sur deux colonnes de polarité très différente donne des renseignements assez précis sur sa structure, l'incrément d'indice pouvant être utilisé pour la caractérisation des dérivés fonctionnels.

(iv) Le volume de rétention absolu est lié au coefficient de partage K du soluté sur la phase fixe par la relation fondamentale:

$$V_N = K \cdot V_L$$

où V_L est le volume de phase fixe utilisé. Ce coefficient de partage est lui-même donné par:

$$K = e^{-\frac{\Delta\mu^\circ}{RT}}$$

où $\Delta\mu^\circ$ représente la différence de potentiel chimique du soluté entre l'état dissous dans la phase fixe et l'état vaporisé. Or, d'après MARTIN, l'enthalpie libre d'une molécule est la somme d'un certain nombre de contributions additives des différents radicaux qui la composent[18]. En particulier, cette enthalpie libre croît linéairement avec le nombre d'atomes de carbone en série homologue, sauf pour les tous premiers termes de certaines séries.

Il est donc normal de voir les indices de rétention de dérivés homologues, qui sont des combinaisons linéaires des logarithmes des volumes de rétention, croître linéairement avec le nombre d'atomes de carbone, l'incrément par atome de carbone (ΔCH_2) étant voisin de 100. Il aurait exactement cette valeur si le principe de l'indépendance des contributions des divers radicaux à l'enthalpie libre était rigoureusement vérifié, mais ce principe n'est qu'approché.

L'utilisation de ces données théoriques facilite considérablement le dépouillement et l'interprétation des résultats et, par consequent, l'identification des composés contenus dans les mélanges étudiés.

Conditions expérimentales

Les caractéristiques des colonnes utilisées sont résumées Tableau I. Les principales propriétés physiques des solutés intéressantes pour la discussion suivante sont données Tableau II. L'appareil utilisé est un Perkin-Elmer modèle 116 E.

La colonne apolaire est destinée principalement à la détermination de ΔI pour la caractérisation des solutés; la solubilité d'un soluté polaire comme ceux utilisés ici est très faible dans une phase apolaire. En effet, dans ce cas, la séparation se fait essentiellement par différence de tension de vapeur. Par contre, la solubilité dans une phase polaire de ces composés très polaires est élevée. Il en résulte un fort incrément d'indice de rétention et des difficultés pour mesurer avec précision l'indice de rétention sur les

TABLEAU I

CARACTÉRISTIQUES DES COLONNES

	Apolaire	*Polaire*
Longueur (cm)	226	227
Diamètre intérieur du tube (cm)	0.4	0.4
Granulométrie du support (téflon-Haloport) (μ)	200–500	200–500
Poids de support utilisé (g)	24	13.8
Phase liquide	Apiézon L	Polyglycol 1500
Poids de phase liquide (g)	1	3.44
Teneur en phase (%)	4.4	20
HETP pour le méthanol (cm)	0.75	0.24
Valeur de k correspondante*	1.2	4.9
HETP pour l'éthylamine (cm)	0.69	0.56
Valeur de k correspondante	0.45	8.04

*$k = K \cdot V_G/V_L$

deux phases à la même température. Ce phénomène est aggravé par la différence de teneur en phase des colonnes apolaire et polaire qui joue dans un sens défavorable, quoique partiellement compensée par la plus grande densité de remplissage de la colonne apolaire. Malheureusement, il n'a pas été possible de préparer une colonne apolaire qui ait une efficacité satisfaisante avec une phase stationnaire contenant plus de 5 % d'Apiézon L.

Nous avons pu, néanmoins, au prix de quelques difficultés, déterminer l'ensemble des indices de rétention à 130°C sur les deux colonnes; les indices sur la colonne apolaire ont été mesurés entre 70° et 130° et l'on a constaté une très faible variation des indices en fonction de la température dans ce domaine: $10(\delta I_A/\delta T)$ dans le cas le plus défavorable est inférieur à 3, alors que l'erreur de mesure est comprise entre 0.5 et 1%, c'est-à-dire est du même ordre de grandeur.

TABLEAU II

PROPRIÉTÉS PHYSIQUES DES SOLUTÉS

	Moment dipolaire[19] *(Debye)*	T_{eb} *(°C)*[20]
Ammoniac	1.44	—33.3
Monométhylamine	1.24	— 6.5
Diméthylamine	1.03	6.9
Triméthylamine	0.67	3.9
Monoéthylamine	1.22	16.6
Diéthylamine	0.92	55.5
Triéthylamine	0.66	89.5
Propylamine	1.17	47.8

Sur la colonne polaire, au contraire, la rétention des amines étant très forte, il serait très difficile de travailler au-dessous de 130°, sauf pour les tout premiers termes des diverses séries. Les mesures ont été faites entre 130° et 150°C.

Résultats expérimentaux

Le Tableau III donne les indices de rétention mesurés à 130° pour les deux colonnes ainsi que l'incrément d'indice résultant. Ces indices ont été mesurés pour les amines primaires dérivés d'hydrocarbures normaux jusqu'à l'octane, les amines secondaires et tertiaires symétriques dérivées d'hydrocarbures normaux jusqu'au butane ainsi que pour les alcools normaux jusqu'au *n*-hexanol et pour l'eau.

TABLEAU III

INDICES DE RÉTENTION MESURÉS À 130°C POUR LES DEUX COLONNES

Soluté	*Indice sur phase polaire*	*Indice sur phase apolaire*	*ΔI*
Eau	1058	317	741
Ammoniac	560	250	310
Alcools			
Méthanol	892	292	600
Ethanol	934	375	559
Propanol-1	1034	497	537
Butanol-1	1140	606	534
Pentanol-1	1245	705	540
Hexanol-1	1342	811	531
Amines primaires			
Méthylamine	709	400	309
Ethylamine	750	441	309
n-Propylamine	826	518	308
n-Butylamine	919	614	305
n-Pentylamine	—	—	—
n-Hexylamine	1145	827	318
n-Heptylamine	1251	912	339
n-Octylamine	1344	1022	322
Amines secondaires			
Diméthylamine	752	458	294
Diéthylamine	763	553	210
Di-*n*-propylamine	925	746	179
Di-*n*-butylamine	1098	948	150
Amines tertiaires			
Triméthylamine	589	536	53
Triéthylamine	765	727	38
Tri-*n*-propylamine	958	917	41
Tri-*n*-butylamine	1187	1163	24

En examinant le Tableau III, on constate, aussi bien pour les alcools que pour les diverses amines, la régularité de variation des indices de rétention sur colonne polaire ou apolaire, avec le nombre d'atomes de carbone de chaque radical, sauf pour les premiers termes (Fig. 1).

Ceci confirme les hypothèses faites sur l'indépendance des contributions des différents radicaux à l'enthalpie libre de la molécule. Sauf pour les valeurs de n inférieures à 3, on peut ainsi mettre l'indice de rétention sous la forme:

$$I = R + n\Delta CH_2$$

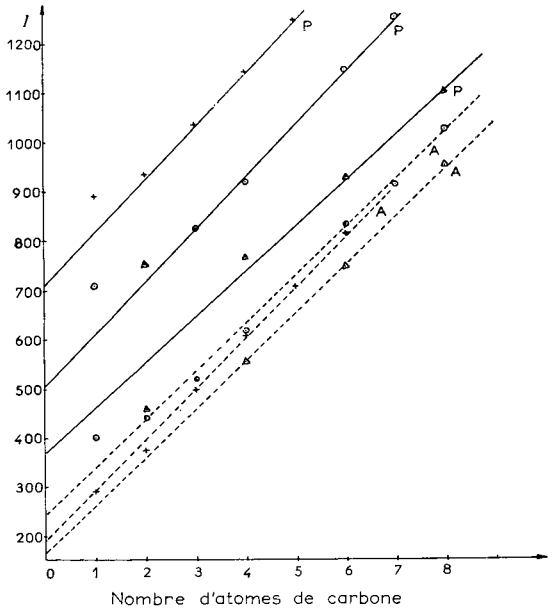

Fig. 1. Variation de l'indice de rétention en fonction du nombre d'atomes de carbone dans la chaîne alkyl. (+) Alcools primaires; (⊙) amines primaires; (△) amines secondaires. P = Colonne polaire (Polyglycol 1500). A = Colonne apolaire (Apiézon L).

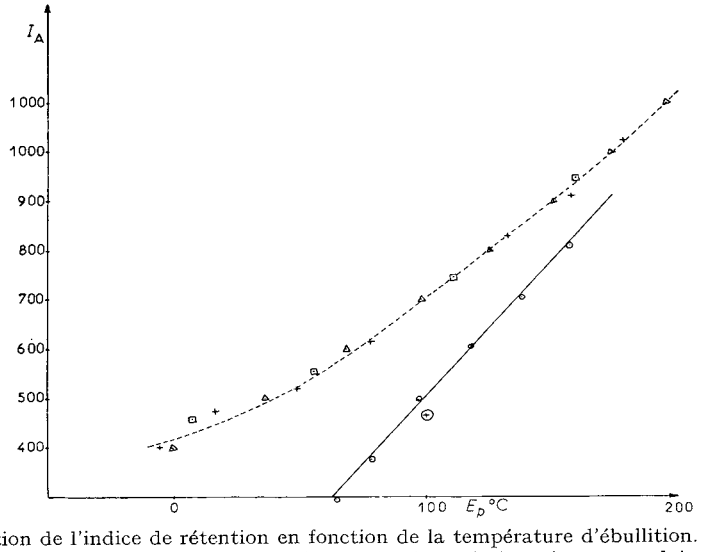

Fig. 2. Variation de l'indice de rétention en fonction de la température d'ébullition. (△) Alcanes normaux; (⊙) alcools normaux; (+) amines primaires; (⊡) amines secondaires; (⊕) eau.

n étant le nombre d'atomes de carbone contenus dans l'ensemble des chaînes. Les valeurs de R et ΔCH_2 sont données Tableau IV. L'expérience acquise en ce domaine montre que l'on peut, en cas de besoin, extrapoler ces valeurs dans une mesure raisonnable[21].

Si l'on trace un graphique en portant l'indice de rétention en fonction de la température d'ébullition, on obtient pour l'indice apolaire une courbe unique pour les hydrocarbures paraffiniques et les diverses amines (Fig. 2). Ce résultat confirme avec des corps très polaires les résultats de KOVATS[15] et HUGUET[22]; il n'est toutefois pas possible de donner les valeurs de R et ΔCH_2 pour les amines tertiaires, la variation de I avec le nombre d'atomes de carbone n'étant pas régulière: il faudrait, pour obtenir un résultat, utiliser des produits plus lourds ayant des chaînes de plus de quatre atomes de carbone. On peut en déduire que l'adsorption résiduelle des amines sur le téflon est faible, sinon on mesurerait des indices apolaires trop forts et on observerait une courbe décalée. Les seules exceptions sont la triméthylamine et surtout l'eau et les alcools, pour lesquels on retrouve un phénomène déjà signalé par KOVATS[15]: les associations moléculaires qui jouent dans le soluté à l'état pur et provoquent un abaissement important de la pression de vapeur n'interviennent pas dans les interactions soluté–solvant à dilution infinie, lorsque le solvant est apolaire. Il en résulte une solubilité beaucoup plus faible que celle qui serait attendue de la seule considération de la tension de vapeur: même une phase fixe apolaire ne classe pas tous les composés selon les pressions de vapeur croissantes.

TABLEAU IV

CONTRIBUTIONS À L'INDICE DE RÉTENTION

	Amines primaires		*Amines secondaires*		*Alcools primaires*	
	ΔCH_2	$R(NH_2)$	ΔCH_2	$R(NH)$	ΔCH_2	$R(OH)$
Colonne polaire	105	505	90	370	104	712
Colonne apolaire	97	245	97	165	103	190
ΔI	8	260	—7	205	1	522

Le Tableau III montre également que les incréments d'indice des corps de chaque famille se groupent autour de valeurs très différentes pour les diverses familles, les différences entre homologues extrêmes d'une famille étant relativement faibles et correspondant à une légère diminution quand le poids moléculaire augmente. Ces valeurs sont réunies Tableau V. On voit que la caractérisation est immédiate à partir

TABLEAU V

INCRÉMENTS D'INDICE CARACTÉRISTIQUES DES DIVERSES FAMILLES ÉTUDIÉES

Famille	$\Delta I_A{}^P$
Alcools aliphatiques primaires	530–600
Amines aliphatiques primaires	300–340
Amines aliphatiques secondaires	150–200*
Amines aliphatiques tertiaires	20–50

* Sauf diméthylamine 294.

de la mesure de l'incrément d'indice pour un pic inconnu. Si l'on compare les indices de rétention des alcools mesurés sur Apiézon L par KOVATS, utilisant la célite comme support, à ceux que nous avons mesurés en utilisant la poudre de téflon, on ne constate que des différences minimes, entièrement imputables aux erreurs d'expériences, sauf peut-être pour le méthanol. En comparant les indices mesurés par KOVATS sur Apiézon L et ceux mesurés par nous[13] sur la même colonne polaire de Carbowax 1500 que celle ayant servi pour la présente étude, on constate que la présence éventuelle d'aldéhydes ou d'esters méthyliques ne pourrait conduire à des interférences qu'avec les amines secondaires.

CONCLUSION

Il ne faut pas s'étonner de trouver une relation linéaire entre indices de rétention et nombre d'atomes de carbone des dérivés homologues. De telles relations avaient déjà été mises en évidence par JAMES ET MARTIN[23] pour les logarithmes de volumes de rétention dont les indices sont des combinaisons linéaires. Il est toutefois remarquable que l on puisse aboutir à des règles simples de calcul a priori des indices de rétention de dérivés homologues, *ce qui constitue un avantage supplémentaire des indices de rétention.* On dispose ainsi d'une méthode rapide de caractérisation et d'identification des amines.

Grâce à un calcul simple, on peut prévoir la différence minimale nécessaire entre les indices de rétention de deux corps pour qu'ils soient séparés sur une colonne donnée[24]. La différence d'indice correspondant à une séparation de 2σ est fournie par l'équation:

$$\delta I(2\sigma) = \frac{T_K}{f_K\sqrt{N}}\left(1 + \frac{V_m}{V_N}\right)$$

où T_K = la température de la colonne (°K),
$f_K = 0.005757\ T_K d_z$,
$d_z = \log V_R^\circ\ (nP_{z+2}) - \log V_R^\circ\ (nP_z)$,
V_m = le volume mort de la colonne,
N = le nombre de plateaux théoriques de la colonne.

On peut chercher par exemple à appliquer cette équation à la séparation des éthylamines. Pour la monoéthylamine on a: $I^P_{130^\circ} = 750$; $\sqrt{N} = 20$; $V_N = 40.2$ cm³.

Par ailleurs, pour la colonne polaire

$V_m = 23.7$ cm³; $T_K = 403$°K; $d_z = 0.42$; donc:

$f_K = 0.005757 \times 403 \times 0.42 = 0.9744$

$$\delta I = \frac{403}{0.9744 \times 20}(1 + 0.59) \sim 33$$

et $\delta I(2\sigma) \sim 33$ unités d'indices.

On ne pourra séparer de la monoéthylamine que des composés ayant un indice différent de 30 points au moins. A 130°C, il est donc impossible de séparer sur la colonne polaire les corps suivants:

monoéthylamine	$I^P = 750$
diméthylamine	$I^P = 752$
diéthylamine	$I^P = 763$
triéthylamine	$I^P = 765$

Toutefois, la séparation de ces corps est possible à 70° sur la colonne polaire, les volumes de rétention étant alors beaucoup plus grands devant le volume mort de la colonne. La détermination de l'indice de rétention sur la colonne apolaire permet l'identification de ces composés à l'exception de la monoéthylamine (441) et de la diméthylamine (458) qui ne sont ainsi séparés sur aucune de nos deux colonnes. Il faudrait pour cela des colonnes ayant au moins quatre fois plus de plateaux théoriques.

La Fig. 3 montre un exemple des séparations que l'on peut obtenir avec la colonne polaire.

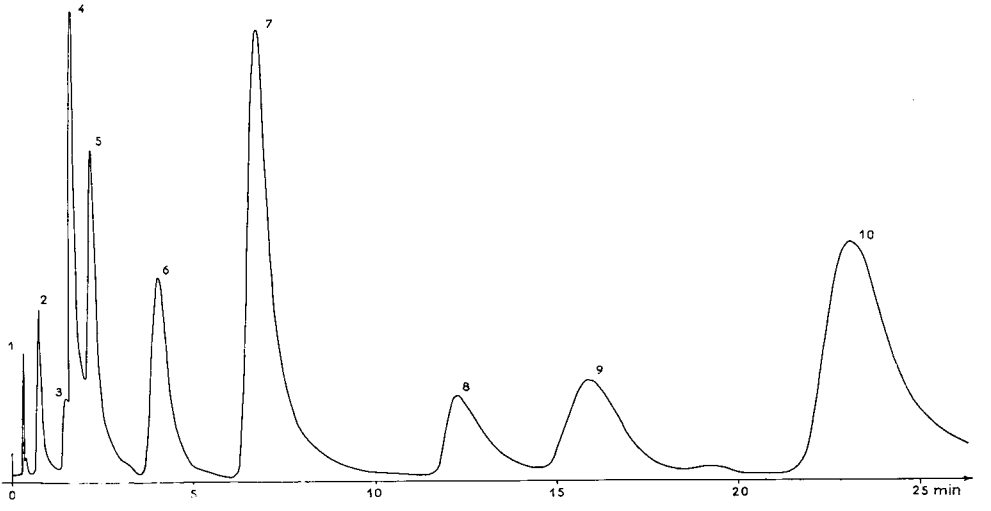

Fig. 3. Séparation d'un mélange d'amines aliphatiques (colonne polaire Tabl. I, 105°C, 70 cm³/min). (1) Air; (2) ammoniac; (3) méthylamine; (4) diéthylamine; (5) propylamine; (6) tripropylamine; (7) eau; (8) hexylamine; (9) tributylamine; (10) morpholine.

RÉSUMÉ

L'application à l'analyse des amines aliphatiques des colonnes utilisant la poudre de téflon comme support permet la séparation et l'identification rapide d'un grand nombre de ces composés. Les indices de rétention des dérivés homologues présentent des régularités que la théorie des solutions explique et qui permettent d'extrapoler dans une certaine mesure les valeurs obtenus et de guider sûrement dans l'interprétation des chromatogrammes.

SUMMARY

In the analysis of aliphatic amines it is possible to separate a great number of these compounds and identify them rapidly by using columns with powdered Teflon as support. The retention indices of homologous derivatives show regularities, which can be explained by the theory of solution, and which make it possible to extrapolate the values obtained, within certain limits. These regularities can also serve as a guide in the interpretation of the chromatograms.

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KETOACID POLYMERS AS GAS–LIQUID CHROMATOGRAPHY SUBSTRATES

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INTRODUCTION

Early analyses of volatile fatty acids depended on the addition of an organic acid to a relatively neutral substrate to reduce tailing of the peaks. Acids such as stearic[1] or sebacic[2] were employed, although bleeding of these materials led to short column life at higher temperatures[3]. Subsequently the introduction of phosphoric acid[4,5] as an additive, and of dimer and trimer acids as a substrate[6] permitted higher temperature operation. More recently formic acid vapor in the carrier gas has been shown to reduce adsorption of fatty acids on the support or in the substrate at both moderate[7] and elevated[8] temperatures.

Prior to the introduction of the latter procedure, and of the non-volatile acid additives or substrates, the need for a material combining high thermal stability with an acidity suitable for the reduction of tailing was evident. The development of keto-acid polymers, formed by techniques such as heating the cadmium salt of sebacic acid[9–12] suggested that this combination of a purely organic polymer with attached carboxyl groups would solve the problem of substrate thermal stability. This paper presents some results of an examination of a ketoacid polymer, primarily in the analysis of volatile fatty acids.

MATERIALS AND APPARATUS

A small sample of ketoacid polymer was prepared from the cadmium salt of sebacic acid by heating at 330° as described by PAQUOT *et al.* [9–12]. The first material crystallized from acetic acid (m.p. 156–159°) was not employed, but instead a second fraction (m.p. 103–110°), probably of much lower molecular weight, was precipitated by the addition of water to the acetic acid mother liquor. The latter sample was preferred since it should have a higher proportion of carboxyl groups. Only a small amount of material was available and the entire sample was used in the preparation of columns for gas–liquid chromatography. The molecular weight and the ratio of ketone to carboxyl groups could thus not be determined.

Two six-foot columns were prepared, of stainless steel tubing $^1/_8$ in. O.D. One contained 2.15 g of packing made up of 20% polymer on 60–80 mesh Chromosorb W, the other 3.59 g made up of 10% polymer on 60–80 mesh Gas-pack F (a teflon-impregnated support obtained from Chemical Research Services, Inc., Addison, Ill.).

Chloroform was used as a solvent in coating the support and was removed with stirring on a steam bath.

The columns were used in an apparatus fabricated in this laboratory based on a Wilkens flame ionization detector and electrometer, with an injection port of the type employed in the Wilkens A-90. Peak areas were measured by a pipping-pen integrator fitted to a 1 mV Minneapolis-Honeywell recorder. Owing to the fewer counts available than in a previous study[7] the deviations from average, although reasonably low in quantitative studies, were in some cases higher than those previously reported.

RESULTS AND DISCUSSION

Analysis of volatile fatty acids

Two mixtures of volatile fatty acids, at two different levels of concentration in water–acetone (2:1) solution, were analysed in triplicate on the two columns, in each case both with and without formic acid vapor in the carrier gas. Very good separations with minimal tailing of the peaks were obtained in all cases (Figs. 1 and 2), with no indication of double peak formation[7]. The average relative area responses given in Table I show that the ketoacid polymer gives quantitative results, comparable with previous analyses[7], on both columns when formic acid vapor is used in the carrier gas, and that with the teflon-impregnated support results can be obtained comparable with the previous data even without formic acid vapor in the carrier gas. The more

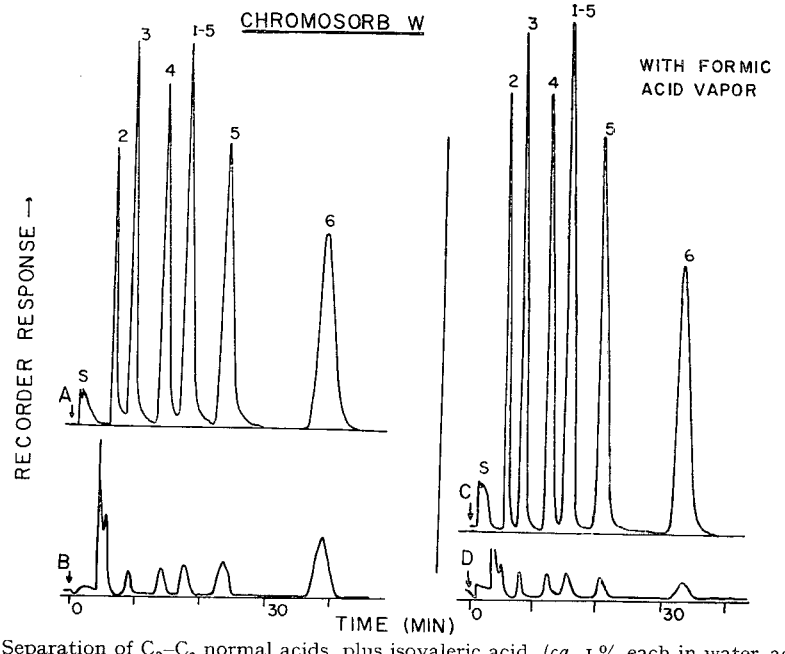

Fig. 1. Separation of C_2–C_6 normal acids, plus isovaleric acid, (*ca.* 1% each in water–acetone) on Chromosorb W column. A: plain carrier gas, 0.001 ml sample. B: subsequent injection of 0.001 ml 1:1 formic acid–water mixture. C: carrier gas containing formic acid vapor, 0.001 ml sample. D: subsequent injection of 0.001 ml 1:1 formic acid–water mixture. Helium carrier gas at approximately 15 p.s.i. in all analyses, column at 150°. Solvent peak (S) attenuated.

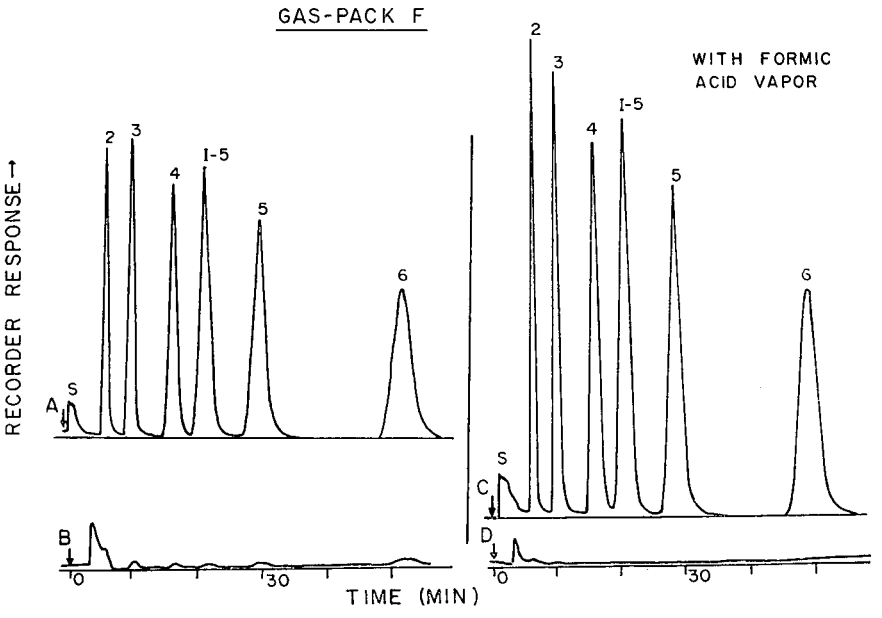

Fig. 2. Separation of C_2–C_6 normal acids, plus isovaleric acid, (*ca.* 1% each in water–acetone) on Gas-pack F column. A: plain carrier gas, 0.001 ml sample. B: subsequent injection of 0.001 ml 1:1 formic acid–water mixture. C: carrier gas containing formic acid vapor, 0.001 ml sample. D: subsequent injection of 0.001 ml 1:1 formic acid–water mixture. Helium carrier gas at approximately 15 p.s.i. in all analyses, column at 130°. Solvent peak (S) attenuated.

TABLE I

AREA RESPONSE, RELATIVE TO ISOVALERIC ACID AS 1.00, OF EQUAL WEIGHTS OF C_2–C_6 VOLATILE FATTY ACIDS, AT TWO DIFFERENT LEVELS OF CONCENTRATION

Analyses on two different ketoacid polymer columns as indicated by support, both with (F) and without (NF) formic acid vapor in the carrier gas

	Carrier gas	*Acid*					
		Acetic	*Propionic*	*Butyric*	*Valeric*	*Caproic*	*Isovaleric*
Acid (g/100 ml)		*1.0096*	*0.8496*	*0.8399*	*1.0286*	*1.0206*	*0.9992*
Chromosorb W	NF	0.33	0.72	0.89	0.97	0.99	1.00
	F	0.41	0.70	0.89	0.98	1.05	1.00
Gas-pack F	NF	0.39	0.70	0.87	0.96	1.05	1.00
	F	0.43	0.71	0.86	0.96	1.08	1.00
Acid (g/100 ml)		*0.061*	*0.053*	*0.065*	*0.083*	*0.089*	*0.077*
Chromosorb W	NF	0.22	0.53	0.89	0.98	1.03	1.00
	F	(0.30)*	0.68	0.86	1.00	1.13	1.00
Gas-pack F	NF	0.36	0.66	0.84	1.00	1.03	1.00
	F	0.35	0.72	0.82	0.97	1.10	1.00

* See discussion in text.

dilute acid solution (<0.1%) cannot be satisfactorily analysed for acetic and propionic acids on the Chromosorb W column unless formic acid vapor is present, although the more concentrated acid solution (*ca.* 1.0%) gives reasonably satisfactory results in repeated analyses on this support without formic acid.

The injection of formic acid–water (1:1) mixture subsequent to each series of analyses (Figs. 1 and 2) shows the extent to which the presence of formic acid vapor suppresses adsorption of acids on the column.

The behavior of the ketoacid polymer on the Chromosorb W support in this respect is somewhat similar to that previously observed with the silicone oil (with stearic acid) column[7]. The displacement of acids, on injection of formic acid–water mixture, even with formic acid vapor in the carrier gas, indicates that slightly more adsorption of acids has occurred. On the other hand, the employment of formic acid vapor in the carrier gas does not give a significant reduction in retention time, suggesting that most of the support is "covered" as with the more polar substrates. With acid solutions of varying concentration the acids displaced by formic acid–water injection are roughly in constant proportion to the preceding normal peak area, indicating that whatever adsorption takes place on the column is proportional to the amount of acid injected. For this reason precautions should be observed, as in all such analyses, to eliminate extraneous peaks from "ghosting"[13] when using a ketoacid polymer on Chromosorb W support in the analysis of volatile fatty acids. Prior to injection of unknown samples the column may be cleared by injection of formic acid–water mixture. With the addition of formic acid vapor to the carrier gas such a column should, however, give satisfactory quantitative results. Moreover the injection of water alone, or of weaker acids such as acetic, as a 1% solution (0.001 ml) failed to displace any acids subsequent to analyses of the mixture carried out with formic acid vapor in the carrier gas. Analysis of mixtures *not* containing formic acid should not therefore present any "ghosting" problems. Both columns contain nearly the same weight of ketoacid polymer. Consequently the fact that far more reversible adsorption occurs on the Chromosorb W column (Figs. 1 and 2) suggests that the substrate itself does not retain any appreciable amount of acid in "solution" or through hydrogen bonding.

After completion of the quantitative analyses the calculations indicated an anomalous relative area response for acetic acid (of 0.66) in the analysis of the weaker acid solution on the Chromosorb W column with formic acid in the carrier gas. Careful examination of the chromatogram (Fig. 3) indicated that this figure, obtained if the approximate baseline was taken as line L_2 (dashes) was erroneous, and the true baseline was actually line L_1 (dots). The same effect could be detected in the analyses of the stronger solution carried out at the same time, but owing to the attenuation of signal and larger acid peak the baseline dip had no serious effect on the interpretation of the chromatogram (Fig. 1). The approximate value for acetic acid of 0.30 given in Table I for the analysis under discussion is based on line L_1, although it is not possible to include that portion of the peak actually below line L_1.

The gradual dip in the chromatogram line after the solvent peak S (acetone) had not previously been noted, and re-examination of earlier analyses[7] indicated that it was not present. A more familiar phenomenon, a sharp dip below baseline, had on occasion been seen after large peaks with vertical trailing edges, or any material coming off the column at a very high concentration. This is also characteristic of carbon disulfide peaks when this material is employed as a solvent, and it has been

suggested that combustion products may in the latter case inhibit ionization[14]. Curiously similar dips in the baseline are shown in certain chromatograms[15] obtained with an argon ionization detector, although water desensitization may be responsible in the latter case. Various experiments apparently ruled out insufficient air supply as

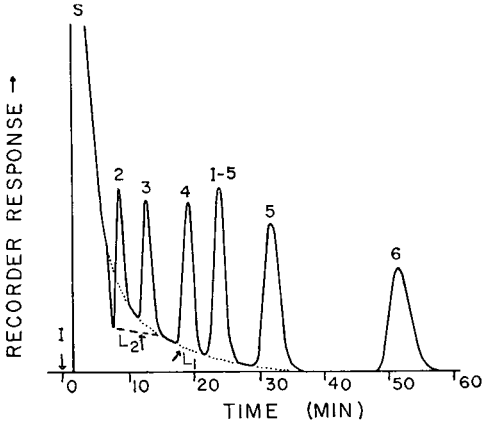

Fig. 3. Injection of 0.001 ml of C_2–C_6 acid mixture (< 0.1% in water–acetone solvent) on Chromosorb W column. L_1 is approximate true baseline, L_2 an erroneous apparent baseline due to dip in curve after acetone peak (S).

a cause of the chromatogram dip under discussion. The injection of the formic acid–water mixture on this column (*cf.* Fig. 1) does, however, give material peaks, the trailing edge of which is very similar in shape and position to the leading edge of the chromatogram dip. It must therefore be concluded that this was due to formic acid, or, more probably, an impurity in formic acid, which desensitized the detector under the particular operating conditions.

The column prepared with the teflon impregnated support approaches ideality of operation for the analysis of volatile fatty acids in that *even with no formic acid in the carrier gas* adsorption of acids, as shown in Fig. 2, was minimal. This type of column would then be suitable with aqueous solutions for use with thermal conductivity detectors, provided the water peak, which should come through rapidly on this combination of hydrophobic support and substrate, does not interfere with the acids under study. Non-aqueous solvents such as acetone are also eluted very rapidly and this type of column could therefore be useful for similar analyses with argon ionization detectors.

Analysis of hydrocarbons

The analysis of hydrocarbons does not fall within the normal scope of this laboratory, and the column dimensions were perhaps not entirely satisfactory for this type of work. Adequate separations of decalins and naphthalene[16] were obtained with the teflon-impregnated support (Fig. 4-A) but considerable tailing occurred with the Chromosorb W column (Fig. 4-B). Cyclohexane appeared before benzene, and on both columns the benzene peak tailed appreciably. When temperature or gas flow were varied to give the cyclohexane the same retention time, the tailing of the latter was

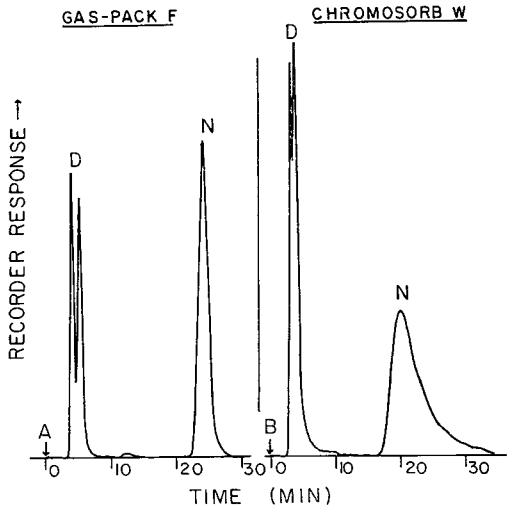

Fig. 4. Separation of decalins (D) and naphthalene (N). A: Gas-pack F column, helium carrier gas at 15 p.s.i., column at 135°. B: Chromosorb W column, helium carrier gas at 15 p.s.i., column at 135°. Sample 0.001 ml CS_2 solution (1 %).

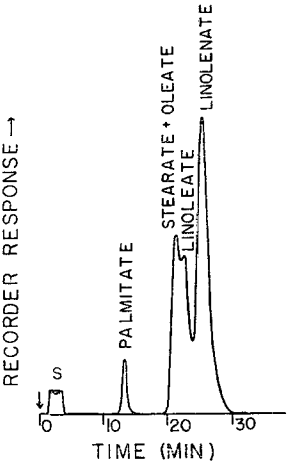

Fig. 5. Separation of methyl esters of linseed oil fatty acids on Chromosorb W column. Helium carrier gas at 15 p.s.i., column at 200°. Sample 0.001 ml CS_2 solution (1 %).

markedly less than that of the benzene. Interaction of the ketone or carboxyl groups with the aromatic double bonds therefore seems possible.

Analysis of higher methyl esters

The performance of this substrate in respect to methyl esters of saturated and unsaturated fatty acids (Fig. 5) is similar to that of one of the less polar polyesters such as neopentyl glycol adipate. In this respect it differs from silane polymers such as SE-30, and Apiezon greases, where the more highly unsaturated materials appear first. There was no appreciable difference in the performance of the two columns with this mixture.

Stability

At temperatures up to 150° the freshly prepared columns did not bleed noticeably or change properties over several weeks of operation. On heating to 220° heavy bleeding lasting several hours was observed. This may have been due in part to removal of cyclic monomeric ketones reportedly formed in association with the polymer, and possibly traces of sebacic acid. Baseline stability thereafter was excellent, but over a period of operation extending over several weeks at temperatures in excess of 200°, the columns gradually lost resolving power with respect to the volatile fatty acid mixture when tested at 150°. A corresponding drastic reduction in retention time indicated that an appreciable proportion of the substrate had been lost from the columns.

The stability of the particular columns tested is probably not typical of this material as a gas–liquid chromatography substrate, since the degree of polymerization

as indicated by the melting point was low. Virtually any range of molecular weight or ketone–carboxylic acid ratio could be obtained by fractionation of a large quantity of polymerization product. The complete removal of all traces of metal used in the preparation of the ketoacid polymer is probably necessary for maximum polymer stability and elimination of side reactions affecting the material under analysis.

CONCLUSIONS

Although particularly suited to analysis of volatile fatty acids, the potentialities of ketoacid polymers in gas–liquid chromatography are extensive. In addition to polymers of different molecular weights obtained from different dicarboxylic acids, chemical modification of the ketone or carboxyl groups, particularly through the insertion of other substituents or functional groups at these sites, could extend the useful range of the basic material into fields other than that of the volatile fatty acids.

SUMMARY

A low molecular weight ketoacid polymer prepared from the cadmium salt of sebacic acid has been demonstrated to be a satisfactory substrate for the separation of the volatile fatty acids. With the addition of formic acid vapor to the carrier gas quantitative results may be obtained employing conventional supports. With a teflon-impregnated support the use of formic acid vapor, while probably helpful at very low concentrations of acids, is not strictly necessary. Separations of certain other materials and the thermal stability of the polymer are discussed. An instance of desensitization of a flame ionization detector has been noted.

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GAS–LIQUID CHROMATOGRAPHY OF SOME CONDENSED-RING AROMATIC HYDROCARBONS ON LIGHTLY-LOADED COLUMNS

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INTRODUCTION

We wished to chromatogram some condensed-ring aromatic hydrocarbons, with boiling points up to 450°, on a conventional commercial apparatus (a Pye Argon Chromatograph) with a maximum column temperature of 225°. It has already been shown[1–4] that by the use of very sensitive detectors and by reduction of the percentage of stationary phase on the inert support, retention times of high boiling materials may be reduced to workable values. SWEETING AND WILSHIRE[3], for instance, successfully chromatographed compounds up to 1,6-diphenylhexane, b.p. 348°, on a 4 ft. column of 5 % apiezon L at 180°. Accordingly we have studied the chromatography of these condensed-ring hydrocarbons on lightly-loaded columns at temperature from 175° to 225°, with both glass beads and Celite 545 as inert supports, on three stationary phases.

EXPERIMENTAL

The apparatus used was a Pye Argon chromatograph. Columns of glass, were all 120 cm × 5 mm (I.D.), packed by tamping on a stone floor. Stationary phases were applied in the usual way; when preparing stationary phases on glass beads, the phase was dissolved in a suitable solvent and then mixed with, and stripped from, the glass beads in as small a vessel as possible, thus minimising the amount of phase lost to the glass vessel. All percentages of stationary phase to inert support are calculated on a w/w basis.

Solutes were applied as 0.1 μl of approx. 10 % solutions in 1-methylnaphthalene; for the higher molecular weight hydrocarbons it was necessary to dissolve them in hot 1-methylnaphthalene.

Retention times are expressed relative to pyrene (100), and retention times relative to pyrene will be denoted by τ. Column efficiencies were measured in terms of theoretical plates[5] and expressed as HETP.

Solutes were reagent chemicals. Stationary phases were as follows: Apiezon L (Edwards High Vac. Ltd.), Silicone oil (May and Baker "Embaphase") and Polystyrene (kindly provided by Mr. M. S. ALGER, and having $\bar{M}v = 6 \cdot 10^4$, and $[\eta] = 0.312$ in toluene at 25°).

RESULTS AND DISCUSSION

(a) *Glass bead supports*

A careful study by LITTLEWOOD[6] has shown that HETP values down to 0.9 mm can be obtained with glass beads as the solid support. LITTLEWOOD found that for a column of 1 % tricresyl phosphate on 150–200 mesh glass beads at temperatures about 80°, the minimum value of HETP, $(HETP)_{min}$, occurred at gas flow rates as low as 10–15 ml/min. Somewhat different results were obtained by FREDERICK, MIRANDA AND COOKE[7] who found that for 0.16 % silicone on glass beads at 74°, $(HETP)_{min}$ occurred at much higher flow rates: 70 ml/min for 70–80 mesh beads, and 69 ml/min for 140–230 mesh beads. Other workers have also used quite high flow rates for analyses on 0.1 % dinonyl phthalate or 0.1 % silicone grease on glass beads (55–90 ml/min)[8] or on 0.125 % apiezon L on glass beads (42–52 ml/min)[2].

We have found that columns of 0.1 to 0.2 % silicone oil or polyethylene glycol adipate on glass beads give very poor chromatograms, but that satisfactory separations could be achieved on columns of 0.1 to 0.2 % apiezon L or polystyrene on 48–66 mesh glass beads. Like LITTLEWOOD, we found that $(HETP)_{min}$ for these columns occurs at very low flow rates (Fig. 1) and this considerably reduces the usefulness of these columns, especially as the HETP increases substantially with increase in flow rate.

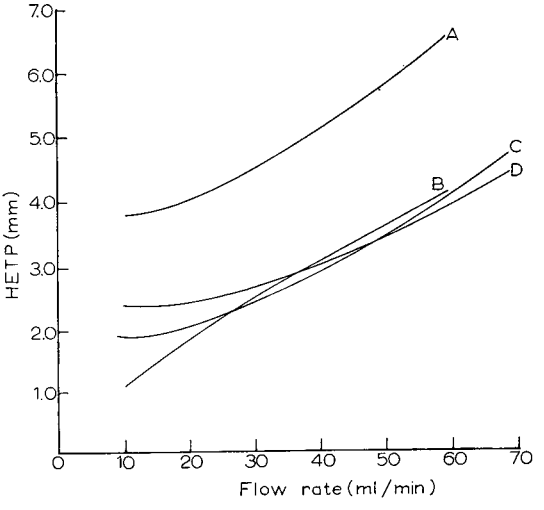

Fig. 1. (A) 0.2 % apiezon L; 225°; pyrene (R_t at 30 ml/min = 2.5 min). (B) 0.2 % polystyrene; 225°; 2:3-benzofluorene (R_t at 30 ml/min = 5.6 min). (C) 0.27 % apiezon L; 175°; anthracene (R_t at 30 ml/min = 11 min). (D) 0.2 % apiezon L; 225°; anthracene (R_t at 30 ml/min = 8 min).

Furthermore, as the packing density of 48–66 mesh glass beads is about six times that of 48–66 mesh celite 545, a given percentage of stationary phase on the glass beads will give retention times equivalent to those obtained with a six times as great a percentage stationary phase on celite. For instance, at 175° with a flow rate of 40 ml/min and on columns of identical lengths and diameters, anthracene is eluted after 13.4 min on 2.0 % apiezon L on celite 545 (48–66 mesh) and after 8.4 min on 0.27 % apiezon L on glass beads (48–66 mesh).

We concluded that for condensed-ring aromatic hydrocarbons, better separations

and more useful analyses could be obtained by the use of lightly-loaded columns with celite 545 as the solid support.

(b) Celite supports

Plots of HETP *vs.* flow rate are shown in Fig. 2 for columns of 2 % apiezon L, 2 % silicone oil and 4 % polystyrene on celite. Solutes were chosen for these determinations so that as far as possible their retention times (at a standard flow rate of 30 ml/min) were the same. Both apiezon L and polystyrene were very satisfactory: not only does $(HETP)_{min}$ occur at a higher flow rate than for the glass bead columns, but also only little loss in efficiency takes place on increasing the flow rate to 50 or 60 ml/min.

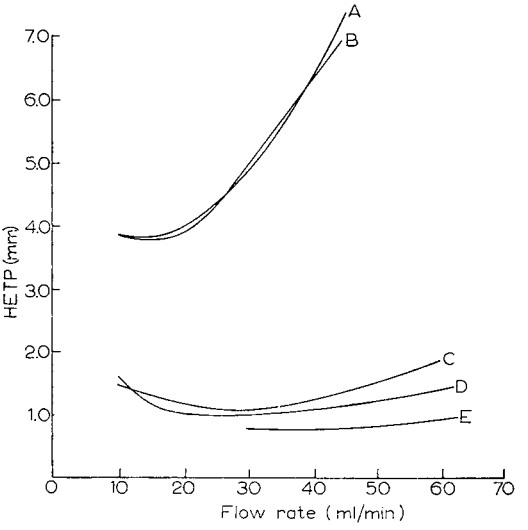

Fig. 2. (A) 2 % silicone oil; 200°; 2:3-benzofluorene (R_t at 30 ml/min = 8.2 min). (B) 2 % silicone oil; 175°; fluoranthene (R_t at 30 ml/min = 8.2 min). (C) 2 % apiezon L; 175°; fluorene (R_t at 30 ml/min = 6.9 min). (D) 4 % polystyrene; 200°; anthracene (R_t at 30 ml/min = 14 min). (E) 2 % apiezon L; 225°; 2:3-benzofluorene (R_t at 30 ml/min = 17.7 min).

Tailing on these two columns was not marked. Columns of 2 % silicone oil on celite were much less satisfactory; however, as the retention times of the condensed-ring aromatics were much lower on silicone oil than on apiezon L or polystyrene, we were able to use higher percentages of stationary phase in the case of silicone oil.

(c) Relative retention times

Table I gives retention times relative to pyrene (100) on columns of 2 % apiezon L, 4 % polystyrene and 2–10 % silicone oil. We observed no change in relative retention times on silicone oil columns with different percentages of stationary phase, and the retention times quoted for silicone oil are the combined averages of results from a 2 % and a 10 % column.

(i) On apiezon L. A plot of log τ *vs.* carbon number for compounds 1 to 13 is linear with some degree of scatter, no doubt because these compounds do not form a homologous series. Both retene and 2-phenylnaphthalene lie well off the best straight line

TABLE I

RETENTION TIMES OF CONDENSED-RING AROMATIC HYDROCARBONS RELATIVE TO PYRENE

No.	Hydrocarbon	B.p. °C	Polystyrene		Apiezon L		Silicone oil	
			200°[a]	225°[b]	175°[c]	225°[d]	175°[e]	200°[f]
1	Naphthalene	218	1.5	—	0.9	—	2.9	4.4
2	1-Methylnaphthalene	245	2.6	3.6	2.1	3.4	4.7	6.3
3	Acenaphthylene	277	5.8	8.0	4.3	6.6	8.6	12.3
4	Fluorene	298	9.5	11.9	8.2	11.2	14.1	18.0
5	Phenanthrene	336	24.6	27.6	23.2	27.0	31.7	36.7
6	Anthracene	340	25.8	28.6	23.4	28.2	31.7	36.7
7	Fluoranthene	383	79.3	79.3	78.8	79.3	84.9	86.2
8	Pyrene	393	100	100	100	100	100	100
9	2:3-Benzofluorene	407	150	139	155	138	153	147
10	Chrysene	440	—	338	—	317	332	294
11	Triphenylene	438	—	344	—	320	—	291
12	1:2-Benzanthracene	437	—	332	—	308	—	307
13	Naphthacene	ca. 450	—	377	—	363	—	324
14	Retene	390	94.5	90.4	133	114	154	147
15	2-Phenylnaphthalene	360	45.9	50.7	47.5	49.4	62.0	65.5
16	Hexadecane	287	2.1	2.5	6.0	7.9	16.5	17.8
17	Octadecane	317	4.4	4.5	16.2	15.8	38.5	39.0

Retention time for pyrene at 45 ml/min:
[a] 4.5% polystyrene; 34.8 min
[b] 4.5% polystyrene; 13.6 min
[c] 2.0% apiezon L; 50.6 min
[d] 2.0% apiezon L; 8.7 min
[e] 2.0% silicone oil; 9.0 min
10.0% silicone oil; 68.3 min
[f] 2.0% silicone oil; 3.6 min
10.0% silicone oil; 26.0 min.

on the side of the hexadecane–octadecane line. However, a plot of log τ *vs.* b.p. gives a straight line for compounds 1 to 17 on which only retene does not lie. Anthracene and phenanthrene are not resolved, and of the C_{18} fused ring aromatics (10–13) only the pair 1:2-benzanthracene/naphthacene are completely resolved at 225°. At a flow rate of 60 ml/min at 225° naphthacene (b.p. *ca.* 450°) has a retention time of only 23 min on 2% apiezon L.

Relative retention times have previously been quoted for compounds[3] 1 to 5 on apiezon L at 180° and for compounds[9] 4, 5, 6 and 10 at 235°; these results agree quite well with ours. CARUGNO AND GIOVANNOZZI-SERMANNI[10] have recorded retention data on apiezon L for compounds 1 to 8 at 230° and compounds 8, 10 and 12 at 260°, as well as retention data for several higher condensed ring systems at 260°. Where comparisons can be made, however, these results do not agree very well with either our results or those found previously.

(ii) On silicone oil. Plots of log τ *vs.* b.p. or carbon number for compounds 1 to 13 again produce a straight line with some scatter. Retention times on silicone oil are much smaller than on apiezon L; at 175° fluoranthene is eluted on 2% apiezon L after 45 min, and on 2% silicone oil after only 6.8 min, the flow rate being 40 ml/min in each case.

DUPIRE AND BOTQUIN[11] have recorded retention times for compounds 1 to 8 and compound 10 on silicone at 235° and 295°, and SOLO AND PELLETIER[9] have chromato-

graphed compounds 4, 5, 6, 10 and 12 at the same temperatures. With the exception of DUPIRE AND BOTQUIN'S result for chrysene at 295°, these results agree with each other and with the retention times that we have obtained.

(iii) On polystyrene. Compounds 1 to 13 show much scatter when plotted as log τ *vs.* carbon number. However, a plot of log τ *vs.* b.p. gives a much better line on which compounds 1 to 15 all lie. As with apiezon L, only the compounds 1:2-benzanthracene and naphthacene are completely resolved at 225°. Polystyrene shows appreciably more selectivity towards aromatics over aliphatics than does apiezon L and we feel that it should make a useful addition to the comparatively small number of high temperature stationary phases that are available. The efficiency of the polystyrene columns, though, dropped drastically below about 190°; possibly this is due to a viscosity effect.

(iv) Identification plots. A plot of log τ (polystyrene) *vs.* log τ (apiezon L) or a plot of log τ (apiezon L) *vs.* log τ (silicone oil) give straight line plots on which compounds 1–13 all lie. The non-condensed aromatic, 2-phenylnaphthalene also lies on both of these straight lines, but the side chain compound, retene (8-methyl-2-isopropyl-phenanthrene) falls just off the straight line. For identification of the condensed-ring aromatic hydrocarbons, a much better method is to plot log τ (polystyrene) *vs.* log τ (silicone oil). As can be seen from Fig. 3, all the hydrocarbons 2–13 lie on the straight

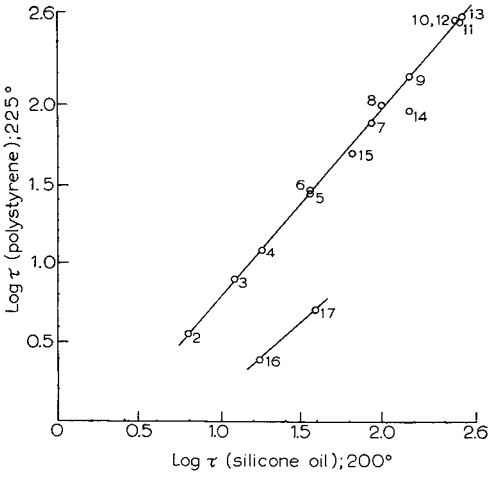

Fig. 3. Retention data taken from Table I.

line, retene is well displaced from the line and 2-phenylnaphthalene also lies just off the straight line.

ACKNOWLEDGEMENT

We thank the Department of Scientific and Industrial Research for aid from a grant.

SUMMARY

Gas–liquid chromatography on lightly-loaded columns with Celite 545 as the support provides a rapid and satisfactory method of identification of condensed ring aromatic hydrocarbons up to and including naphthacene, at elution temperatures of 175° to 225°. No special equipment is required for these analyses.

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GAS–LIQUID CHROMATOGRAPHY OF THE TETRAHYDRO DERIVATIVES OF CORTISOL ISOLATED FROM URINE*

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VANDENHEUVEL, SWEELEY AND HORNING[1] reported that various androstane, pregnane and cholestane derivatives, containing oxygen atoms in the 3, 11, or 20 positions or as 17-ketosteroids, could be separated by gas chromatography on Chromosorb W coated with 2 to 3 % silicone gum (SE-30) at 222°. Cortisol and other pregn-4-en-17, 21-diol-3, 20-dione compounds appeared as the corresponding 17-keto-steroids[2]. LIPSKY AND LANDOWNE[3] found no evidence of epimerization of hydroxyl groups or of hydrogen on carbon position 5 of various steroids, but noted that compounds of the pregnan-21-ol-20-one series were unstable. In earlier work in this laboratory, DALLMAN AND GOULD showed that the chief cortisol metabolites, reduced at carbons 3, 4 and 5, could be separated by gas chromatography, each giving a single peak with the retention time of the corresponding 17-ketosteroid.

In fractions obtained from liquid-liquid chromatography of extracts of urine, the principal metabolites of cortisol, pregnane-3α,17α,21-triol-11,20-dione (tetrahydrocortisone), allopregnane-3α,11β,17α,21-tetrol-20-one (allotetrahydrocortisol), and pregnane-3α,11β,17α,21-tetrol-20-one (tetrahydrocortisol) are eluted sequentially, but the first two may not be regularly or completely separated. The present studies were undertaken to determine whether these metabolites could be separated and measured by gas liquid chromatography as individual standards, in mixtures, and in fractions eluted during liquid-liquid chromatography of urine extracts.

METHODS

A model 15 Barber-Coleman gas chromatograph was used. The glass U-tube (8 ft. long × 5 mm internal diam.) was packed with Chromosorb-W (acid washed), which had been silanized and coated with SE-30 (0.75 %). Temperature around the column was 217°, and at the vaporizer 250°. Argon flow rate was 50 ml/min. The ionization detector utilizing ^{90}Sr was operated at 750 volts.

Authentic reference steroids were obtained from Dr. W. KLYNE (through the courtesy of Mr. M. GRAFF and the Endocrinology study Section, N.I.H.) and from U.S.P. Reference Standards. The neutral steroid fraction, extracted from urine incubated with β-glucuronidase, was transferred to a Celite column (60 cm long × 1 cm internal diam.) with 50% methanol as the stationary phase and a mobile phase

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of one part of ethyl acetate in 15 parts of toluene. An aliquot of each fraction, which contained material reducing blue tetrazolium[4] and giving the SILBER-PORTER reaction[5], was dried in a stream of air and rinsed down into the point of a conical, glass stoppered tube. Measured amounts of cholestane and ethanol were added to each standard or sample to bring the concentration of cholestane to 1.0 μg/μl and the concentration of steroid to between 2 and 4 μg/μl. Aliquots of 2 to 8 μl were injected from a Hamilton 10 μl syringe into the vaporizer chamber.

Areas under recorded peaks of detector response were recorded by a disc integrator and calculated from peak height. Agreement was satisfactory with standard steroids or when urine fractions consisted of well defined and separated components.

RESULTS

Retention time of standards

As noted in Table I, tetrahydrocortisone, tetrahydrocortisol, and allotetrahydrocortisol yielded peaks with retention times identical with the corresponding 17-ketosteroid analogs. As the pregnane compounds showed no greater tendency to

TABLE I

RETENTION TIME AND MOLAR AREA OF STANDARD STEROIDS RELATIVE TO CHOLESTANE

Standard	*Relative retention time*[a]	*Relative molar area*[b]
Pregnan-3α,17α,21-triol-11,20-dione (tetrahydrocortisone)	0.47	0.33
Etiocholan-3α-ol-11,17-dione	0.47	0.50
Pregnan-3α,11β,17α,21-tetrol-20-one (tetrahydrocortisol)	0.62	0.30
Etiocholan-3α,11β-diol-17-one	0.61	0.50
Allopregnan-3α,11β,17α,21-tetrol-20-one (allotetrahydrocortisol)	0.69	0.33
Androstan-3α,11β-diol-17-one	0.70	0.50
Cholestane	1.00[a]	1.00[b]

[a] Observed retention time 18.6 $\pm$ 0.7 min for cholestane under operating conditions described.
[b] Area under recorded peak per micromole steroid divided by area per micromole cholestane. In each case, area represents integrated detector response under operating conditions described.

trailing, it seems likely that loss of side chain occurs almost immediately after injection into the vaporizer. The order of appearance (retention time shorter for 11-ketone than for the corresponding 11β-OH compound, and shorter for 5β-H than for 5α-H) conforms with findings on other steroids[1,3,6].

Detector response to standards

The greatest response per microgram was given by cholestane, followed by the 17-ketosteroids (Fig. 1). The lowest response was observed in the pregnane series, especially with tetrahydrocortisol. Results, presented in Table I as integrated detector response per micromole of steroid, supported the thesis of SWEELEY AND CHANG[6] that increasing oxygen content was associated with decreasing relative detector response,

and that the lowest relative response was obtained from pregnane derivatives which lost carbons 20 and 21 during gas chromatography, even when conditions which minimized variations in molar response were selected.

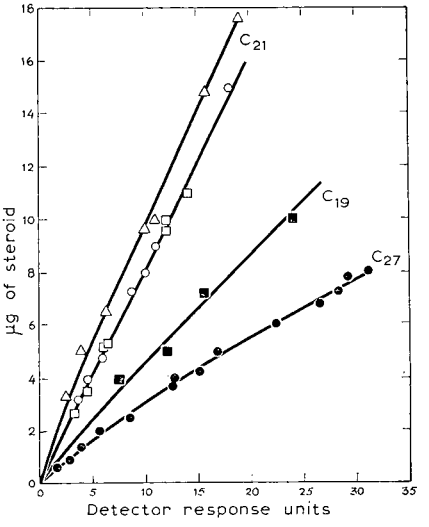

Fig. 1. Area under recorded peak of detector response after injection of various steroids. Cholestane (C_{27}) is indicated by solid circles; 17-ketosteroids (C_{19}) by solid squares; and in the pregnane series (C_{21}), tetrahydrocortisone by open circles, allotetrahydrocortisol by open squares, and tetrahydrocortisol by open triangles.

Fractions from partition chromatograms of urine extracts

Fig. 2 presents records of detector response during four gas-liquid chromatograms. In each case, the double spike on the left represents air and solvent and the last peak on the right is due to cholestane. In the pair of records on the left side, standard tetrahydrocortisone (above) is compared with a fraction obtained from chromatography of urine extract (below). The responses are quite similar and indicate that tetrahydrocortisone is the chief component of each sample. The pair of records on the right side of Fig. 2 represent gas-liquid chromatograms of standard allotetrahydrocortisol (above), and a urine fraction (below), containing tetrahydrocortisone (large peak), allotetrahydrocortisol (smaller peak corresponding to peak in standard run above), and several unidentified components.

Estimates of the quantity of reduced cortisol metabolites in fractions of urine extracts by gas-liquid chromatography agree well with results of colorimetric analysis when the quantity of the cortisol metabolite is sufficient to give an easily measurable peak, well separated from other components of the fraction. When these conditions are not satisfied, one or more of the following problems may interfere with the analysis or with recovery of known steroid added to urine extracts: elevated or irregular baseline of detector response may make quantitation difficult; or retention time may vary when a large amount of contaminant is present; or a small amount of a known pregnane compound added to a heavy residue (obtained by pooling a number of urine fractions) may fail to give the expected peak, suggesting delay or interference

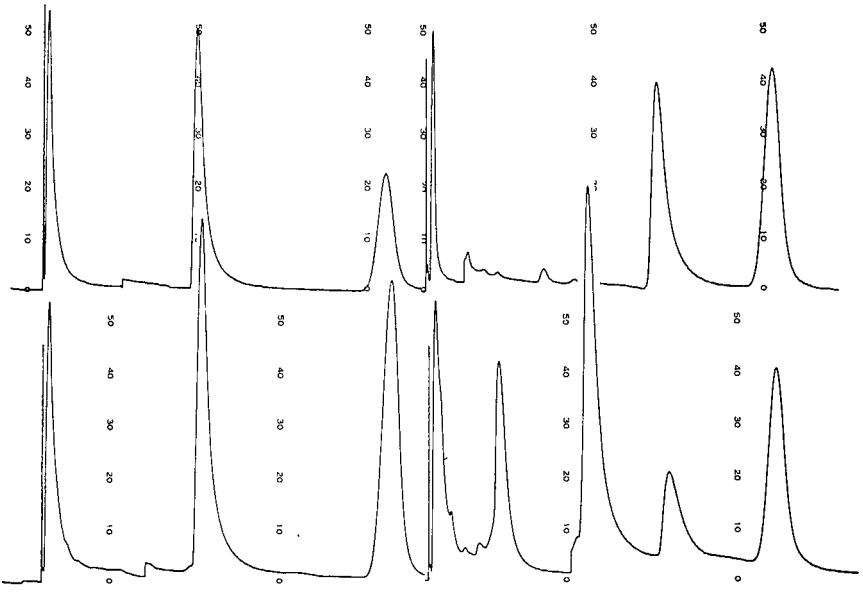

Fig. 2. Detector responses recorded during four gas-liquid chromatograms. See text for explanation.

with the rapid and reproducible conversion to 17-ketosteroid observed when a standard compound is injected.

The technique described has proved quite useful in monitoring fractions from liquid-liquid partition columns in order to differentiate between tetrahydrocortisone and allotetrahydrocortisol, which are not easily separated on the liquid-liquid column, but which separate readily on gas chromatography.

SUMMARY

Tetrahydrocortisone, tetrahydrocortisol, and allotetrahydrocortisol can be separated by gas-liquid chromatography and quantitated with an argon ionization detector. The retention times of these cortisol metabolites are identical with those of their 17-ketosteroid analogs. This technique has been used to monitor the composition of fractions from liquid-liquid chromatograms of urine extracts.

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THE PERFORMANCE OF A COMMERCIAL HIGH-TEMPERATURE IONIZATION CHAMBER FOR RADIOISOTOPE ANALYSIS OF GAS CHROMATOGRAPHY VAPORS*

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(Received May 28th, 1963)

The technique of gas liquid chromatography as a tool for chemical research has found numerous applications in recent years. In biological research it is often desirable to separate a sample into its components and to determine the amount of radioactivity that might have been incorporated into the various components. A common procedure now used is to trap the effluent as it emerges from the column and count it separately or, as reported recently, continuously as it bubbles into liquid scintillator[1]. The disadvantages of these procedures are discussed by JAMES AND PIPER[2].

Methods have been developed in which the detector is an integral part of the system. Proportional counters have been used in which the effluent from the gas chromatograph is first combusted to CO_2[2]. Proportional counters have been designed for use at temperatures up to 200°[3]. WINKELMAN AND KARMAN[4] used a 275 c.c. ionization chamber to determine the activity in the products of the combusted effluent.

CACACE AND INAM-UL-HAQ[5] used a 100 ml ionization chamber which did not require heating for compounds that boiled under 150°. These authors used nitrogen as a purge gas. MASON *et al.*[6] constructed an ionization chamber with a working volume of 9 ml which was insulated with teflon. The ionization chamber was not included in the oven but was heated separately to 240°.

GANT[7] included a teflon insulated ionization chamber in the gas chromatographic oven but found that noise greatly increased at temperatures above 125°.

Sapphire insulated high temperature ionization chambers have been developed[8] that are suitable for inclusion in gas chromatographic ovens. This ionization chamber is used in the commercially available Loenco-Cary Model 70.

We wish to describe a complete system which employs a Cary high temperature ionization chamber and has proved useful in the detection of small amounts of radioactivities in the system.

EXPERIMENTAL

Apparatus and reagents

The gas chromatograph was constructed by the authors for inclusion of an ionization chamber. Both the injection and exit blocks had provision for heating and the oven temperature was maintained by an Assembly Products controlling pyrometer.

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Brooks rotameters model 1100 were used to control the gas flows. For helium the capacity was 14.4–144 c.c./min and for argon 110–1103 c.c./min. Five foot copper columns were packed with 16% LAC 296 (Chrom Line Laboratories, Kansas City, Missouri) on acid washed fire brick 42/60, 84% (Wilkens Instrument and Research, Inc.).

The gas chromatograph bridge circuit was similar to that used in the Aerograph A-90 and the detector cell was an XA-222 Aerograph stainless steel detector cell. Better stability was obtained with the bridge circuit separated from the oven. The electrometer was a Cary Model 31 Vibrating Reed Electrometer with critical damping. The high resistance leak method for measuring ionization current was used with a single $10^{12}\,\Omega$ resistor. The ionization chamber was a Cary No. 3295700 High Temperature Ion Chamber Assembly with a 12 in. stainless steel rigid line and adaptor. The output from the gas chromatograph and the electrometer was fed into a Weston Model 6807 two pen recorder. Twenty-five mV was used for the Vibrating Reed Electrometer and 1 mV for the gas chromatograph. The carriage speed on both channels was 1 sec and the chart speed was 30 in./h. The first channel was connected to the electrometer and the second to the gas chromatograph bridge. Connected in this manner the two peaks were made to coincide even though the signal reached the gas chromatograph channel first.

A 90 V DC battery was used to provide the polarizing voltage for the ionization chamber. Insulation from the rest of the system was provided by Kovar to glass seals. The glass seals were protected from strain by the use of a coiled $^1/_8$ in. copper tubing in the system.

Benzene-^{14}C uniformly labelled (Nuclear-Chicago Corporation) was diluted with reagent grade benzene such that final activities of 20,700, 2060 and 206 disintegrations/min/μl were obtained. A Packard-tri Carb Model 314EX Automatic Liquid Scintillator Spectrometer was used to check the radioactivity of the injected and recovered samples.

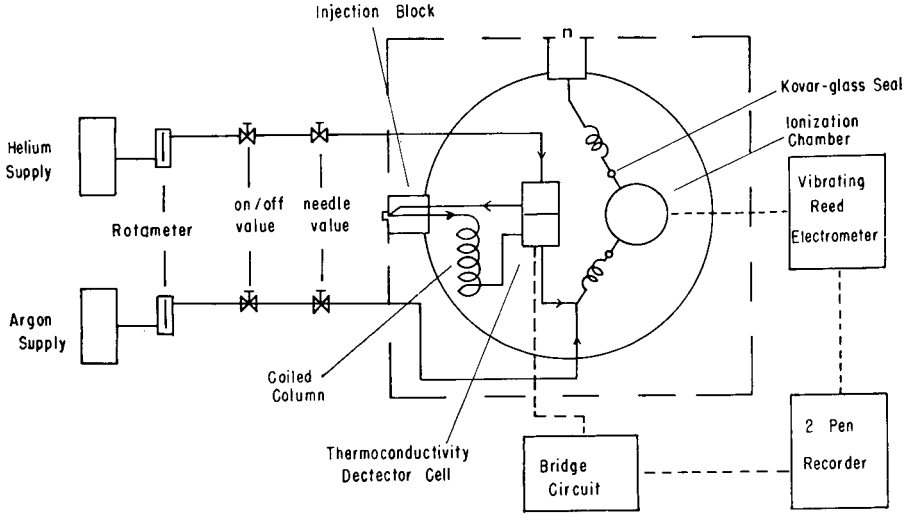

Fig. 1. System flow diagram.

The components were arranged according to the diagram in Fig. 1. With a helium valve pressure of 15 p.s.i., the full range of the gas chromatograph flow rates could be obtained (28–140 c.c./min). With the argon valve set at 10 p.s.i. flow rates of from 97 to 940 c.c./min were obtained. The helium flow rate was set for 95 c.c./min. Both the injection and exit block heaters were set at 150°. Injections of from 0.5 μl to 3 μl of the samples were made using a 10 μl syringe. The gas chromatograph was was run at an attenuation of 32–128 times.

An impingement type trap in dry ice–ethanol was used to trap the vapor from the system. The vapor was passed from the trap to a secondary glass coil which was also in the coolant. The trap was constructed with a cone at the bottom such that the condensate could be withdrawn with a capillary pipet. Increased recoveries were obtained if 0.5 ml of toluene was added to the bottom of the trap. Where liquid scintillation counting of the condensate was desired, 3.5 ml of toluene in portions was used to rinse the trap. This arrangement allowed a recovery of better than 95 % of the activity added to the system.

RESULTS

In order to determine the optimum flow rate for the purge gas, a series of injections of 20,700 d.p.m. of benzene-^{14}C in 1 μl volumes was made. The series was started with 197 c.c./min of helium and argon and the latter gas was increased in step-wise manner until a final flow rate of 1041 c.c./min was reached. These flow rates represent chamber constants of from 84 to 15.8 sec. At the lower flow rates the ionization chamber required 4 min to return to the background level. As the argon flow rate was increased the base of the peak narrowed. At flow rates above 689 c.c./min (600 on the argon rotameter) the shape of the base of the curve was changed only to a slight degree. The ratio of radioactivity to thermoconductivity peak heights remained constant up to 689 c.c./min. With increased flow rates above 689 c.c./min the ratio was found to decrease slightly. A flow rate of 689 c.c./min represents a chamber constant of 23.9 sec, under these conditions 1.5 min were required for the ionization chamber to return to the background level.

Fig. 2 shows a simultaneous measurement of radioactivity and thermoconductivity. The three peaks represent activities of 515, 1030 and 2060 d.p.m. respectively.

Fig. 3 shows that a straight line relationship exists between the area under the peaks and the amount of radioactivity injected. Fig. 4 shows the results of a plot of peak height *versus* d.p.m. of ^{14}C injected. At the higher activities, peak height is proportional to the amount of ^{14}C injected. At the lower activities, however, there is no linear relationship between peak height and the amount of ^{14}C injected. Although each point in Figs. 3 and 4 represents the average of five determinations, peak heights particularly at low activities would be higher than expected due to "bursts" of activity. WINKELMAN AND KARMAN[4] also found that peak area was more linearly related to the amount of radioactivity injected than was peak height.

Fig. 5 shows the excellent resolution that can be obtained with the system. A mixture of benzene-^{14}C and toluene were injected into the system (oven temperature 70°). Although the benzene and toluene were not completely separated all of the radioactivity was shown to be associated with the benzene peak.

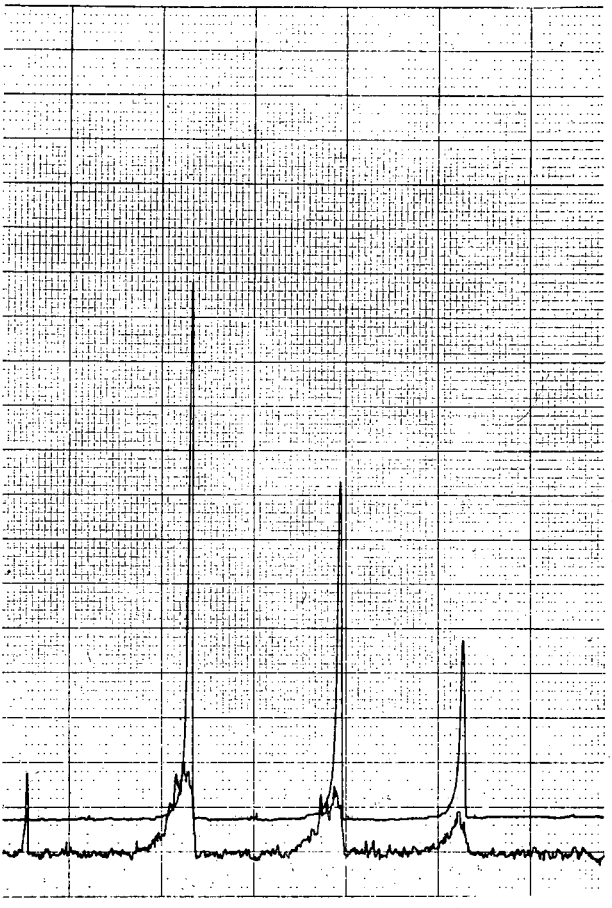

Fig. 2. Simultaneous measurement of radioactivity (lower curve) and thermoconductivity (upper curve) of benzene-^{14}C.

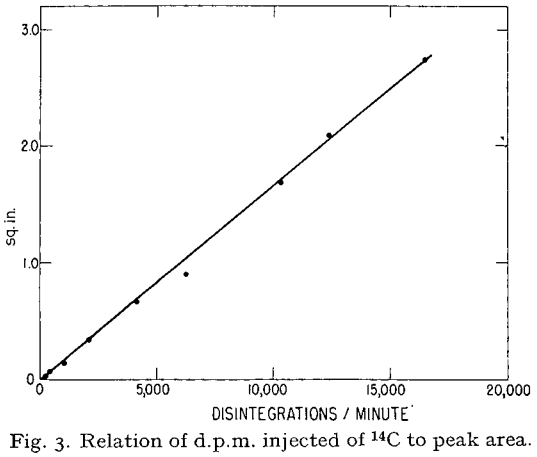

Fig. 3. Relation of d.p.m. injected of ^{14}C to peak area.

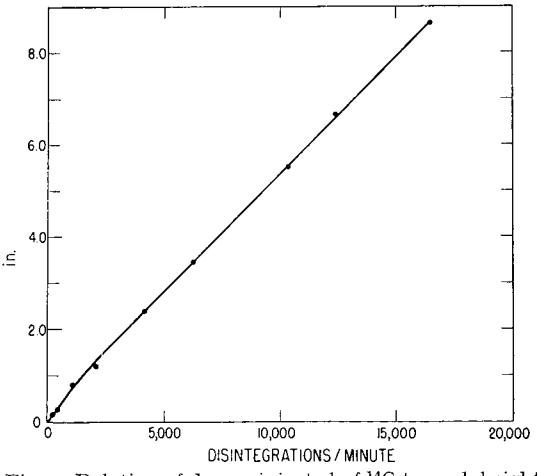

Fig. 4. Relation of d.p.m. injected of ^{14}C to peak height.

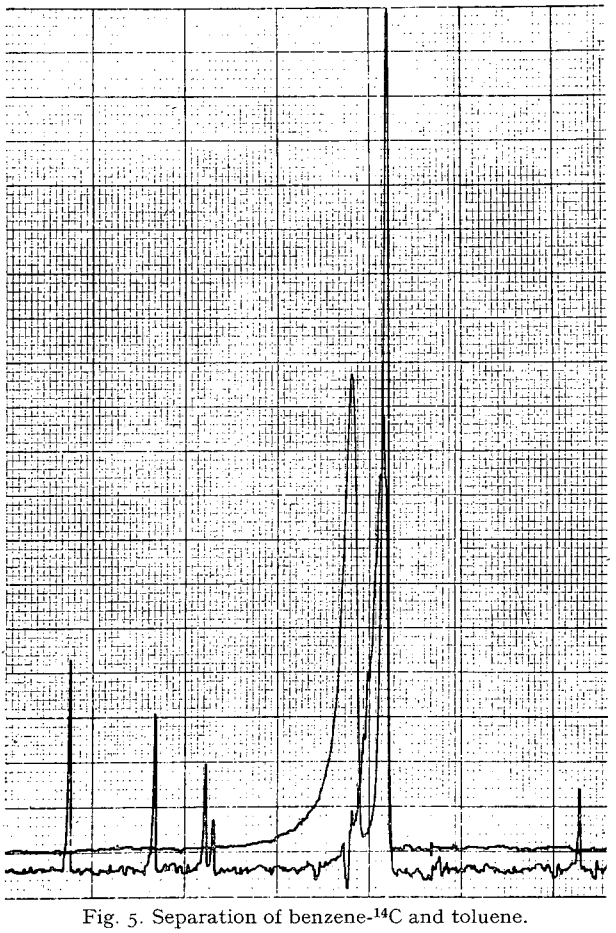

Fig. 5. Separation of benzene-^{14}C and toluene.

DISCUSSION

A number of factors are involved in the ability to detect radioactivity in a sample injected into a system as described in this paper. The ionization chamber should be of sufficient size such that most of the energy from a disintegration is absorbed by the gas rather than the walls of the chamber[4]. A 275 c.c. ionization chamber without the use of a purge gas would give very poor resolution. Argon has been shown[1,9] to be a better ionization gas in this application than helium.

It is important that the noise level of both the ionization chamber and the electrometer be low particularly if one desires to detect small amounts of radioactivity. Although benzene-^{14}C was used in the experiments reported here, similar results were obtained at temperatures above 200° using lauric acid-1-^{14}C methyl ester. Benzene-^{14}C was used because of its short retention time.

Both peak height and peak area are affected by the manner in which the peak reaches the ionization chamber and the chamber constant. The ideal situation is one in which the peak reaches the ionization chamber in a very small volume and is quickly purged from the chamber. Where the sample enters the chamber in a larger volume, *i.e.* over a longer period of time, the peak height is lower and the area is spread over a longer time interval even under an optimum chamber constant. The sensitivity of the system is lowered under these conditions. Thus a sample with low activity could pass through the ionization chamber undetected.

Under the conditions employed in these experiments it was possible to detect as little as 200 d.p.m. although at this level of activity the normal fluctuations of the background excluding α-disturbances nearly equaled the peak height. At 400–500 d.p.m. the peak representing the radioactivity could be distinguished from the background noise. It would of course be desirable to use larger amounts of radioactivity in order to use less sensitive electrometer settings. In biological research the experimenter is often required to analyze samples with very low activities.

The problem of trapping the effluent from this system is much greater than those experienced with conventional gas chromatograph flow rates. The trap system that was used was found to be very efficient at the flow rate used. With the use of a trap, the experimenter is able to recover samples from the system. This represents a real advantage over methods in which the samples are combusted or otherwise changed in detection.

The main problem in using this system for quantitative work is one of being able to inject accurate amounts into the system. The ratios of the peak heights (thermoconductivity to radioactivity) were nearly constant above 2060 d.p.m. in the data from which Figs. 3 and 4 were derived. However, in this series the individual thermoconductivity peaks varied by ± 2–3%. In preliminary work several syringes were used to inject the same volume, however, when the charts were compared there was a greater variation than should be expected. The most consistent results were obtained by using one syringe and, by the use of dilutions, injecting similar volumes.

ACKNOWLEDGEMENT

The technical assistance of ALFRED WILSON and ERNEST BURTON was greatly appreciated.

SUMMARY

A system is described for the simultaneous analysis of the effluent of a gas chromatograph column for thermoconductivity and radioactivity. A gas chromatograph was constructed for inclusion of a 275 ml high temperature ionization chamber. The effluent from the thermoconductivity cell passes directly into the ionization chamber providing coincident peaks on two pen recorder. Argon is used as a purge gas for the system. The sensitivity of the system was such that 400 disintegrations per minute of carbon-14 could be detected. Under ideal conditions 206 d.p.m. could be distinguished from background noise.

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SINGLE DETECTOR AND FORECOLUMN TRAP FOR SERIES GAS-CHROMATOGRAPHY ANALYSIS

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INTRODUCTION

The purpose of this investigation was to establish a reproducible chromatographic technique for the analysis of fixed gases related to a pilot plant operation at Iowa State University in which gaseous sulfur dioxide is produced by the reductive decomposition of gypsum. The gases of relative interest to the process are oxygen, nitrogen, hydrogen, carbon monoxide, methane, hydrogen sulfide, carbon dioxide, and sulfur dioxide. The mixture is usually saturated with water vapor.

PREVIOUS INVESTIGATIONS

Several references[1-4] can be cited for the separation of oxygen, nitrogen, methane, and carbon monoxide on columns of Types 5A or 13X molecular sieves (The Linde Co., Tonawanda, New York). The separation of carbon dioxide from the same mixture was usually obtained by use of a silica gel column. It has been reported[5] that carbon dioxide could be separated on a molecular sieve column by linear temperature programming.

MURAKAMI[3] developed a method for the analysis of these gases by using an arrangement of two columns in series. He obtained a continuous chromatogram by passing the gases through a silica gel column first, then through the reference side of a thermal conductivity detector, then through a molecular sieve column and finally through the sample side of the detector. The peaks from the silica gel column were detected by reversing the polarity of the recorder input and the peaks from the molecular sieve column were detected with normal polarity. The chromatogram contained a peak for the mixture of oxygen, nitrogen, methane, and carbon monoxide, followed by a carbon dioxide peak from the silica gel column. Then the individual peaks of oxygen, nitrogen, methane, and carbon monoxide appeared from the molecular sieve column. One objectionable feature of the method was that carbon dioxide contaminated the molecular sieve column by permanent adsorption on the active sites.

OTTENSTEIN[6] made the same separations with similar arrangements of dual columns in series except that he used two separate detectors. His separation, depending on the columns used, also included hydrocarbons, sulfur dioxide, and hydrogen sulfide. The column used to separate carbon dioxide, hydrogen sulfide, and sulfur dioxide was a 9 ft. by $^1/_4$ in. column of 10 % di-2-ethylhexylsebacate (commercially available as Octoil-S Vacuum Pump Fluid, Consolidated Vacuum Corp., Rochester, N.Y.) on 40–60 mesh Teflon-6. It gave a peak for the mixture of oxygen, nitrogen, methane, and

carbon monoxide followed by individual peaks of carbon dioxide, hydrogen sulfide, and sulfur dioxide. The other column in the arrangement had two parts. It was composed of 5 ft. by $^1/_4$ in. of uncoated Chromosorb-P (60–80 mesh) followed by 7 ft. by $^1/_4$ in. of Type 13X molecular sieve (40–60 mesh). The uncoated Chromosorb-P "forecolumn" which preceded the molecular sieve column made no separation; it delayed the composite mixture from the first column so that the oxygen, nitrogen, methane and carbon monoxide peaks from the second column would appear at the proper time. The disadvantages of the system were that (1) two detectors were required, and (2) the molecular sieve column became contaminated with the three compounds (carbon dioxide, hydrogen sulfide, and sulfur dioxide) that were separated on the first column.

Other columns[7,8] have been reported for the separation of sulfur dioxide and various sulfur compounds in the absence of many of the fixed gases.

PRESENT CHROMATOGRAPHIC ARRANGEMENT

For the pilot plant gases a multiple-column gas chromatographic analysis system is used so that all the desired separations can be obtained from a single gas sample. Although only two columns perform the actual separation, a series of three columns is used. The extra column, in the middle position, traps the gases that are separated on the first column and provides the proper time delay for peaks appearing from the third column. The time delay permits the use of one thermal conductivity cell to obtain a continuous chromatogram without having any peak overlap.

Column 1 is a 20 ft. long section of $^1/_4$ in. O.D., 20 gauge, Type 304, stainless steel tubing filled with 10 wt. % dibutyl sebacate on the $-20 + 80$ U.S. standard screen fraction of Fluoropak (Wilkins Instrument and Research Corp., Walnut Creek, California). This column gives a peak of the mixture of hydrogen, oxygen, nitrogen, methane, and carbon monoxide followed by individual peaks of carbon dioxide, hydrogen sulfide, and sulfur dioxide in that order.

Column 2, a "forecolumn trap", is a 11 ft. long section of $^1/_4$ in. O.D. copper tubing filled with 25 wt.% potassium hydroxide on the $-30 + 60$ U.S. standard screen fraction of Chromosorb W (Johns Manville Corp., Manville, New Jersey). The packing is prepared by adding a solution of 5 g of potassium hydroxide in 100 ml of methanol to 20 g of Chromosorb W and then evaporating the methanol while stirring the entire mixture continuously. The potassium hydroxide in the column permanently absorbs carbon dioxide, hydrogen sulfide, sulfur dioxide, and water vapor. The length of the column creates the proper time lag for the remaining gas mixture to enter Column 3.

Column 3 is a 7 ft. long section of $^1/_4$ in. O.D., 20 gauge, Type 304, stainless steel tubing filled with the $-14 + 30$ U.S. standard screen fraction of Type 13X molecular sieves (The Linde Co., Tonawanda, New York). After the column was activated at 300° for 12 h, it separated hydrogen, oxygen, nitrogen, methane, and carbon monoxide in that order.

The detector used was a Model TR-II-B Gow-Mac (Gow-Mac Instrument Co., Madison, N. J.) thermal conductivity cell, geometry 9193, with a Model 9293-B Gow-Mac power supply control unit.

The column arrangement, Fig. 1, uses the reference side of the thermal conductivity cell to detect gases from Column 1 and the sample side to detect gases from Column 3. Peaks from either side of the thermal conductivity cell can be recorded in a

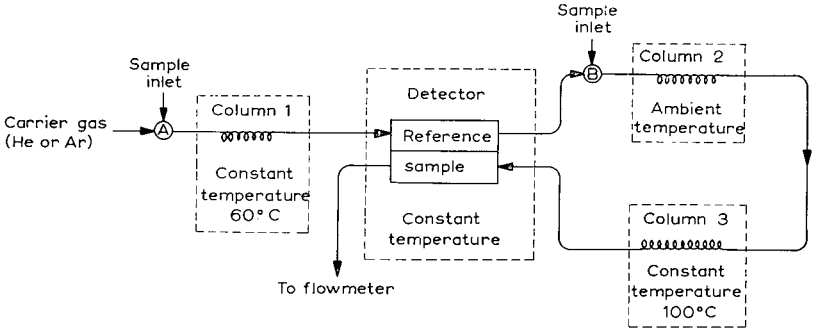

Fig. 1. Column arrangement.

continuous chromatogram by reversing the polarity of the signal from the detector cell with a double-pole, double-throw switch on the recorder input leads. The recorder input signal must be adjusted to zero potential at the beginning of a chromatogram so reversal of polarity does not affect the base line.

Separation of all components requires a helium flow rate of 40 ml/min. Column 1 is operated at 60°, Column 2 at ambient temperature, and Column 3 at 100°. With the polarity of the signal reversed, Column 1 gives a peak of the mixture of hydrogen, oxygen, nitrogen, methane, and carbon monoxide followed by individual peaks of carbon dioxide and hydrogen sulfide. The polarity is then switched to normal to obtain peaks of hydrogen, oxygen, nitrogen, methane, and carbon monoxide from Column 3. Finally the polarity is reversed again for the sulfur dioxide peak from Column 1. Water vapor appears from Column 1 about 5 min. after the final peak, but appears only as a very low hump with a seven minute base width. It consequently does not interfere

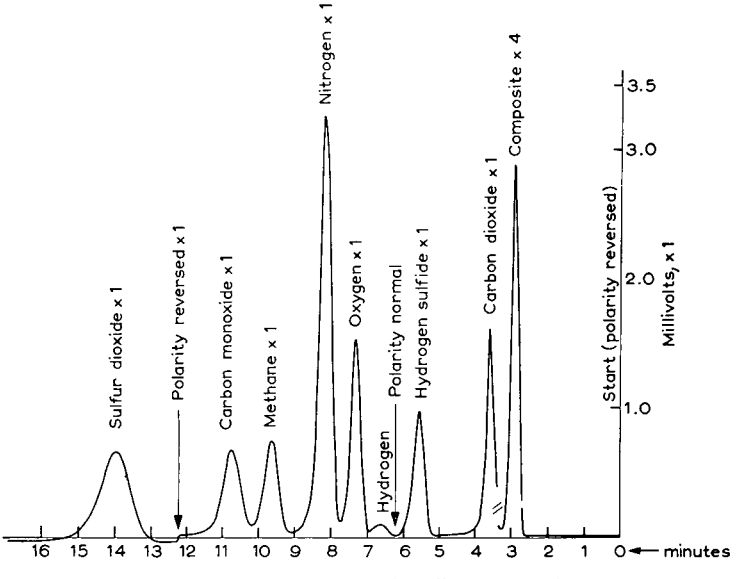

Fig. 2. Sample chromatogram for all components.

with the chromatogram and is trapped on Column 2 along with carbon dioxide, hydrogen sulfide, and sulfur dioxide.

Fig. 2 shows a chromatogram for the separation of all components in 15 min. The sample contained 50 μl of all components except for 250 μl of hydrogen. Usually the pilot plant gases do not contain all of the components. In this case the flow rate is increased to 80 ml of helium/min and an analysis is completed in 8 min. Fig. 3 is a

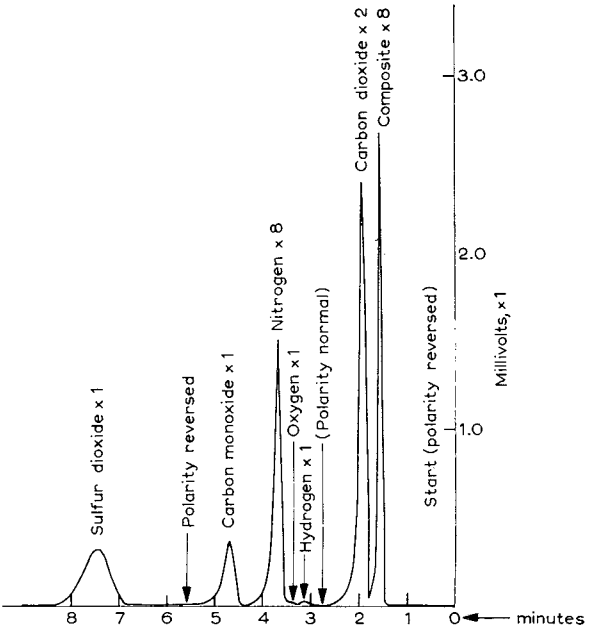

Fig. 3. Sample chromatogram for limited number of components

typical chromatogram of a 1-ml sample. The sensitivity of hydrogen is poor when helium is used as the carrier gas; so argon is used as the carrier gas for the determination of small concentrations of hydrogen. The separation of hydrogen takes place on the molecular sieve column; so the sample is injected just preceding Column 2 (inlet B) when hydrogen is analyzed alone.

ACKNOWLEDGEMENT

The work reported here was supported by the Iowa Engineering Experiment Station.

SUMMARY

A gas chromatography system is described for analysis of carbon dioxide, hydrogen sulfide, hydrogen, oxygen, nitrogen, methane, carbon monoxide, and sulfur dioxide from a single gas sample. The system consists of three columns in series. Column 1 separates the polar gases which are then irreversibly absorbed on Column 2. Column 2 delays but does not absorb the other gases which are subsequently separated on Column 3. Eluted peaks from Column 1 are detected in the reference side of the thermal conductivity cell and peaks from Column 3 in the sample side of the cell.

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COLLECTION OF GAS-CHROMATOGRAPHIC EFFLUENTS FOR INFRARED SPECTRAL ANALYSIS

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INTRODUCTION

In recent years, high-temperature gas–liquid chromatography has become the method of choice in the analysis of high-boiling liquids, particularly in the field of lipid chemistry. This is to be expected, since the method is rapid, reproducible, and nearly quantitative. However, the results obtained must be interpreted with caution. Particularly in the case of unknown mixtures, the analyst must have some means of qualitatively identifying the components he has separated and quantified. In many cases the amount of the pure components available is so small that classical methods of analysis are impractical, but valuable information may be obtained from an infrared spectrum.

For this purpose, the vaporized sample in the effluent from the chromatographic column is generally condensed in a chilled glass tube and later transferred to a suitable cell. The methods presently in common use for non-volatile liquids include smearing the material on a salt plate (AgCl, KBr, or NaCl), or transferring it with solvent to a standard micro-cell[1–4]. A technique has recently been developed which eliminates the necessity of transferring the sample, by condensing it on the same medium that is used to support the sample in the infrared spectrophotometer. For this purpose, a material is required which can efficiently remove the sample from the exit stream of a gas-chromatographic column without interrupting the flow of gas; furthermore, the material must be somewhat transparent in the infrared region of the spectrum.

To meet these requirements, what is needed is essentially a filter or thin, porous membrane. SLOANE has previously shown that specially made, thin (25 μ) cellulosic membranes, of the type manufactured by Millipore, can be used in a differential technique for infrared analysis[5]. While the cellulose esters, of which the filter is composed, have four strong absorption bands in the infrared (see Fig. 1a), these can be effectively nullified in a double-beam instrument (Fig. 1b), allowing the material to be used as a sample support. This fact, combined with the high porosity and uniformity of the Millipore® membrane, makes it ideally suited to the purpose. Tests on this membrane indicate that the fine, uniform pores (0.45 $\pm$ 0.02 μ in diameter) constitute up to 70% of the membrane volume. Thus it is fine enough to trap effectively small droplets, and porous enough to permit a very high flow rate per unit area. This is supported by the

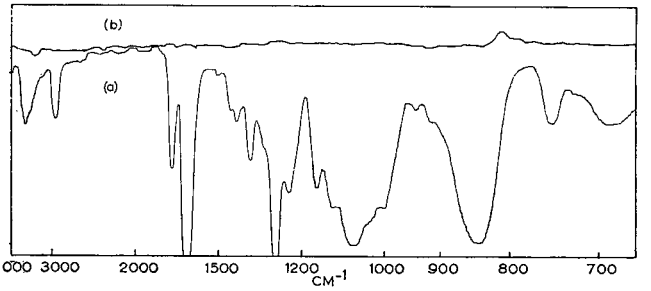

Fig. 1. (a) Infrared spectrum of thin Millipore membrane filter uncompensated. (b) Differential spectrum of compensating filters.

fact that standard thickness Millipore filters have previously been used as a supplementary collection device in gas–liquid chromatography[6].

EXPERIMENTAL

To collect samples, a $^1/_2$ in. filter disk in a Millipore "Swinny" Adapter was tried initially. The exit of a gas-chromatographic column (F & M Scientific Co., Avondale, Pa., Model 500) was fitted with a male luer-taper adapter which provided a leak-proof seal to the filter holder while permitting quick changes. Studies with methyl 1-^{14}C-stearate indicated, however, that only 10% of the radioactive material injected into the column was collected on the filter. Cooling the filter produced no improvement. For efficient collection, the filter and its support screen must be kept cold, but the passages upstream from the filter must be hot to prevent condensation of the sample before reaching the filter. This apparent dilemma was solved by making the front half of the holder from Teflon® (see Fig. 2), which prevented heat transfer from the heated

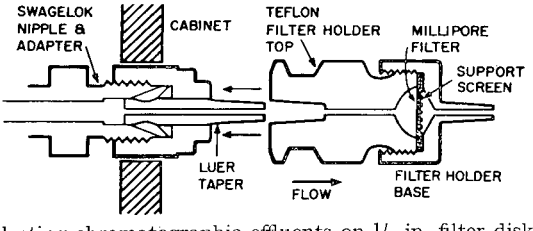

Fig. 2. Holder for collecting chromatographic effluents on $^1/_2$-in. filter disk, showing adapter on column exit.

exit port to the filter. The base of the holder was then cooled by wrapping it with a strip of cloth which dipped into an acetone–dry ice bath in a Dewar flask. With this arrangement, collection efficiency was increased significantly. To determine this collection efficiency 1 μl of methyl 1-^{14}C-stearate was injected into the chromatographic column and an equal quantity was placed in a counting vial, No. 1. The effluent from the column was collected on a filter as described and the filter was then placed in vial No. 2. A second filter was used to collect effluent following emergence of the stearate; this filter was placed in vial No. 3. Fifteen ml of polyether scintillation fluid was added

to each, and to a blank vial No. 4, and all were counted in a Packard liquid scintillation counter. The results are shown in Table I.

To record spectra of samples after collection, the filters were mounted in the spectrophotometer using a simple holder designed for the $^1/_2$ in. KBr disc. A blank filter of the same size and thickness was mounted in the reference beam, and the spectra were recorded in the usual manner on a Perkin-Elmer Infracord®.

In experiments using minimal amounts of sample, greater sensitivity was attained by reducing the effective area of the filter, to conform with the effective beam area of the spectrophotometer. This was done most simply by pressing a mask of gummed paper over the filter; a more elaborate holder using a specially-designed Teflon® insert was also used (Fig. 3).

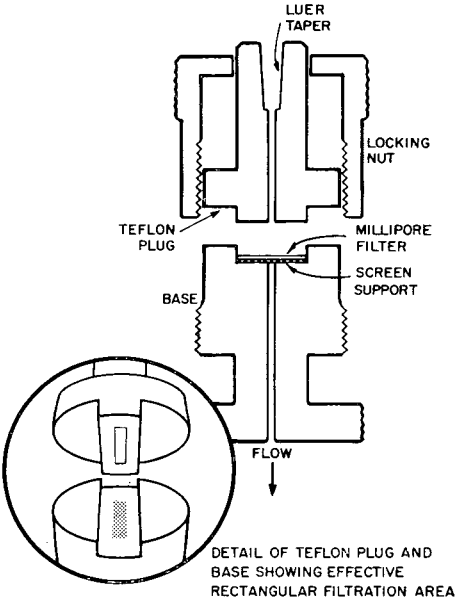

Fig. 3. Improved holder which concentrates sample on 2 mm by 10 mm area, for greater efficiency.

RESULTS AND DISCUSSION

The data from the experiment to determine collection efficiency are shown in Table I. They indicate that about 45% of the injected material was collected on this filter. Since recovery from chromatographic columns rarely exceeds 80% these were results considered quite favorable.

TABLE I

No.		*c.p.m.*	*% of standard corrected*
1	Standard	3400	100
2	Collected on filter	1620	44
3	Column background	130	—
4	Counter background	30	—

Fig. 4a shows the spectrum of diethyl malonate collected on a filter. The sample of the pure compound was separated from 1 μl of a 30% solution in benzene, on a silicone rubber column at 120°. Fig. 4b is the spectrum of an equivalent amount of pure

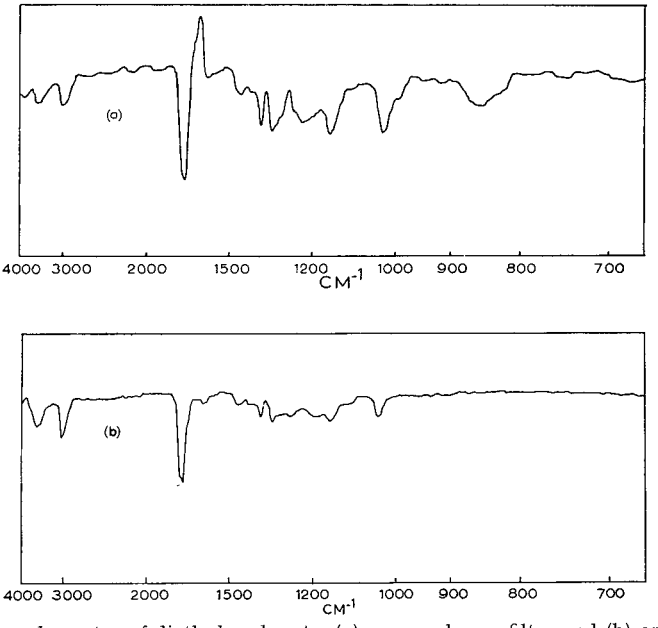

Fig. 4. Infrared spectra of diethyl malonate, (a) on membrane filter and (b) on salt plate.

diethyl malonate smeared on a salt plate. The absorption is weaker because not all of the sample is concentrated in the beam area, and because some evaporation occurred. The main differences between the two spectra occur at 1650 cm^{-1} and 850 cm^{-1}, frequencies at which the filter is very strongly absorbing (see Fig. 1). This is an example of imperfect compensation.

Fig. 5a shows the spectrum obtained from 2-hydroxydodecanoic acid (methyl ester), a component of the fatty acids in the bound lipids of *Azotobacter vinelandii*, collected on a filter from a silicone rubber column at 190°. Although these fatty acids are normally separated on a polyester column, we found new absorption bands in samples collected from a polyester column at 190°; these were shown to be due to substrate bleeding from the column. Readers are cautioned to be aware of this possibility when collecting from columns at high temperatures.

Fig. 5b shows the spectrum of synthetic 2-hydroxydodecanoic acid (methyl ester) smeared on a salt plate. The spectrum obtained on the filter in this case differs in two respects: (1) the additional band at 850 cm^{-1}, due to strong filter absorption and (2) the gradual lowering of the base line at higher frequencies. The most probable explanation for the latter result is the formation of tiny crystals on the filter, which disperse the incident radiation of short wavelength.

From the results of these and other tests which have been run it appears that the membrane filter technique is a useful supplement to the standard methods of collecting gas-chromatographic effluents for infrared spectral analysis. The technique is best

applied to relatively non-volatile liquids. Good spectra cannot be obtained from crystalline solids owing to the dispersion of the incident beam. Because of the energy of the infrared beam, however, some low-melting solids may become liquid, and provide characteristic spectra.

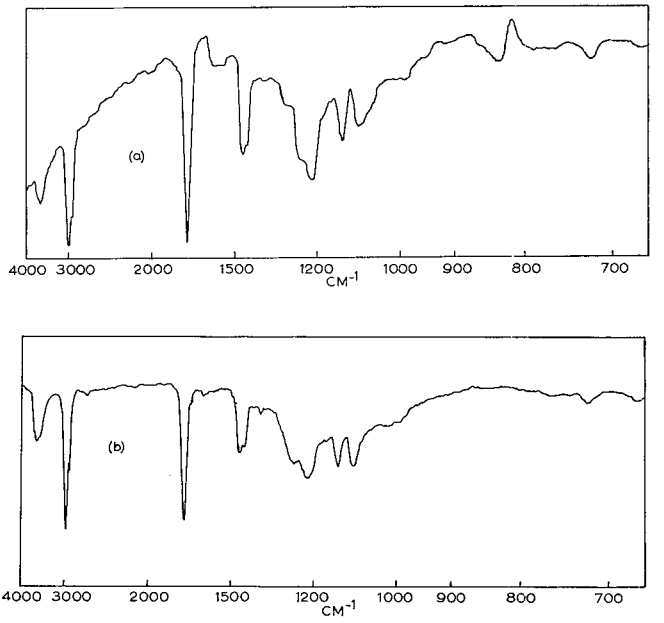

Fig. 5. Infrared spectra of 2-hydroxydodecanoic acid (methyl ester), (a) on membrane filter and (b) on salt plate.

As with any differential technique, matching of the two beams is important. In this case, the blank filter must have the same thickness as the sample filter, within 1%. Since the tolerance cannot be maintained within this range in the manufacturing, it is necessary to cut the two filters from adjacent areas of the same piece. Attempts are being made to improve the variation in manufacturing, but for the best results at present it is advisable to match the filters. If they are cut with a die having a constant area, they can be matched with respect to thickness simply by weighing them on a micro-balance.

The spectra shown in Fig. 4 and 5 were obtained from samples collected with the specially designed holder, but the results obtained with the Teflon Swinny adapter are equally good. These holders and the filters are available from Millipore. Adapters to fit most chromatographs can be furnished on request if the details of the fitting are supplied.

ACKNOWLEDGEMENTS

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SUMMARY

Thin, porous, cellulosic membrane filters were used to collect components of the effluent from a high temperature gas-chromatographic column. These filters were then mounted directly in the sample beam of an infrared spectrophotometer, while a blank filter of the same thickness was mounted in the reference beam. Characteristic spectra were obtained from the samples.

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ANALYSIS OF ALKYL ESTERS OF PHOSPHORIC ACID BY GAS CHROMATOGRAPHY

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INTRODUCTION

Mono- and di-alkyl phosphoric acids are now widely used for the extraction of metal ions from aqueous solution[1]. Mono- and di-*n*-butyl phosphoric acid (H_2MBP and HDBP respectively) are of importance[2] in commercial processes using tri-*n*-butyl phosphate (TBP) for the separation and purification of metals, because these acidic esters are formed by radiolysis and hydrolysis and lead to reduced separation factors, deposition of metal-complexes as solids, and emulsification problems.

A rapid paper-chromatographic method of analysis of H_3PO_4, H_2MBP and HDBP in TBP has been developed previously[3]. This method was essentially qualitative, but was later developed[4,5] into a method suitable for the quantitative determination of these impurities in solutions of TBP in hydrocarbon diluents.

Gas chromatography should provide a more rapid and sensitive method of analysis but the main problem in the past has been the difficulty of making these compounds migrate through the gas–liquid column owing to their low volatility and their thermal decomposition. However, gas chromatography has been used to analyse butyl nitrate[6] and water[7] in TBP, and alkyl hydrogen *phosphites* have been analysed[8] directly by gas chromatography. Diazomethane has been widely used for the conversion of fatty acids to their more volatile methyl esters (see references given in review[9]). It has now been shown that mixtures of alkyl esters of phosphoric acid can be converted into their more volatile methyl esters by diazomethane and that high separation factors can be obtained in simple gas–liquid columns. The method is applicable to the analysis of impurities in TBP and also to the analysis of alkyl phosphoric acids in general.

EXPERIMENTAL

The diazomethane was obtained by the reaction of nitrosomethylurea (NMU) (supplied by Dr. E. S. LANE, Chemistry Division, A.E.R.E.) in ether with an equal volume of 40 % w/v aqueous potassium hydroxide at room temperature, followed by drying the separated ether solution with pellets of solid KOH. The solid NMU is not very stable at room temperature and was stored at 0°. A more stable reagent for future work is *p*-tolyl-sulphonylnitrosamide (available from L. Light & Co. Ltd., Colnbrook, England); an ethereal solution of CH_2N_2 can be obtained by adding an ethereal solution of the nitrosamide to warm alcoholic alkali and distilling the ethereal CH_2N_2 as soon as it is formed[10].

The ethereal solution of CH_2N_2 was added to a 0.05–0.1 ml sample of the mono- or di-alkyl phosphoric acid, with or without TBP present, until the yellow colour of CH_2N_2 just persisted, indicating that an excess was present; the reaction was fast at room temperature, *i.e.* complete in less than 1 min, with the evolution of nitrogen gas. The ether and excess CH_2N_2 were removed by warming the solution and bubbling dry air through it. A sample of the remaining methyl esters, alone or with TBP present, was taken up in a calibrated hypodermic syringe and 1–10 μl injected into the gas-chromatographic column through a rubber septum.

A standard Griffin and George Mark III A gas chromatograph was used with 3 ft. $\times$ $^1/_4$ in. diameter glass columns containing (i) 13.6 g Perkin Elmer "C" material (Dow Corning silicone DC 200 on 80–100 mesh firebrick) at 188°, 2.3 l/h helium carrier gas; (ii) 1.5 g silicone E301 (Griffin and George Ltd.) on 6.8 g 80–100 mesh celite at 197°, 1.7–2.5 l/h helium; and hot-wire thermal conductivity detectors at the column temperature.

Relative retention volumes (referred to TBP as internal standard) were calculated from retention distances measured on the recorder chart from the maximum of the air peak to the maxima of the solute peaks. Samples of H_2MBP and HDBP were separated from a commercially available mixture[3]; tributyl phosphate was purified[11] by steam-distillation of volatile impurities from its mixture with dilute aqueous alkali, separation from the aqueous phase (which contained the sodium salts of acidic impurities), and vacuum drying. A.R. ortho-phosphoric acid was used as received. Other alkyl phosphoric acids were obtained from Albright & Wilson Ltd., Oldbury, England.

RESULTS

Separation of H_3PO_4, H_2MBP, HDBP and TBP

Retention volumes of the various esters were measured for 2 μl samples of solutions in TBP of the methyl esters of H_2MBP, HDBP and H_3PO_4 relative to TBP = 10 (see Table I).

TABLE I

RETENTION VOLUMES (RELATIVE TO TBP = 10) OF ALKYL PHOSPHATES

	Liquid phase: silicone	
	DC 200, 188°	*E 301, 197°*
Monomethyl di-*n*-butyl phosphate	4.1	4.4
Dimethyl mono-*n*-butyl phosphate	1.6	1.9
Trimethyl phosphate	~ 0.4 and	~ 0.4 and
(2 peaks, see text)	0.55	0.6
Ether	~ 0.01	~ 0.01

The water present in the methyl esters after the diazomethane reaction was not completely removed by treatment with pellets of solid KOH for 15 min, and appeared on the chromatograms as a low tailing peak between relative retention volumes 0.5 and 3.

It is interesting to note that the retention volume increases by an average factor of about 2.5 for each butyl group present. Three small sharp peaks appeared before

the trimethyl phosphate peak and were due to air, ether, and an unidentified impurity, in order of increasing retention volume. Two peaks were often obtained from the reaction of H_3PO_4 with CH_2N_2. One was trimethyl phosphate and the other may have been dimethyl phosphoric acid or some impurity. The ratio of the two peaks obtained on treatment of a sample of H_3PO_4 with a slight excess of CH_2N_2 did not alter significantly on further treatment with CH_2N_2. No products having low retention volumes were observed in a "blank" experiment in which a solution of CH_2N_2 in ether was (a) shaken with aqueous alkali, (b) the ether solution decanted off and dried with KOH, (c) the ether and CH_2N_2 evaporated off and (d) the residue taken up in pure TBP and a sample injected into the column. This indicates that volatile materials (which might be formed by the reaction of CH_2N_2 with CO_2 or impurities in the aqueous alkali) are not formed in the methylation of acidic phosphate esters by this method.

The feasibility of quantitative analysis of H_2MBP, HDBP and H_3PO_4 in TBP

A sample of 2 μl of a solution of $\approx$ 5 % w/w of H_2MBP and of HDBP in TBP was injected into the silicone E 301 column at a temperature of 197°, a sensitivity $\times$ 3, and a bridge-current of 200 mA. The areas of the peaks on the chromatogram were measured by counting squares and the weight percentage of the components calculated by the method of "internal normalisation", *i.e.* the percentage of a component is given by 100 $\times$ the area of the peak for that component divided by the sum of the areas of the peaks for all the components:

Component	% *w/w present*	% *w/w found*
TBP	89.8	90.0
HDBP	4.9	4.9
H_2MBP	5.3	4.5
H_3PO_4		0.6

The H_2MBP contained about 10 % of H_3PO_4 impurity, and the HDBP a trace.

The injection of 5 μl of a solution in TBP of 1 % w/w of each of the components HDBP, H_2MBP, and H_3PO_4 is calculated to give peaks of the following heights (at a maximum sensitivity of $\times$ 1 and 200 mA bridge current).

	TBP	*HDBP*	*H_2MBP*	*H_3PO_4*
Amount (%)	97	1	1	1
Peak-height (mm)	200	30	10	60

It therefore seems feasible with the present apparatus to determine 1 % w/w of HDBP and H_3PO_4 in TBP to about $\pm$ 5 % and of 1 % H_2MBP to about $\pm$ 10 %.

Experiments on other alkyl phosphoric acids and alkyl phosphates

Samples of impure mono-*n*-octyl phosphoric acid and di-*n*-octyl phosphoric acid were separately treated with diazomethane in ether and injected (with and without TBP

present) into the silicone DC200 column at 204°. The 2 μl sample of impure mono-*n*-octyl phosphoric acid gave a main peak due to dimethyl mono-*n*-octyl phosphate and five small peaks at lower retention volumes due to unidentified impurities (one of which was probably trimethyl phosphate from phosphoric acid). The 2 μl sample of di-*n*-octyl phosphoric acid gave one broad peak due to methyl di-*n*-octyl phosphate and a small sharp peak which could have been due to trimethyl-phosphate.

A 2 μl sample of equal parts of tri-*n*-propyl phosphate (TPP) and TBP was chromatographed at 204° for comparison of their retention volumes and to test the possible use of TPP as an internal standard for the analysis of mixtures of alkyl phosphoric acids which have components with retention volumes similar to that of TBP. The two phosphates were easily separated (see Table II).

TABLE II

RETENTION VOLUMES (RELATIVE TO TBP = 10) OF ALKYL PHOSPHATES

Tri-*n*-propyl phosphate (TPP)	3.7
Dimethyl mono-*n*-octyl phosphate	7.9
Methyl di-*n*-octyl phosphate	73

CONCLUSIONS

Mono- and di-alkyl phosphoric acids can readily be converted to their methyl esters by treatment with diazomethane in ether. The diazomethane can be prepared from nitrosomethylurea but the use of *p*-tolyl-sulphonylnitrosamide is recommended for future work because of its greater stability. A high separation factor (2.5) can be obtained between the methyl butyl phosphates on a 3 ft. column containing silicone DC 200 at 188°, or silicone E301 at 197°.

Further work will be required to demonstrate that the diazomethane reaction is quantitative, and to obtain the optimum separation of the products by gas chromatography. The lower limit of detection will be set by the size of sample, the type of column and the type of detector employed. It is feasible to determine these impurities at the 1% level in TBP to about $\pm$ 5% with the apparatus described, but a more sensitive detector should allow a considerable improvement on the accuracy at this level and the limit of detection.

SUMMARY

Mono- and di-alkyl phosphoric acids can be converted to volatile methyl esters by treatment with diazomethane in ether. The separation of the mixed methyl alkyl phosphates can readily be achieved by gas chromatography on a 3 ft. column of silicone DC200 on a firebrick support at 188° or silicone E301 on a celite support at 197°. The method is applicable to the quantitative analysis of mixtures of alkyl esters of phosphoric acid in TBP.

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SUBSTANCE RECOVERY IN PREPARATIVE GAS CHROMATOGRAPHY

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A serious problem in preparative gas chromatography is the recovery of the separated substances. Difficulties can arise because of only partial condensation or because the condensation produces an aerosol. In each case some of the material is not held back in the collecting trap and is swept away by the carrier gas. Various methods have been advanced to solve this problem, such as electrical precipitation[1,2]; centrifugation of the collector bottle during the chromatography[3]; the use of specially heated or gradient cooled collectors[4,5] to prevent fogging; filling the collecting bottles with asbestos, glass or cotton wool to trap the aerosols[6]. A 100% efficient recovery system has not been devised and it seems improbable that this will ever be the case.

In this paper some factors are discussed which have an influence on the recovery percentage and ways of improvement are investigated.

EXPERIMENTAL

The collector trap

Apart from special effects like centrifugation or electrical precipitation, the form of the collector could have some influence. We tried several models and some are depicted schematically in Fig. 1. Of these, model E was most efficient and we did not succeed in improving it by prolonging the tube inlet, drawing the tube inlet to a fine point, or by using thin walled vessels. As reported in the literature, the efficiency of these traps is greatly improved by filling them with cotton wool, asbestos wool or glass wool. In

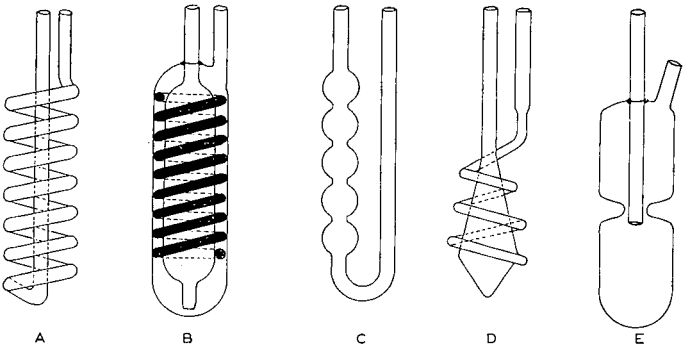

Fig. 1. Collecting bottle types. A = spiral; B = Widmer; C = ball condenser; D = cyclone; E = Aerograph 700.

this case, however, the resistance to carrier gas flow increases and the connection between the column exit end and the trap must be adequate to prevent losses at this point.

Volatility of the substances

Two hundred microlitres of ether, cyclohexane, iso-octane and *trans*-decalin were chromatographed at their respective boiling temperatures on an Aerograph Autoprep 700 (6 m $^3/_8$ in. column, filled with 30 % SE 30 on chromosorb P; 200 ml/min hydrogen) and collected at —20° (ice–salt mixture*). The recoveries were 0, 39, 44.6 and 88 % respectively. This shows clearly the influence of the volatility of the samples.

Concentration of the substances in the gas phase

The concentration of the substances in the gas phase plays an important role in the recovery, which increases with increased concentration. This concentration is determined by column parameters such as the volume of mobile phase in the column (column length and diameter) and the separation efficiency of the column expressible in plate numbers (more plates per metre gives narrower bands and higher concentrations). For a given column, however, these are not very variable. Other factors directly related to the concentration are the amount of material put on the column, and more particularly the temperature of elution**. Large samples chromatographed at a high temperature will show the highest concentration in the eluate. Unfortunately, however, the most interesting are nearly always the rarest and most complicated chemical mixtures which are therefore difficult to separate, so that in this case only small samples and long retention times (low temperatures) can be used. The recovery percentage will be low although it is just with such mixtures that high recovery is most interesting. That the sample size has a direct influence on the concentration of the eluted substances is obvious, but the influence of the temperature of elution is shown by the results given in Table I.

TABLE I

PERCENTAGE RECOVERY OF *trans*-DECALIN AS A FUNCTION OF ELUTION TEMPERATURE (ISOTHERMAL CHROMATOGRAPHY)

Quantity (μl)	*Column temperature*					
	200°	*180°*	*160°*	*140°*	*120°*	*100°*
200	93.1	87.8	72.7	71.3	66.7	47.0
100			69.0			

Table I gives the mean value of five determinations. The collecting bottles were tared and the collected sample weight was compared with the weight of the injected sample as delivered by a syringe. Other conditions were: on column injection on Aerograph Autoprep 700; gas rate 200 ml/min H_2; collecting bottles cooled in ice water.

* It is most efficient to use the same salt water solution again and again, by simply freezing it in the Dewar flask by repeated addition of liquid air with vigorous stirring.

** Unpublished work shows that, as stated, the temperature at elution and not the retention time is important in this connection[7].

High separation effect, long retention times and high temperature at elution can be obtained together by programming the gas chromatographic separation. That programming results in better recovery has been noted before[8].

An alternative is to run the chromatogram at a lower temperature for the desired time, stop the flow of carrier gas, increase the temperature of the column oven and then restore the gas flow. In this way the substances are chromatographed long enough to be separated and are eluted at a high temperature. That recovery is better by this technique is shown by the figures for 200 μl ether, cyclohexane, iso-octane and *trans*-decalin which were run in the conditions as described before, but with an additional temperature increase of 70° with the gas flow stopped just before elution. The recovery was respectively 14.4, 42.4, 61.5 and 95.4 %, which is decidedly higher than without temperature increase.

Gas flow rate

High speeds of the gas flow through the collecting trap must be detrimental to the recovery. One way to ascertain this is by diminishing the gas flow rate just before elution of a peak. In a recovery experiment on 200 μl ether, cyclohexane, iso-octane and *trans*-decalin, as described above, the temperature was increased by programming and the gas flow was reduced to 25–30 ml/min just before elution of the peaks. This was very easily effected without changing any control setting of the instrument, by inserting a suitable bleeding capillary through the injection gasket at the appropriate moment.

The recovery results were now respectively 31.8, 73.4, 84.6 and 97.0 %. While such a drastic gas rate reduction would have a marked influence on baseline stability in normal analyses with high sensitivity, this is not the case in preparative work, since the sensitivity of the instruments is much reduced with regard to the high sample load. Comparative gas chromatograms on decalin are shown in Fig. 2.

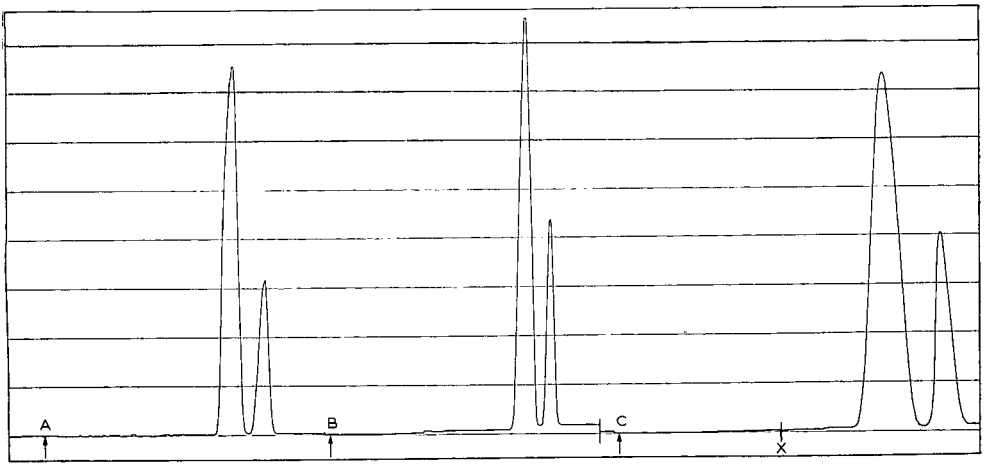

Fig. 2. Chromatograms of decalin on Autoprep 700. Sample load 200 μl; bridge current 150 mA. Attenuator 32 for A and B, 64 for C. Collecting bottles cooled in ice water. Other conditions: (A) isotherm at 170°—200 ml/min H_2; (B) programmed from 150 to 190° at peak elution; 200 ml/min H_2; (C) programmed from 150 to 200° at peak elution. At point X the gas rate was changed from 200 to 25 ml/min H_2.

A and B have been run in such conditions that the retention times are about the same. The elution temperature for the programmed run is, however, higher and therefore gives narrower and more concentrated peaks (higher signal for identical sample). The recorded band broadening of C is of course due to the lower gas rate while eluting the peaks.

Another possibility seemed to be to collect the eluate of very volatile materials in inflatable toy balloons and then to press the gas very slowly through a collecting bottle. Several attempts in this direction gave no interesting results.

The nature of the carrier gas

Three gases can be considered for general use in preparative gas chromatography, hydrogen, helium and nitrogen. Helium is very expensive, especially in Europe, and therefore often out of the question. Hydrogen is dangerous, although it may be pointed out that this should not be exaggerated since hydrogen–air mixtures become inflammable only above 4 % hydrogen concentration.

Nitrogen, on the other hand, compared with hydrogen, produces a serious efficiency drop in long columns with high resistance to gas flow. From the point of view of recovery discussed in this paper, hydrogen and nitrogen were compared in experiments on 200 μl ether. The starting temperature was 35° and programming produced a 50° increase to the elution point. The gas rate was 200 ml/min at the start of each run. The instrument was again a Aerograph Autoprep 700 with a 6 m column filled with 30 % SE30 on chromosorb P and the collector bottles were cooled at —15° in an ice–salt mixture.

The recovery was slightly better with nitrogen than with hydrogen. The mean value for five determinations with each gas was 16.5 and 13.0 % respectively. The difference between hydrogen and nitrogen with regard to the recovery percentages is therefore not significant.

Temperature of the cooling bath

In the experiments described above the collector bottles were cooled by immersion in a bath containing ice water (0°) or an ice–salt mixture (—15° to —20°). The effect of further cooling on the recovery of ether was studied. 200 μl of ether was chromatographed isothermally at 70° on an Aerograph 700 with hydrogen as carrier gas, the other conditions being as described above. The mean of five recovery determinations per temperature are found in Table II. Adequate cooling is therefore very important for the recovery in preparative gas chromatography.

Availability of the recovered substances

Efficient recovery is only possible when using glass wool scrubbers in the collecting

TABLE II

PERCENTAGE RECOVERY OF ETHER AS A FUNCTION OF COOLING BATH TEMPERATURE

	Temperature of the cooling bath					
	0°	*—8°*	*—14°*	*—25°*	*—65°*	*—80°*
Recovery %	0	2.3	16.7	24.2	83.3	89.4

bottles. High boiling substances can be rinsed quantitatively from the glass wool with suitable solvents, but low boiling substances cannot be processed in this way and losses through wetting of the glass wool are unavoidable. Centrifuging, however, forces most of the material to the bottom of the collecting bottle, where it can be sucked out with a syringe or a pipette. This problem was investigated with increasing amounts of iso-octane chromatographed at 120° and with cooling of the collecting bottles in an ice–salt mixture, the instrument and other conditions being as described before. For 50, 100, 200, 500 and 1000 μl of iso-octane the recovery in the collecting bottles increased in the same order from 80 to 97%. The amount which could be drawn out with a syringe was then 31, 37, 74, 78 and 86% respectively. This is rather low for the 50 and 100 μl samples, and such small samples are indeed necessary with difficult separations. The separation will have to be repeated on a large number of small samples to obtain the desired amount of the substances. With such repeated separations and collection in the same bottle over and over again the recovery increases markedly. Five injections of 100 μl iso-octane gave a recovery in the collector of 79% while 67% could be sucked out with a syringe.

CONCLUSION

With higher boiling substances, the recovery in preparative gas chromatography is not a big problem. This is not so with substances boiling below 100° and in this case preparative gas chromatography should be run, with cooling in a dry ice–acetone mixture or even in liquid air. Improvement of the recovery percentage can also be obtained by programming the temperature and diminishing the gas flow rate at the moment of peak elution. This gas rate reduction is something which could easily be controlled automatically by the signal switch on the recorder which actuates the movement of the collecting bottles. As shown, linear programming of the temperature should be better than asymptotic programming. It seems thus that high (> 85%) recovery is possible with all liquids using simple collecting vessels.

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SUMMARY

Ways to improve recovery in preparative gas chromatography were systematically investigated. The results show that good recovery is possible in all cases with simple collecting recipients.

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ANALYSE RAPIDE PAR CHROMATOGRAPHIE EN PHASE GAZEUSE
SÉPARATION DU MÉLANGE OXYGÈNE–AZOTE–MÉTHANE–OXYDE DE CARBONE

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La chromatographie gaz–solide ne semble pas avoir bénéficié des progrès faits récemment dans la construction des colonnes de chromatographie gaz–liquide. La croyance trop répandue que les résistances aux transferts de masse en phase adsorbée sont élevées et s'opposent à l'obtention de bonnes efficacités semble avoir freiné les développements de cette technique. Pourtant, on sait depuis longtemps obtenir de bonnes efficacités avec de l'alumine[1], peut-être parce qu'un simple tamisage suffit à éliminer l'essentiel des particules trop fines auxquelles on attribue généralement la mauvaise efficacité des colonnes.

Ce travail illustre les avantages considérables que l'on peut obtenir d'une forte amélioration de la qualité des colonnes. Un gain d'efficacité important se traduit par une diminution très sensible de la durée nécessaire pour effectuer l'analyse. Comme les coefficients d'adsorption des gaz permanents sont dans la plupart des cas très différents il devrait être possible d'effectuer l'analyse sur une colonne très courte, ce qui permettra d'utiliser facilement ou un fort débit ou un remplissage de faible granulométrie et d'accélérer encore l'analyse. De plus par la diminution simultanée de la durée de l'analyse et de la longueur des colonnes nécessaires, on obtient une diminution considérable de la largeur des pics, donc une forte augmentation de la sensibilité de l'analyse. En effet l'appareil et les conditions opératoires restant les mêmes, l'aire du pic est constante, par conséquent sa hauteur se trouve augmentée.

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La séparation des gaz permanents sur tamis moléculaires 5 A est bien connue[2]. Elle est sensiblement plus facile que leur séparation par chromatographie gaz–liquide qui doit être faite à basse température, dans des conditions difficiles.

Ainsi, d'après PORTER ET JOHNSON[3], la séparation qualitative de l'azote, de l'oxygène et de l'oxyde de carbone peut se faire sur une colonne de 30 m de longueur, en utilisant comme phase stationnaire le *n*-heptane sur celite à —80°; la durée de l'analyse est de 32 min.

Sur tamis moléculaire 5 A, BOREHAM ET MARHOFF[4] ont pu obtenir en 35 min une analyse complète du mélange oxygène–azote–méthane–oxyde de carbone, en utilisant une colonne de 2 m de long. Récemment, ZHUKOVITSKY *et al.*[5] ont utilisé une colonne de tamis moléculaire 5 A, en série avec une colonne de poudre de brique imprégnée

de 8% de sang de cheval, cette seconde colonne assurant la séparation oxygène–argon; la séparation obtenue est bonne, mais la durée d'une analyse complète est longue, puisque le temps nécessaire pour éluer l'oxyde de carbone est de 44 min.

De meilleurs résultats[2] ont été obtenus à l'aide de la chromatographie à température programmée; en employant une colonne de tamis moléculaire 5 A de 122 cm, dont la température croît de 35° à 239°C à la vitesse constante de 4°/min, la séparation du mélange O_2–N_2–CH_4–CO demande environ 14 min. L'élution du gaz carbonique est alors possible au bout d'un temps très élevé.

Les résultats peu concordants obtenus par les premiers chercheurs ont conduit à étudier l'influence, sur la résolution des colonnes, des conditions d'activation ou plutôt de déshydratation du tamis moléculaire 5 A. D'après HAMILTON ET KORY[6], la longueur de la colonne nécessaire pour obtenir la résolution optimum du mélange O_2–N_2 dépend en grande partie des conditions d'activation du tamis moléculaire; ainsi, si l'on chauffe à 300° pendant 24 h, cette longueur est de 280 cm. JAY ET WILSON[7] activent le tamis moléculaire à 350° pour faire des analyses d'oxygène dans divers gaz. SMITH ET CLARK[8] ont obtenu de bons résultats par activation entre 500° et 600°.

Les divergences entre résultats expérimentaux peuvent s'expliquer en partie par l'emploi de techniques différentes pour activer le tamis moléculaire. De toutes façons, une activation à température relativement élevée paraît souhaitable. Un gain considérable sur la rapidité des analyses (14 min dans le cas le plus favorable) paraît nécessaire si l'on veut pouvoir suivre des phénomènes rapidement variables.

CONDITIONS EXPÉRIMENTALES

Le tamis moléculaire 5 A acheté en granulés a été d'abord broyé et tamisé entre les tamis de 315 et 400 μ (No. 25 et 26 AFNOR), puis on lui fait subir une élutriation sous contrecourant d'eau pendant 10 min, en faisant varier le débit d'eau jusqu'à obtenir une élimination complète des particules fines. En raison de la forte enthalpie d'adsorption de l'eau sur le tamis moléculaire, il est à conseiller de jeter le tamis moléculaire dans l'eau par petites fractions. Après élutriation le tamis moléculaire placé dans un cristallisoir est progressivement déshydraté, puis activé par chauffage, d'abord 3 h à 80° pour éliminer l'eau non adsorbée, ensuite 12 h à 250° sous une pression de 1 mm de mercure dans une étuve sous vide; enfin, pour désorber à peu près complètement l'eau, le tamis moléculaire est chauffé à 400° pendant 12 h dans un four, sous courant d'air sec. La colonne est remplie par la méthode classique puis montée sur un chromatographe Perkin-Elmer modèle 116-E, avec détecteur à thermistances. Le gaz vecteur utilisé est l'hydrogène. Les échantillons analysés ont été injectés au moyen d'une vanne à gaz munie d'un calibre de 0.25 cm^3 de capacité. L'enregistreur utilisé est un galvanomètre à suiveur de spot* dont le temps de réponse est environ 0.2 sec.

Pour assurer une durée de vie importante aux colonnes, il est bon de mettre en série avec elle, avant l'injecteur, un piège contenant du tamis moléculaire, qui purifie le gaz vecteur.

Pour corriger les volumes de rétention du volume gazeux de la colonne on utilise le volume de rétention de l'hélium mesuré à 100°C.

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ÉTUDE DES COLONNES

Nous avons préparé deux colonnes en cuivre de longueurs 20 et 200 cm, de diamètre 4 mm, remplies de tamis moléculaire 5 A, 315–400 μ, préparé comme décrit ci-dessus. Les caractéristiques de ces colonnes sont données Tableau I.

TABLEAU I

CARACTÉRISTIQUES DES COLONNES UTILISÉES

Colonne	*No. 1*	*No. 2*
Longueur (cm)	20	200
Diamètre (cm)	0.4	0.4
Granulométrie (μ)	315–400	315–400
Poids de phase stationnaire (g)	2.1	17
Section de la veine gazeuse (cm^2)	0.032	0.08
Efficacité maximale (plateaux)		
N_2	212	1330
CO	206	1510
H.E.T.P. (cm)		
N_2	0.093	0.154
CO	0.096	0.130
Débit optimum (cm^3/min)		
N_2	38	37
CO	50	62
Facteur de résolution O_2–N_2 à 22°, 200 cm^3/min	5	41
Durée d'élution (sec)	14	84

Nous avons déterminé les variations de la hauteur équivalente à un plateau théorique en fonction de la vitesse de passage du gaz vecteur pour l'oxyde de carbone et l'azote sur les deux colonnes (Fig. 1). Les valeurs trouvées sont très satisfaisantes et sensiblement inférieures aux valeurs publiées antérieurement pour des colonnes de cette nature. Cette efficacité analogue à celle obtenue sur des colonnes de chromatographie gaz–liquide faites avec un support de même granulométrie peut être attribuée au traitement d'élutriation que nous avons fait subir au tamis moléculaire. En effet, ce traitement élimine complètement les fines qui sont connues pour diminuer considérablement l'efficacité des colonnes. En même temps, l'élimination de ces particules fines permet d'obtenir des colonnes dont la perte de charge est très faible. On pourra donc, suivant les cas et sans difficultés, soit utiliser de forts débits de gaz, soit employer des colonnes de grande longueur, soit diminuer sensiblement les dimensions moyennes des particules de phase stationnaire.

L'efficacité de la colonne de 20 cm est telle qu'on doit l'utiliser au débit optimum du gaz vecteur pour effectuer à la température ambiante une analyse quantitative du mélange O_2–N_2–CH_4–CO, ce qui nécessite un facteur de séparation de 1 pour le couple le moins bien séparé. Dans ces conditions, la durée totale de l'analyse est de 3 min environ (Fig. 2).

Nous avons cherché s'il était possible de diminuer la durée de l'analyse en aug-

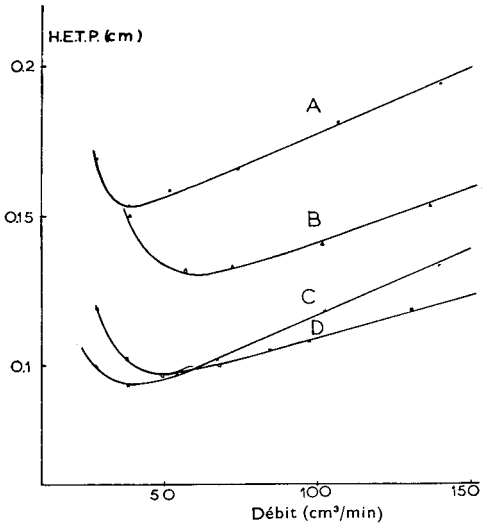

Fig. 1. Courbes de Van Deemter à 22°. C, D: colonne 1; A, B: colonne 2; B, C: N_2; A, D: CO.

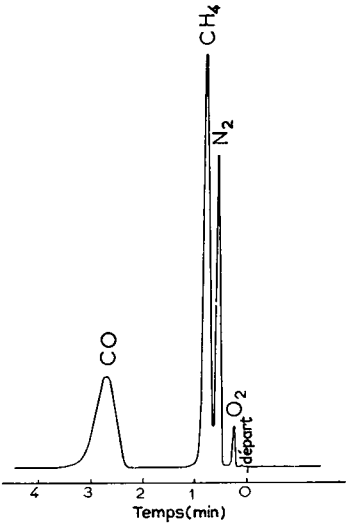

Fig. 2. Séparation du mélange O_2–N_2–CH_4–CO sur la colonne No. 1. T = 22°, débit 53 cm³/min.

mentant simultanément le nombre de plateaux de la colonne, donc sa longueur, et la vitesse du gaz vecteur. Cela est toujours possible d'après PURNELL ET QUINN[9] lorsque le terme C est assez faible et donc que H.E.T.P. augmente lentement avec le débit. La durée de l'analyse à la température ambiante avec la colonne de 200 cm, pour un débit de 333 cm³/min, est d'environ 7 min. Il paraît difficile de diminuer beaucoup la durée de l'analyse en augmentant davantage le débit. Le facteur de résolution est alors considérable et permettrait aisément de procéder à l'analyse de très faibles traces de trois des gaz étudiés dans le quatrième. Comme il n'est pas possible de modifier, comme en chromatographie gaz–liquide, le taux de phase stationnaire, nous avons cherché à diminuer les temps de rétention en augmentant la température. Le Tableau II donne à la température ambiante les valeurs des termes de l'équation de Van Deemter:

$$H = A + \frac{B}{u} + Cu$$

pour les deux premières colonnes et les valeurs de C mesurées pour la colonne de 200 cm à différentes températures. Ce terme ne varie que faiblement, ce qui confirme les idées de GIDDINGS[10] que les résistances au transfert de masse provoquées par la structure aléatoire du remplissage l'emportent de beaucoup dans les colonnes classiques sur les résistances dues à la cinétique de transfert en phase fixe. La Fig. 3 montre comment varient, avec la température, les volumes de rétention spécifiques sur tamis moléculaire des quatre gaz étudiés. On constate que la séparation serait obtenue dans les meilleures conditions vers 80°, température pour laquelle les volatilités relatives des couples N_2–CH_4 et CH_4–CO sont égales et un peu inférieures à la volatilité relative du couple O_2–N_2, ce qui est favorable car il est toujours plus difficile de séparer les paires les moins retenues.

TABLEAU II

COEFFICIENTS DE L'ÉQUATION DE VAN DEEMTER

Colonne	*Gaz*	*T(°C)*	*A (cm)*	*B (cm²/sec)*	*C (sec)*	H_{min} *(cm)*
No. 1	N_2	22	$7.7 \cdot 10^{-2}$	$5.1 \cdot 10^{-3}$	$1.3 \cdot 10^{-2}$	0.093
No. 1	CO	22	$8.5 \cdot 10^{-2}$	$4.6 \cdot 10^{-3}$	$6.6 \cdot 10^{-3}$	0.096
No. 2	N_2	22	$1.4 \cdot 10^{-1}$	$4.3 \cdot 10^{-3}$	$1.1 \cdot 10^{-2}$	0.153
No. 2	CO	22	$1.1 \cdot 10^{-1}$	$1.03 \cdot 10^{-2}$	$9.7 \cdot 10^{-3}$	0.130
No. 2	CO	50			$2.6 \cdot 10^{-2}$	
No. 2	CO	100			$2.6 \cdot 10^{-2}$	
No. 2	CO	150			$1.5 \cdot 10^{-2}$	

Nous avons trouvé cependant plus intéressant de travailler à une température un peu supérieure où les temps de rétention sont plus courts, ce qui compense la nécessité d'obtenir une efficacité plus grande de la colonne et donc de fonctionner avec un débit plus faible. Les essais effectués nous ont montré que, à 100°, on pourrait faire l'analyse complète en 24 sec sur la colonne de 2 m, la pression d'entrée étant de 2.5 kg/cm² et le débit de 700 cm³/min. Néanmoins, l'utilisation de débits si élevés

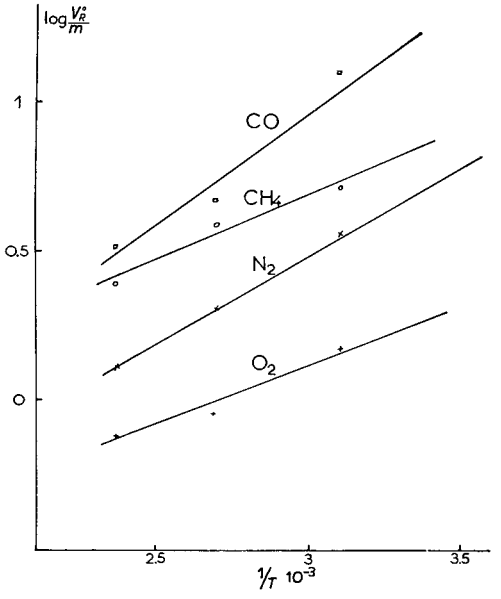

Fig. 3. Variation avec la température des volumes de rétention spécifique des gaz étudiés sur tamis moléculaire 5 Å élutrié à l'eau.

diminue fortement la sensibilité du détecteur et risque de provoquer une détérioration des thermistances. Un meilleur résultat du point de vue efficacité et sensibilité est obtenue à 100° et 428 cm³/min, avec une durée totale d'analyse de 32 sec à peine supérieure (Fig. 4a). Dans les mêmes conditions (T = 100°, débit 400 cm³/min) une colonne de 1 m de long remplie de la même phase (tamis moléculaire 5 A, 315–400 μ) permet d'obtenir une excellente séparation en 13 sec (Fig. 4b).

Les résultats obtenus à 150° sont moins bons; la séparation du couple CO–CH_4 devenant difficile, il faut travailler avec des débits relativement faibles et la durée des analyses devient plus grande. Par ailleurs, à cette température, on n'a pas une amélioration de la sensibilité, le gain apporté par la réduction du débit étant compensé

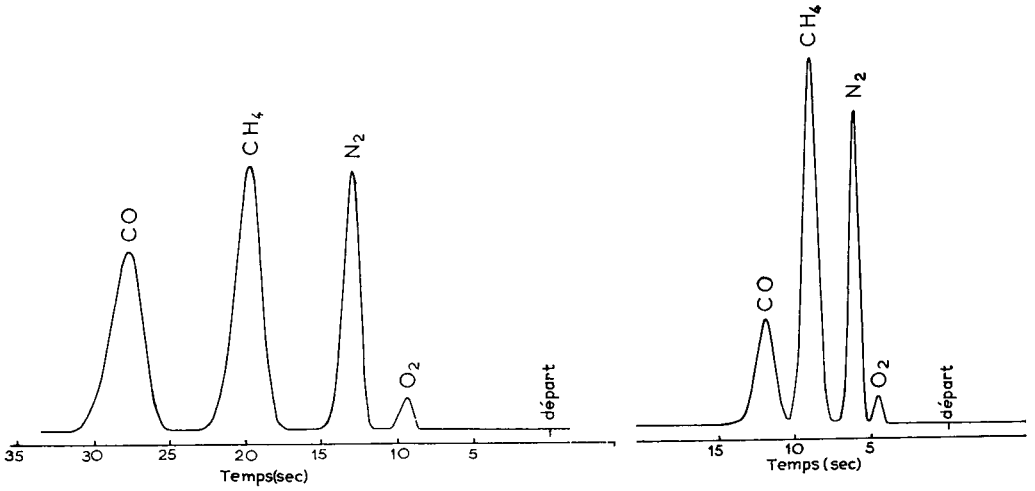

Fig. 4. (a) Séparation du mélange O_2–N_2–CH_4–CO sur la colonne No. 2. T = 100°, débit 428 cm³/min. (b) Séparation du mélange O_2–N_2–CH_4–CO sur une colonne de 1 m tamis moléculaire 5 A, 345–400 μ. T = 100°, débit 400 cm³/min.

par la perte résultant de l'élévation de température. De même à 100° avec la colonne de 20 cm la durée de l'analyse est très raccourcie mais la séparation, surtout celle de l'oxygène et de l'azote, est médiocre.

Les valeurs des enthalpies d'adsorption calculées à partir des pentes des droites de la Fig. 3 sont données Tableau III. Ces valeurs sont plus faibles que celles mesurées

TABLEAU III

ENTHALPIES D'ADSORPTION

Gaz	$-\Delta H$ *(kcal/mole)*
O_2	1.76
N_2	2.67
CH_4	1.98
CO	3.55

par GREENE ET PUST[11]. Il semble raisonnable d'attribuer cette différence soit à une activation insuffisante, soit plutôt à une perte d'activité du tamis moléculaire au cours de l'élutriation sous contrecourant d'eau. Si cette seconde interprétation est vérifiée, ce phénomène, lorsqu'il est gênant, ce qui n'est pas toujours le cas, pourrait être

éliminé en pratiquant l'élutriation dans le cyclohexane, voire sous courant d'air, à moins que des phénomènes électrostatiques ne provoquent l'agglomération des fines autour des particules plus grossières.

La bonne efficacité de ces colonnes nous a aussi permis d'étudier le cas de la séparation du mélange N_2–O_2. Les facteurs de séparation* et les durées d'élution sur les deux colonnes sont donnés Tableau I. De l'examen de ces résultats, on peut déduire qu'avec une colonne de quelques centimètres, il est possible de faire des analyses quantitatives du mélange N_2–O_2 à la température ambiante en un temps très inférieur à 10 sec avec un débit de 200 cm³/min environ et un facteur de résolution encore supérieur à 1, dans la mesure où les temps de rétention sont proportionnels aux longueurs des colonnes, ce qui néglige en première approximation de facteur de correction de JAMES ET MARTIN. Les premiers résultats expérimentaux sur la colonne de 20 cm (Fig. 5a) donnaient une durée d'analyse conforme aux prévisions, mais le

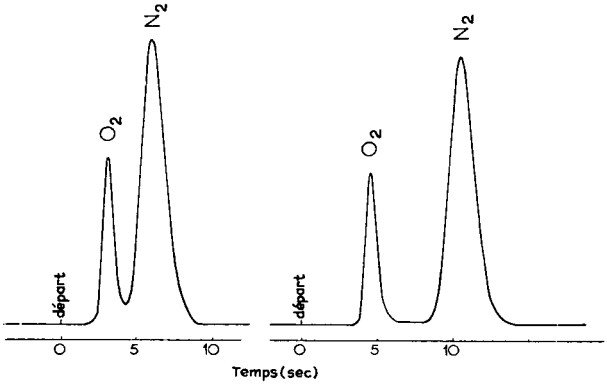

Fig. 5. Séparation de l'air sur la colonne No. 1. (a) Après utilisation sans purification du gaz vecteur. (b) Après régénération.

facteur de résolution était médiocre ($F = 1.5$). L'origine de cette anomalie était un empoisonnement du tamis moléculaire au cours du fonctionnement de la colonne, par des impuretés probablement amenées par le gaz vecteur. La régénération du tamis moléculaire peut se faire simplement par chauffage direct de la colonne avec un chalumeau à gaz et air pendant quelques minutes. La Fig. 5b montre les résultats trouvés après une telle régénération. Le Tableau IV donne les caractéristiques de la colonne dans les deux cas. Lorsqu'un piège contenant du tamis moléculaire est interposé sur le courant de gaz vecteur en avant de l'injecteur, cet empoisonnement ne se produit plus. On souligne encore l'importance, en chromatographie gaz–solide, des conditions d'activation du solide actif, comparable, dans une certaine mesure, à l'importance de la teneur en phase stationnaire en chromatographie gaz–liquide. L'importance qu'il y a à maintenir constante cette activation est particulièrement grande lorsque des analyses systématiques ou des travaux de routine doivent être entrepris.

* $F = 2(t_A - t_B)^2/w_A^2 + w_B^2$ où t_A et w_A sont le temps de rétention et la largeur à la base du pic du corps A.

TABLEAU IV

INFLUENCE DE L'EMPOISONNEMENT DU TAMIS MOLÉCULAIRE SUR LES PERFORMANCES DE LA COLONNE NO. I

	Avant régénération	*Après régénération*
Température (°C)	22	22
Débit (cm^3/min)	200	200
H.E.T.P. pour O_2 (cm)	0.324	0.158
H.E.T.P. pour N_2 (cm)	0.324	0.123
Facteur de résolution N_2–O_2	1.54	5.00
Temps de rétention N_2 (sec)	7	11
Durée d'élution du pic N_2 (sec)	9	14

Une dernière méthode d'obtention de durées d'analyse très réduites consiste à raccourcir les colonnes utilisées et à prendre comme phase stationnaire des particules de diamètre moyen aussi faible que possible, de façon qu'au débit optimum la perte de charge soit égale à la pression maximum à laquelle peut travailler l'appareil[12]. Cette méthode repose sur l'hypothèse que H est proportionnel au diamètre moyen d_p des particules. Nous avons cherché à réaliser des colonnes avec une poudre de diamètre nettement plus faible que celle de 315–400 μ utilisée pour les deux premières colonnes. En prenant une granulométrie de 100–125 μ, on obtient pour une colonne de 1 m de long une valeur minimale de H de 0.011 cm, 1.4 fois plus faible que pour la poudre de 315–400 μ, mais une perméabilité près de 10 fois inférieure. On ne peut pas obtenir une analyse plus rapide comme le voudrait le calcul de KNOX[12] parce que H n'est pas proportionnel à d_p dans les conditions de remplissage de nos colonnes.

Quoique nos résultats marquent un progrès sensible sur les travaux antérieurs, il nous semble qu'il est encore possible de réduire appréciablement la durée des analyses en améliorant encore le remplissage, en particulier celui des colonnes faites avec les poudres fines et en utilisant un appareillage mieux adapté à l'analyse rapide: détecteur et enregistreur de plus faible temps de réponse, injecteur automatique rapide, canalisations de plus faibles volumes morts. Avec l'appareillage utilisé il serait très difficile d'enregistrer correctement des analyses plus rapides que celles obtenues.

REMERCIEMENTS

Nous sommes très reconnaissants envers le Prof. L. JACQUÉ pour les encouragements qu'il nous a apportés dans la réalisation de ce travail. L'un de nous (F.F.-R.) remercie le Ministère de l'Éducation Nationale d'Espagne pour la bourse qu'il lui a accordée.

RÉSUMÉ

Une préparation soigneuse du tamis moléculaire 5 A utilisé pour séparer le mélange O_2–N_2–CH_4–CO permet d'obtenir des colonnes beaucoup plus efficaces et plus perméables qu'il n'était possible jusqu'à maintenant. Avec une colonne de dimensions convenables on peut obtenir l'analyse complète de ce mélange en moins de 15 sec, avec une excellente résolution.

SUMMARY

By careful preparation of molecular sieve 5 A powder it is possible to obtain much more efficient and permeable columns than were previously available. With such columns a mixture of O_2, N_2, CH_4 and CO can be resolved in less than 15 sec.

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APPLICATION OF THIN-LAYER CHROMATOGRAPHY TO THE STEROIDS OF THE ANDROSTANE SERIES

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Thin-layer chromatography utilizing Silica gel G with a binder has been successfully employed for the separation of several classes of steroids, *e.g.* etianic acids[3], oestrogens[19], Δ^4-3-oxo-C_{21}-steroids[18] and 19-norsteroids[12].

For a number of steroids of the androstane series, thin-layer chromatography has been applied without a binder using alumina[6], or with a binder using silica gel–starch[27], silica gel G[11,23] or alumina G[23].

The variation in techniques employed and the small number of steroids studied, does not permit extensive application of these results, *e.g.* for ΔR_M-function calculation.

Our experience has been with more than 50 steroids of the androstane series, and this paper describes the application of nine solvent systems to twenty-nine steroids of this series, nearly all of them saturated, and the calculation of the function ΔR_{Mg} for any hydroxyl and ketogroup. Forthcoming communications will cover the Δ^4-3-oxo- and Δ^5-3-hydroxy-steroids of this series.

MATERIALS

Reagents

All reagents used were of analytical grade, and the solvents were redistilled through fractionating columns before use. Sulphuric acid (batch No. 731) glacial acetic acid (batch No. 60), acetic anhydride (batch 42) and *m*-dinitrobenzene (batch No. 3114) were obtained from Merck A.G., Darmstadt. Anisaldehyde(*p*-methoxybenzaldehyde) (S 5209), chromic acid anhydride (A 229), bismuth subnitrate (A 1878) and osmium tetroxide (3616) were from Kebo A.B., Stockholm. Pyridine was purchased from the Gas Works of Stockholn, potassium iodide (batch No. 3162) from Baker Chemical Co., Philipsburg, U.S.A. and potassium hydroxide from E.K.A. (Elektrokemiske A.B., Bohus), Sweden.

Steroids

The sources of the steroids used in this investigation are given in Table I. In addition to their systematic names, trivial names are given, whenever indicated. The abbreviations *ol* and *one* are used to designate one or more hydroxyl- and oxo-groups, respectively.

METHODS AND RESULTS

General methods

The method used in this investigation has been described in a previous paper[19]. In this series of experiments, one-dimensional chromatograms were run on Silica

TABLE I

SYSTEMATIC NAMES, TRIVIAL NAMES, ABBREVIATIONS, SOURCES AND COLOURS OBTAINED WITH ANISALDEHYDE–SULFURIC ACID REACTION FROM THE 29 STEROIDS STUDIED IN THIS EXPERIMENT

Systematic name	*Trivial name*	*Abbreviation*	*Source**	*Colour***
5α-Androstan-11-one	Androstan-11-one	5α A 11 one	(e)	No colour
5β-Androstan-11-one	Etiocholan-11-one	5β A 11 one	(e)	No colour
5α-Androstan-17-one	Androstan-17-one	5α A 17 one	(d)	Blue
5α-Androstane-3,17-dione	Androstanedione	5α A 3,17 one	(c)	Olive
5β-Androstane-3,17-dione	Etiocholanedione	5β A 3,17 one	(d)	Brown
5α-Androst-1-ene-3,17-dione	1-Dehydro-androstanedione	Δ^1 5α A 3,17 one	(d)	Violet blue
Androst-4-ene-3,17-dione	Androstenedione	Δ^4 A 3,17 one	(c)	Salmon
Estr-4-ene-3,17-dione	19-Norandrostenedione	19-nor Δ^4 A 3,17 one,	(d)	Orange-brown
5α-Androst-2-ene-7,17-dione		Δ^2 5α A 7,17 one	(a)	Olive
5β-Androstane-3,11,17-dione	11-Oxo-etiocholanolone	5β A 3,11,17 one	(e)	Red brown
5α-Androstan-17-ol		17β ol 5α A	(b)	Blue
17β-Hydroxy-5α-androstan-3-one	5α-Dihydrotestesterone	17β ol 5α A 3 one	(d)	Brown-olive to blackish-olive
17β-Hydroxy-5β-androstan-3-one	5β-Dihydrotestosterone	17β ol 5β A 3 one	(c)	Bright violet
17β-Hydroxy-androst-4-en-3-one	Testosterone	17β ol Δ^4 A 3 one	(c)	Red-orange to violet-purple
3α-Hydroxy-5α-androstan-17-one	Androsterone	3α ol 5α A 17 one	(c)	Green
3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	3β ol 5α A 17 one	(c)	Green
3α-Hydroxy-5β-androstan-17-one	Etiocholanolone	3α ol 5β A 17 one	(c)	Green
3β-Hydroxy-5β-androstan-17-one	5β-Epiandrosterone	3β ol 5β A 17 one	(c)	Green
11β-Hydroxy-5α-androstane-3,17-dione	11β-Hydroxy-androstanedione	11β ol 5α A 3,17 one	(e)	Brown
17β-Hydroxy-5α-androstane-3,17-dione	7-Oxo-5α-dihydrotestosterone	17β ol 5α A 3,7 one	(a)	Brown
3α-Hydroxy-5β-androstane-11,17-dione	11-Oxo-etiocholanolone	3α ol 5β A 11,17 one	(e)	Brown-olive
3α-Hydroxy-5α-androstane-7,17-dione	7-Oxo-androsterone	3α ol 5α A 7,17 one	(a)	Green-olive
3β-Hydroxy-5α-androstane-7,17-dione	7-Oxo-epiandrosterone	3β ol 5α A 7,17 one	(a)	Green-olive
5α-Androstane-3α,17β-diol		3α,17β ol 5α A	(b)	Blue
5α-Androstane-3β,17β-diol		3β,17β ol 5α A	(c)	Blue
Androst-4-ene-3β,17β-diol		3β,17β ol Δ^4 A	(d)	Dark blue
5β-Androstane-3α,17β-diol		3α,17β ol 5β A	(d)	Blue
3β,11β-Dihydroxy-5α-androstan-17-one	11β-Hydroxy-epiandrosterone	3β,11β ol 5α A 17 one	(e)	Green
3α,11β-Dihydroxy-5β-androstan-17-one	11β-Hydroxy-etiocholanolone	3α,11β ol 5β A 17 one	(e)	Dark grey-blue

* Source (a) Dr. J. Joska, Academy of Sciences, Prague, Czechoslovakia; (b) Dipl. Ing. I. Könyves, Hälsingborg, Sweden; (c) A. G. Schering, Berlin, Germany; (d) Steraloids Inc., Flushing 52, N.Y., U.S.A.; (e) U.S.P. Steroid Reference Substance.

** Anisaldehyde-sulfuric acid reaction.

gel G (Merck A.G., Darmstad, batches No. 132307 and No. 62631). The plates were prepared, using the apparatus for thin-layer chromatography described by STAHL[28], from a mixture obtained by homogenizing the Silica gel for 4–5 min with 80 ml of distilled water; 45 g of batch No. 132307 or 30 g of batch No. 62631 were used. The plates were dried in a stream of hot air followed by heating for 30 min at 100–105°.

Every chromatogram was run in completely saturated tanks. By totally covering three of the walls and the fourth wall partially with a double layer of thick filter paper soaked in solvent, complete saturation was obtained. The chambers must be equilibrated for at least 4 h prior to each chromatogram. Reproducible R_F values could only be obtained by maintaining complete saturation of the chambers in this manner. Under these experimental conditions, comparable R_F values were obtained for chromatoplates kept in desiccators, over anhydrous silica gel (Blaugel, Silica Gel Ges. Hamburg, Germany), or exposed to the air for over 24 h.

During the introduction of the chromatoplate into the chamber great care must be taken. The plate is introduced into the chamber with the silica gel facing the wholly covered walls by raising, just as much as is necessary, that part of the cover of the chamber above the partially covered wall.

In spite of all these precautions, the upper layers of the chamber become unsaturated and this cannot be overcome during the short period of development. To avoid the influence of this unsaturation on the R_F values of extremely weakly polar steroids, the run should be terminated when the solvent front is 15 cm from the starting line. However, the calculated ΔR_{Mg} and ΔR_{Mr} values with an R_F over 0.75 are only approximations because of this effect. 1–25 μg of samples was dissolved in 5–15 μl of ethanol or methanol–chloroform (1:1) mixture using a 0.01 Blaubrand pipette (Colodur, Hamburg, Germany). The origin was 2.5–3 cm from the lower edge of the plate and at least 2 cm from the lateral border, to prevent variation in R_F values due to capillary action. The edges of the silica gel on the plate were marked in each case.

Detection of the steroids

For detection of the spots, the following reactions were used:

(a) *Anisaldehyde–sulphuric acid reaction*[9,21]. The plates were sprayed with a 1 % (v/v) solution of anisaldehyde in a 2 % (v/v) solution of concentrated sulphuric acid in glacial acetic acid. After spraying, the chromatoplates were heated to 95–100° for 12–15 min.

The colours developed are recorded in Table I.

(*b*) *The Zimmermann reaction*[32]. This reaction for methylene groups, activated by an oxo-group in *ortho* position, was used for the detection of 17- and 3-oxo-steroids. After being sprayed with a freshly prepared mixture of equal parts of a 2 % ethanolic solution of *m*-dinitrobenzene and 1.25 *N* ethanolic potassium hydroxide, the plates were exposed to a stream of hot air. 3-Oxosteroids appear immediately as blue spots while 17-oxo-steroids with an unsubstituted 16 position give the classical violet colour after 3 to 6 min.

(*c*) *Dragendorff's reagent*. This was suggested by PELCOVA[26] for steroids, especially α,β-unsaturated ketosteroids. It was prepared in the following modified manner: 10 ml of 0.3 % bismuth subnitrate solution in 50 % (v/v) sulphuric acid was added with constant agitation to 30 ml of 10 % KI in 70% (v/v) ethanol. This

reagent must be prepared every second day and kept at a low temperature (about $+4°$).

After spraying the plates with this reagent, the Δ^4-3-oxosteroids almost immediately developed an orange colour. This reaction is not specific, and a yellow or yellow-orange colour occurred with several steroids. It was primarily used here for the identification of etiocholan-11-one and androstan-11-one, which are difficult to identify by other methods.

(*d*) *Conversion of alcoholic steroids to ketosteroids, in situ.* The oxosteroid so formed was detected by the Zimmermann reaction (KUPFER *et al.*[17], PAN[24]). Plates were sprayed with a 0.25 % solution of chromic acid anhydride in glacial acetic acid and heated for 15 min at 90–95°. The plates were then subjected to the Zimmermann reaction as previously described.

(*e*) *Osmium tetroxide.* Double bonds were detected by the exposure of the plates to the vapour of osmium tetroxide under sealed conditions[10]. Isolated double bonds such as Δ^2-5-androstene-7,17-dione, form an osmate ester after 5 to 10 min exposure. Conjugated double bonds, as found in 1-dehydro-androstanedione and testosterone react after 20 to 40 min, and $\Delta^{1,4}$-conjugated bonds (17-hydroxy-$\Delta^{1,4}$-androstadien-3-one and $\Delta^{1,4}$-androstadiene-3,17-dione) after 1 h. Osmium tetroxide is a very sensitive reagent for 2-hydroxyoestrogens[19a] and reacts immediately with it.

Solvent systems and chromatographic results

(*a*) *Solvent systems and symbols.* In this study the following systems, which have been described previously[18,19] were employed:

System A: ethyl acetate 45 parts, cyclohexane 45 parts and abs. ethanol, 10 parts.
System C: ethyl acetate 50 parts, cyclohexane 50 parts.
System D: chloroform 90 parts, abs. ethanol 10 parts.
System E: ethyl acetate 72 parts, water saturated *n*-hexane 13.5 parts, abs. ethanol 4.5 parts and glacial acetic acid 10 parts.
System H: benzene 40 parts, abs. ethanol 10 parts.
System K: benzene 90 parts, abs. ethanol 10 parts.
System L: chloroform 19 parts, abs. ethanol 1 part.
System N: benzene 19 parts, abs. ethanol 1 part.

In addition, the following new systems were used:

System M: ethyl acetate 75 parts, *n*-hexane 20 parts, and acetic acid 5 parts.
System O: *n*-hexane 75 parts, ethyl acetate 25 parts.

With the exception of system O, developed for steroids of extremely high polarity R_F- and R_S-values (S = testosterone), the standard deviation S.D. and the function R_M of almost all the steroids studied have been calculated. However, values for androstan-11-one and etiocholan-11-one were only calculated in five systems: N, C, L, K and O.

The R_M-function was calculated according to the definition given by BATE-SMITH AND WESTALL[4]:

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

Symbols ΔR_{Mg}, ΔR_{Mr} and ΔR_{Ms} are employed here with the same meaning as that used by BUSH[5]. The ΔR_{Mg} of a radical is a ΔR_M resulting from the substitution of

a hydrogen atom in the molecule by this radical. $\Delta R_{Mr} = \Delta R_{M_1} - \Delta R_{M_2}$ and is the ΔR_M resulting from the substitution of one radical for another, *e.g.*, by means of the reduction of an oxo group. The ΔR_M of a steroid in two solvent systems is called ΔR_{Ms}. The R_M concept is being employed here in adsorption systems although its general validity is usually only recognised for partition systems.

(*b*) *Chromatographic results.* The chromatographic results of the application of ten systems to twenty-nine steroids of the androstane series are summarised in Tables II to XI. It can be seen from these Tables that the separation of isomers which differ in the 5α-(androstane) and 5β-(etiocholane or testane) configuration, without substitution in ring A, is difficult and occasionally impossible. Androstan-11-one and etiocholan-11-one could not be separated in any of the systems, while the separation of androstanedione and etiocholanedione could only be achieved in systems K, L, N, M and O.

(Text continued on p. 401)

TABLE II

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY FIVE STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM A*. THIN LAYER CHROMATOGRAPHY CARRIED OUT UNDER COMPLETE SATURATION ON SILICA GEL G (MERCK A.G., DARMSTADT, BATCH NO. 132307)

Steroid	n***	R_F	S.D.	F.L.	R_M	R_S	S.D.	F.L.
5α A 17 one	12	0.75	0.03	0.68–0.82	—0.477	1.52	0.03	1.45–1.59
Δ^2 5α A 7,17 one	8	0.67	0.02	0.62–0.72	—0.308	1.43	0.08	1.23–1.63
17β ol 5α A	10	0.64	0.03	0.58–0.70	—0.250	1.31	0.04	1.22–1.40
5β A 3,17 one	12	0.64	0.03	0.58–0.70	—0.250	1.29	0.06	1.17–1.41
5α A 3,17 one	9	0.63	0.03	0.57–0.69	—0.231	1.28	0.07	1.13–1.43
Δ^1 5α A 3,17 one	8	0.61	0.02	0.56–0.66	—0.194	1.30	0.08	1.10–1.50
3β ol 5β A 17 one	9	0.57	0.02	0.51–0.63	—0.122	1.16	0.04	1.08–1.24
17β ol 5α A 3 one	12	0.57	0.02	0.52–0.62	—0.122	1.16	0.03	1.10–1.22
11β ol 5α A 3,17 one	8	0.57	0.03	0.49–0.65	—0.122	1.15	0.10	0.82–1.38
3α ol 5α A 17 one	11	0.55	0.04	0.47–0.63	—0.087	1.19	0.04	1.11–1.27
17β ol 5β A 3 one	9	0.55	0.04	0.47–0.63	—0.087	1.12	0.08	0.93–1.31
3β ol 5α A 17 one	9	0.52	0.03	0.46–0.58	—0.035	1.06	0.03	1.00–1.12
5β A 3,11,17 one	7	0.52	0.04	0.43–0.61	—0.035	1.04	0.08	0.84–1.24
3α 17β ol 5α A	9	0.50	0.03	0.41–0.55	0.000	1.02	0.04	0.94–1.10
3β 17β ol Δ^4 A	12	0.50	0.03	0.43–0.57	0.000	1.00	0.05	0.89–1.11
3α ol 5β A 17 one	11	0.49	0.03	0.41–0.57	0.017	1.06	0.04	0.98–1.14
17β ol Δ^4 A 3 one	69	0.48	0.03	0.42–0.54	0.035			
3β 17β ol 5α A	30	0.48	0.03	0.41–0.55	0.035	0.99	0.03	0.93–1.05
3β 11β ol 5α A 17 one	9	0.44	0.03	0.37–0.53	0.105	0.90	0.07	0.73–1.07
3α 17β ol 5β A	12	0.44	0.03	0.38–0.50	0.105	0.88	0.04	0.80–0.96
3α 11β ol 5β A 17 one	9	0.43	0.04	0.34–0.52	0.122	0.88	0.08	0.70–1.06
17β ol 5α A 3,7 one	8	0.40	0.02	0.37–0.43**	0.176	0.86	0.05	0.74–0.98
3α ol 5α A 7,17 one	8	0.40	0.02	0.35–0.45**	0.176	0.85	0.06	0.70–1.00
3α ol 5β A 11,17 one	9	0.38	0.04	0.29–0.47	0.213	0.78	0.07	0.61–0.95
3β ol 5α A 7,17 one	8	0.33	0.01	0.30–0.36	0.308	0.71	0.05	0.58–0.84

* System A (cyclohexane: 45, ethylacetate: 45, ethanol: 10).

** The employment of S.D. with three digits in the calculation of the F.L. results in slightly different F.L. in instances of identical R_F values, based on the same number of experiments and exhibiting the same S.D.

*** The number of experiments is n; S.D. indicates the standard deviation of a single estimation and is given in the tables to only two significant digits, whereas values with three digits were employed in the calculation of the fiducial limits (F.L.) — fiducial limits of error are those within which 95% of individual R_F values are expected to fall. These limits have been calculated as being on both sides of the mean R_F values are expected to fall. These limits have been calculated as being on both sides of the mean R_F-value to a distance of t × S.D., where t is the 95% probability for the corresponding degrees of freedom. The R_M value is defined as log $(1/R_F - 1)$.

TABLE III

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-SEVEN STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM C*

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	12	0.73	0.02	0.68–0.78	—0.432	3.11	0.15	2.79–3.43
5α A 11 one	5	0.69	0.01	0.50–0.88	—0.338	3.19	0.11	2.88–3.50
5β A 11 one	5	0.69	0.02	0.64–0.74	—0.338	3.18	0.15	2.77–3.59
17β ol 5 α A	6	0.54	0.02	0.48–0.60	—0.070	2.36	0.20	1.86–2.86
Δ^2 5α A 7,17 one	6	0.52	0.03	0.43–0.61	—0.035	2.19	0.08	1.99–2.39
5α A 3,17 one	8	0.46	0.02	0.42–0.50	0.070	1.87	0.13	1.54–2.20
5β A 3,17 one	12	0.43	0.02	0.38–0.48	0.122	1.83	0.09	1.63–2.03
Δ^1 5α A 3,17 one	6	0.38	0.03	0.30–0.46	0.213	1.61	0.09	1.38–1.84
3β ol 5β A 17 one	14	0.36	0.03	0.30–0.42	0.250	1.49	0.12	1.24–1.74
17β ol 5α A 3 one	18	0.35	0.03	0.29–0.41	0.269	1.48	0.09	1.28–1.68
3α ol 5α A 17 one	21	0.33	0.04	0.25–0.41	0.308	1.44	0.11	1.14–1.74
3β ol 5α A 17 one	24	0.30	0.03	0.23–0.37	0.368	1.23	0.11	1.00–1.46
17β ol 5β A 3 one	14	0.30	0.03	0.24–0.36	0.368	1.23	0.11	0.99–1.47
11β ol 5α A 3,17 one	19	0.27	0.04	0.19–0.35	0.432	1.13	0.08	0.96–1.30
3α 17β ol 5α A	5	0.25	0.02	0.21–0.29	0.477	1.16	0.14	0.76–1.56
3β 17β ol 5α A	12	0.25	0.02	0.20–0.30	0.477	1.05	0.07	0.90–1.20
3β 17β ol Δ^4 A	12	0.25	0.02	0.19–0.31	0.477	1.05	0.07	0.90–1.20
5β A 3,11,17 one	8	0.25	0.03	0.22–0.28	0.477	0.99	0.08	0.79–1.19
3α ol 5β A 17 one	21	0.24	0.03	0.18–0.30	0.501	0.92	0.09	0.74–1.10
17β ol Δ^4 A 3 one	54	0.23	0.02	0.19–0.27	0.525			
3α 17β ol 5β A	12	0.17	0.01	0.14–0.20	0.689	0.71	0.07	0.55–0.87
3β,11β ol 5α A 17 one	8	0.14	0.02	0.10–0.18	0.788	0.60	0.08	0.44–0.76
3α ol 5α A 7,17 one	6	0.12	0.02	0.08–0.16	0.865	0.50	0.08	0.30–0.70
17β ol 5α A 3,7 one	6	0.12	0.02	0.08–0.16	0.865	0.49	0.06	0.32–0.65
3α,11β ol 5β A 17 one	8	0.12	0.01	0.10–0.14	0.865	0.49	0.04	0.40–0.58
3α ol 5β A 11,17 one	8	0.09	0.01	0.06–0.12	1.005	0.38	0.05	0.27–0.49
3β ol 5α A 7,17 one	6	0.06	0.01	0.04–0.08	1.195	0.28	0.05	0.16–0.40

* System C: (ethyl acetate: 50, cyclohexane: 50).
** See footnote *** to Table II.

TABLE IV

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-FIVE STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM D*

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	11	0.78	0.02	0.72–0.84	—0.550	1.35	0.04	1.30–1.40
Δ^2 5α A 7,17 one	7	0.77	0.03	0.71–0.82	—0.520	1.32	0.07	1.14–1.50
Δ^1 5α A 3,17 one	7	0.76	0.03	0.68–0.84	—0.501	1.30	0.08	1.11–1.49
5β A 3,17 one	12	0.74	0.04	0.66–0.82	—0.454	1.28	0.06	1.15–1.41
5α A 3,17 one	19	0.74	0.02	0.71–0.77	—0.454	1.27	0.07	1.13–1.41
5β A 3,11,17 one	12	0.69	0.05	0.58–0.80	—0.338	1.19	0.08	1.02–1.36
17β ol 5α A	6	0.64	0.02	0.60–0.68	—0.250	1.04	0.03	0.97–1.11
11β ol 5α A 3,17 one	12	0.63	0.04	0.54–0.72***	—0.231	1.08	0.06	0.95–1.21
3α ol 5α A 17 one	12	0.63	0.04	0.55–0.71***	—0.231	1.08	0.06	0.95–1.21
3β ol 5β A 17 one	12	0.62	0.03	0.56–0.68	—0.213	1.07	0.04	0.99–1.15
17β ol 5α A 3 one	12	0.61	0.04	0.52–0.70	—0.194	1.05	0.07	0.92–1.18
17β ol 5β A 3 one	12	0.61	0.02	0.56–0.66	—0.194	1.05	0.04	0.95–1.10
17β ol Δ^4 A 3 one	74	0.58	0.03	0.52–0.64	—0.140			
3α ol 5β A 17 one	12	0.58	0.03	0.51–0.65	—0.140	1.00	0.06	0.88–1.12

(continued on p. 397)

TABLE IV (*continued*)

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
3β ol 5α A 17 one	12	0.58	0.03	0.52–0.64	—0.140	1.00	0.05	0.89–1.11
17β ol 5α A 3,7, one	7	0.56	0.05	0.44–0.68	—0.105	0.96	0.07	0.80–1.12
3α ol 5α A 7,17 one	7	0.53	0.05	0.40–0.66	—0.052	0.92	0.07	0.75–1.09
3α ol 5β A 11,17 one	12	0.52	0.03	0.45–0.59	—0.035	0.90	0.05	0.78–1.02
3β ol 5α A 7,17 one	7	0.50	0.05	0.37–0.63	0.000	0.86	0.07	0.70–1.02
3α 17β ol 5α A	12	0.50	0.03	0.43–0.57	0.000	0.86	0.04	0.78–0.94
3β,17β ol 5α A	23	0.47	0.04	0.39–0.55	0.052	0.81	0.07	0.67–0.95
3β,17β ol Δ^4 A	12	0.47	0.04	0.37–0.57	0.052	0.81	0.05	0.70–0.92
3α,11β ol 5β A 17 one	12	0.44	0.03	0.38–0.50	0.105	0.76	0.04	0.68–0.84
3β,11β ol 5α A 17 one	12	0.43	0.03	0.37–0.49	0.122	0.74	0.04	0.65–0.83
3α,17β ol 5β A	12	0.40	0.04	0.31–0.49	0.176	0.69	0.06	0.55–0.83

* System D (chloroform: 90, ethanol: 10).
** See footnotes** and *** Table II.

TABLE V

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-FIVE STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM E*

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	12	0.87	0.02	0.81–0.93	—0.826	1.29	0.03	1.24–1.34
Δ^2 5α A 7,17 one	8	0.81	0.02	0.76–0.86	—0.630	1.22	0.02	1.18–1.26
17β ol 5α A	8	0.78	0.04	0.69–0.87	—0.550	1.20	0.03	1.13–1.27
5β A 3,17 one	12	0.78	0.02	0.74–0.82	—0.550	1.15	0.03	1.09–1.21
5α A 3,17 one	9	0.77	0.04	0.58–0.86	—0.528	1.17	0.03	1.11–1.23
11β ol 5α A 3,17 one	9	0.75	0.03	0.67–0.83	—0.477	1.14	0.06	0.99–1.29
Δ^1 5α A 3,17 one	8	0.75	0.03	0.68–0.82	—0.477	1.18	0.04	1.04–1.22
3α ol 5α A 17 one	16	0.74	0.03	0.67–0.81	—0.454	1.14	0.04	1.06–1.22
17β ol 5α A 3 one	12	0.74	0.02	0.69–0.79	—0.454	1.10	0.03	1.03–1.17
3β ol 5β A 17 one	9	0.73	0.03	0.65–0.81	—0.432	1.11	0.02	1.08–1.14
17β ol 5β A 3 one	9	0.73	0.04	0.61–0.85	—0.432	1.11	0.04	1.02–1.20
3β ol 5α A 17 one	8	0.72	0.03	0.65–0.79	—0.410	1.08	0.02	1.04–1.12
3α ol 5β A 17 one	16	0.71	0.03	0.64–0.78	—0.389	1.09	p.03	1.05–1.13
5β A 3,11,17 one	9	0.70	0.03	0.63–0.77	—0.368	1.07	0.06	0.92–1.22
3α,17β ol 5α A	8	0.70	0.04	0.62–0.78	—0.368	1.05	0.03	0.98–1.12
3β,17β ol Δ^4 A	12	0.70	0.03	0.64–0.76	—0.368	1.03	0.04	0.94–1.12
3β,11β ol 5α A 17 one	9	0.68	0.04	0.59–0.77	—0.327	1.04	0.07	0.87–1.21
3β,17β ol 5α A	23	0.68	0.04	0.60–0.76	—0.327	1.04	0.04	0.97–1.11
3α,11β ol 5β A 17 one	9	0.66	0.03	0.59–0.73	—0.288	1.00	0.06	0.88–1.12
17β ol Δ^4 A 3 one	69	0.66	0.03	0.59–0.73	—0.288			
3α,17β ol 5β A	12	0.65	0.03	0.59–0.71	—0.269	0.97	0.04	0.89–1.05
3α ol 5β A 11,17 one	9	0.61	0.03	0.53–0.69	—0.194	0.93	0.05	0.81–1.05
17β ol 5α A 3,7 one	8	0.57	0.04	0.49–0.65	—0.122	0.86	0.04	0.77–0.95
3α ol 5α A 7,17 one	8	0.54	0.03	0.46–0.62	—0.070	0.82	0.04	0.63–0.91
3β ol 5α A 7,17 one	8	0.49	0.04	0.40–0.58	0.017	0.73	0.05	0.61–0.85

* System E (Ethyl acetate: 72, *n*-hexane: 13.5, ethanol: 4.5, acetic acid: 10).
** See footnote *** to Table II.

TABLE VI

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-FIVE STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM H

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	12	0.75	0.01	0.72–0.78	—0.477	1.34	0.05	1.23–1.45
Δ^2 5α A 7,17 one	8	0.70	0.04	0.62–0.78	—0.368	1.27	0.06	1.12–1.42
5β A 3,17 one	12	0.67	0.03	0.60–0.74	—0.308	1.21	0.04	1.11–1.31
Δ^1 5α A 3,17 one	8	0.66	0.02	0.61–0.71	—0.288	1.21	0.04	1.12–1.29
5β A 3,11,17 one	9	0.66	0.03	0.56–0.74***	—0.288	1.18	0.08	1.00–1.36
5α A 3,17 one	9	0.66	0.03	0.60–0.72***	—0.288	1.17	0.05	1.06–1.28
11β ol 5α A 3,17 one	9	0.64	0.04	0.56–0.72	—0.250	1.14	0.08	0.96–1.32
17β ol 5α A	6	0.62	0.02	0.56–0.68	—0.213	1.15	0.06	0.99–1.31
3β ol 5β A 17 one	17	0.59	0.03	0.54–0.64***	—0.158	1.03	0.04	0.95–1.11
17β ol 5β A 3 one	17	0.59	0.03	0.53–0.65***	—0.158	1.03	0.04	0.95–1.11
3α ol 5α A 17 one	22	0.58	0.02	0.53–0.65	—0.140	1.04	0.05	0.93–1.15
17β ol 5α A 3 one	20	0.57	0.01	0.54–0.60	—0.123	1.03	0.03	0.96–1.10
17β ol Δ^4 A 3 one	58	0.56	0.04	0.48–0.64	—0.105			
3α ol 5β A 17 one	22	0.56	0.04	0.48–0.64	—0.105	1.00	0.04	0.91–1.09
3β ol 5α A 17 one	17	0.55	0.02	0.50–0.60	—0.087	0.97	0.04	0.88–1.06
3α ol 5β A 11,17 one	9	0.55	0.02	0.49–0.61	—0.087	0.95	0.04	0.84–1.04
3α,11β ol 5β A 17 one	9	0.52	0.02	0.46–0.58	—0.035	0.89	0.05	0.78–1.00
3β,11β ol 5α A 17 one	9	0.51	0.03	0.44–0.58	—0.017	0.90	0.05	0.79–1.01
3β,17β ol Δ^4 A	12	0.50	0.02	0.45–0.55	0.000	0.89	0.03	0.82–0.96
17β ol 5α A 3,7, one	8	0.49	0.04	0.40–0.58	0.017	0.90	0.04	0.81–0.99
3β,17β ol 5α A	14	0.49	0.02	0.45–0.53	0.017	0.88	0.03	0.81–0.95
3α,17β ol 5α A	8	0.48	0.03	0.42–0.54	0.035	0.88	0.03	0.80–0.96
3α,17β ol 5β A	12	0.48	0.02	0.42–0.64	0.035	0.86	0.03	0.79–0.93
3α ol 5α A 7,17 one	8	0.47	0.04	0.38–0.56	0.052	0.85	0.06	0.71–0.99
3β ol 5α A 7,17 one	8	0.45	0.04	0.37–0.53	0.087	0.81	0.06	0.68–0.94

* System H (benzene: 40, ethanol: 10).
** and *** See footnotes ** and *** to Table II.

TABLE VII

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-SEVEN STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM K*

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	6	0.78	0.04	0.69–0.87	—0.550	2.16	0.19	1.68–2.64
5β A 11 one	6	0.73	0.02	0.68–0.78	—0.432	1.86	0.07	1.68–2.04
5α A 11 one	5	0.72	0.02	0.66–0.78	—0.432	1.85	0.08	1.62–2.08
5α A 3,17 one	7	0.63	0.04	0.53–0.73	—0.231	1.77	0.15	1.38–2.16
Δ^2 5α A 7,17 one	8	0.61	0.05	0.50–0.72	—0.194	1.70	0.18	1.24–2.16
5β A 3,17 one	18	0.60	0.03	0.53–0.67	—0.176	1.61	0.11	1.47–1.85
Δ^1 5α A 3,17 one	8	0.58	0.04	0.50–0.66	—0.140	1.61	0.13	1.30–1.92
17β ol 5α A	8	0.57	0.04	0.48–0.66	—0.122	1.27	0.08	1.06–1.48
5β A 3,11,17 one	18	0.50	0.04	0.41–0.59	0.000	1.32	0.09	1.13–1.51
3β ol 5β A 17 one	18	0.44	0.04	0.36–0.52***	0.105	1.16	0.03	1.10–1.22
11β ol 5α A 3,17 one	18	0.44	0.04	0.35–0.53***	0.105	1.15	0.03	1.09–1.21
3α ol 5α A 17 one	12	0.43	0.04	0.35–0.51	0.122	1.23	0.05	1.11–1.35
17β ol 5α A 3 one	6	0.42	0.03	0.35–0.49	0.140	1.18	0.07	1.00–1.36
17β ol 5β A 3 one	18	0.42	0.03	0.32–0.46	0.140	1.10	0.10	0.91–1.29
3β ol 5α A 17 one	18	0.39	0.03	0.32–0.46	0.194	1.04	0.06	0.91–1.17
17β ol Δ^4 A 3 one	49	0.39	0.04	0.31–0.49	0.194			

(continued on p. 399)

TABLE VII (*continued*)

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
3α ol 5β A 17 one	12	0.38	0.04	0.30–0.46	0.213	1.09	0.05	0.98–1.20
3α ol 5β A 11,17 one	18	0.33	0.04	0.24–0.42	0.308	0.88	0.07	0.73–1.03
3α,17β ol 5α A	18	0.32	0.04	0.24–0.40	0.327	0.84	0.06	0.66–1.02
3β,17β ol Δ^4 A	15	0.30	0.06	0.18–0.42	0.368	0.75	0.09	0.56–0.94
17β ol 5α 3,7 one	8	0.29	0.04	0.19–0.39	0.389	0.80	0.09	0.58–1.02
3α,11β ol 5β A 17 one	18	0.29	0.04	0.20–0.38	0.389	0.77	0.07	0.62–0.92
3β,11β ol 5α A 17 one	18	0.28	0.04	0.19–0.37	0.410	0.74	0.08	0.57–0.91
3α ol 5α A 7,17 one	8	0.27	0.04	0.19–0.35	0.432	0.74	0.07	0.57–0.91
3β,17β ol 5α A	12	0.25	0.02	0.21–0.29	0.477	0.70	0.06	0.56–0.84
3β ol 5α A 7,17 one	8	0.23	0.03	0.15–0.31	0.525	0.65	0.07	0.48–0.82
3α,17β ol 5β A	6	0.23	0.01	0.20–0.26	0.525	0.65	0.02	0.60–0.70

* System K (benzene: 90, ethanol: 10).
** and *** See footnotes** and *** to Table II.

TABLE VIII

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-SEVEN STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM L*

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	12	0.76	0.03	0.69–0.83	—0.501	1.80	0.12	1.76–2.04
5α A 11 one	6	0.72	0.02	0.68–0.76	—0.410	1.65	0.12	1.31–1.99
5β A 11 one	6	0.71	0.03	0.63–0.79	—0.389	1.63	0.13	1.25–2.01
Δ^2 5α A 7,17 one	7	0.70	0.03	0.63–0.77	—0.368	1.58	0.11	1.30–1.86
5α A 3,17 one	18	0.69	0.03	0.63–0.75	—0.338	1.64	0.15	1.33–1.95
5β A 3,17 one	12	0.67	0.04	0.57–0.77	—0.308	1.55	0.10	1.34–1.75
Δ^1 5α A 3,17 one	8	0.67	0.02	0.65–0.69	—0.308	1.51	0.10	1.29–1.73
5β A 3,11,17 one	17	0.58	0.04	0.50–0.66	—0.140	1.36	0.12	1.10–1.66
17β ol 5α A	6	0.57	0.03	0.50–0.64	—0.122	1.30	0.04	1.19–1.41
3β ol 5β A 17 one	18	0.50	0.03	0.43–0.57	—0.000	1.20	0.12	0.96–1.44
3α ol 5α A 17 one	15	0.50	0.05	0.39–0.61	0.000	1.18	0.10	0.97–1.49
17β ol 5α A 3 one	12	0.49	0.03	0.42–0.56	0.017	1.19	0.07	1.04–1.34
11β ol 5α A 3,17 one	18	0.46	0.04	0.39–0.51	0.070	1.10	0.10	0.90–1.30
17β ol 5β A 3 one	17	0.46	0.04	0.38–0.54	0.070	1.10	0.11	0.87–1.33
3β ol 5α A 17 one	18	0.44	0.04	0.36–0.52	0.105	1.04	0.06	0.92–1.16
3α ol 5β A 17 one	15	0.43	0.05	0.31–0.55	0.122	1.02	0.10	0.71–1.23
17β ol Δ^4 A 3 one	64	0.43	0.08	0.27–0.59	0.122			
17β ol 5α A 3,7 one	7	0.36	0.05	0.23–0.49	0.250	0.81	0.10	0.56–1.06
3α,17β ol 5α A	18	0.32	0.05	0.22–0.42	0.327	0.77	0.11	0.55–0.99
3β,17β ol Δ^4 A	21	0.32	0.04	0.25–0.39	0.327	0.75	0.06	0.59–0.91
3α ol 5α A 7,17 one	7	0.32	0.03	0.24–0.40	0.327	0.70	0.05	0.57–0.83
3α ol 5β A 11,17 one	18	0.31	0.04	0.24–0.38	0.348	0.71	0.06	0.59–0.83
3β 17β ol 5α A	12	0.29	0.03	0.22–0.36	0.389	0.70	0.06	0.56–0.84
3β ol 5α A 7,17 one	7	0.27	0.05	0.16–0.38	0.432	0.61	0.09	0.39–0.83
3α,17β ol 5β A	12	0.23	0.02	0.18–0.28	0.525	0.56	0.04	0.47–0.64
3α,11β ol 5β A 17 one	18	0.23	0.03	0.16–0.30	0.525	0.54	0.11	0.31–0.77
3β,11β ol 5α A 17 one	18	0.22	0.03	0.16–0.28	0.550	0.53	0.07	0.38–0.68

* System L (chloroform: 19, ethanol: 1).
** See footnote *** to Table II.

TABLE IX

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-FIVE STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM M*

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	8	0.80	0.03	0.73–0.87	—0.602	1.56	0.06	1.43–1.69
Δ^2 5α A 7,17 one	8	0.74	0.03	0.64–0.81	—0.454	1.43	0.05	1.30–1.56
17β ol 5α A	6	0.73	0.01	0.70–0.76	—0.432	1.44	0.09	1.21–1.67
5α A 3,17 one	8	0.72	0.02	0.68–0.76	—0.410	1.35	0.08	1.21–1.49
5β A 3,17 one	8	0.69	0.02	0.64–0.73	—0.338	1.33	0.07	1.13–1.49
Δ^1 5α A 3,17 one	8	0.67	0.04	0.58–0.76	—0.308	1.30	0.04	1.20–1.40
3β ol 5β A 17 one	8	0.66	0.02	0.61–0.71	—0.288	1.26	0.05	1.15–1.37
17β ol 5β A 3 one	8	0.63	0.02	0.58–0.68	—0.231	1.19	0.05	1.08–1.30
17β ol 5α A 3 one	8	0.62	0.02	0.58–0.66	—0.213	1.20	0.05	1.09–1.31
3α ol 5α A 17 one	8	0.62	0.03	0.55–0.69	—0.213	1.20	0.06	1.06–1.34
3β ol 5α A 17 one	8	0.61	0.04	0.51–0.71	—0.194	1.17	0.04	1.07–1.27
11β ol 5α A 3,17 one	8	0.59	0.02	0.55–0.63	—0.158	1.15	0.04	1.06–1.24
3β,17β ol Δ^4 A	8	0.58	0.01	0.55–0.61	—0.140	1.12	0.05	1.00–1.24
3α ol 5β A 17 one	8	0.57	0.02	0.53–0.61	—0.122	1.11	0.05	0.98–1.20
3α,17β ol 5α A	8	0.56	0.02	0.52–0.60	—0.105	1.09	0.05	0.98–1.18
5β A 3,11,17 one	8	0.56	0.01	0.54–0.58	—0.105	1.08	0.04	0.92–1.18
3β,17β ol 5α A	8	0.54	0.02	0.49–0.59	—0.070	1.05	0.06	0.92–1.18
17β ol Δ^4 3 one	34	0.52	0.02	0.47–0.57	—0.035			
3β,11β ol 5α A 17 one	8	0.51	0.01	0.48–0.53	—0.017	0.98	0.05	0.87–1.09
3α,17β ol 5β A	8	0.50	0.02	0.45–0.55	0.000	0.96	0.04	0.86–1.06
3α,11β ol 5β A 17 one	7	0.48	0.01	0.45–0.51	0.035	0.92	0.05	0.80–1.04
3α ol 5β A 11,17 one	7	0.43	0.02	0.39–0.47	0.122	0.82	0.05	0.70–0.94
17β ol 5α A 3,7 one	8	0.42	0.02	0.37–0.47	0.140	0.82	0.04	0.74–0.90
3α ol 5α A 7,17 one	8	0.40	0.02	0.35–0.46	0.176	0.77	0.03	0.71–0.83
3β ol 5α A 7,17 one	8	0.34	0.02	0.29–0.39	0.288	0.65	0.03	0.58–0.72

* System M (ethyl acetate: 75, *n*-hexane: 20, acetic acid: 5).
** See footnote *** to Table II.

TABLE X

R_F, R_S (S = TESTOSTERONE) AND FUNCTION R_M OF TWENTY-SEVEN STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM N*

Steroid	*n***	R_S	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
5α A 17 one	13	0.66	0.04	0.58–0.74	—0.288	2.89	0.24	2.37–3.41
5β A 11 one	5	0.66	0.03	0.58–0.74	—0.288	2.87	0.31	2.00–3.74
5α A 11 one	5	0.66	0.03	0.57–0.75	—0.288	2.86	0.32	1.97–3.75
Δ^2 5α A 7,17 one	8	0.55	0.02	0.46–0.64	—0.087	2.41	0.21	1.91–2.91
5α A 3,17 one	8	0.53	0.06	0.44–0.70	—0.052	2.06	0.15	1.71–2.41
5β A 3,17 one	8	0.49	0.04	0.40–0.58	0.017	2.11	0.22	1.59–2.63
17β ol 5α A	10	0.46	0.06	0.31–0.61	0.070	1.75	0.15	1.42–2.08
Δ^1 5α A 3,17 one	8	0.45	0.02	0.41–0.49	0.087	1.97	0.15	1.61–2.33
5β A 3,11,17 one	8	0.36	0.04	0.27–0.45	0.250	1.60	0.18	1.18–2.02
3β ol 5β A 17 one	9	0.33	0.04	0.23–0.43	0.308	1.27	0.10	1.05–1.49
17β ol 5α A 3 one	10	0.31	0.02	0.26–0.36	0.348	1.31	0.12	1.04–1.58
3α ol 5α A 17 one	21	0.29	0.03	0.22–0.36	0.389	1.29	0.16	0.96–1.62
17β ol 5β A 3 one	8	0.28	0.02	0.23–0.33	0.410	1.11	0.09	0.90–1.32
3β ol 5α A 17 one	10	0.27	0.02	0.22–0.32	0.432	1.15	0.10	0.92–1.38
11β ol 5α A 3,17 one	8	0.27	0.03	0.19–0.35	0.432	1.22	0.05	1.10–1.36
17β ol Δ^4 A 3 one	47	0.24	0.03	0.19–0.29	0.501			

(continued on p. 401)

TABLE X (*continued*)

Steroid	*n***	R_F	*S.D.*	*F.L.*	R_M	R_S	*S.D.*	*F.L.*
3α ol 5β A 17 one	11	0.23	0.03	0.17–0.29	0.525	1.07	0.11	0.85–1.29
3α,17β ol 5α A	18	0.18	0.02	0.14–0.22	0.659	0.80	0.07	0.65–0.95
17β ol 5α A 3,7 one	8	0.18	0.02	0.13–0.23	0.659	0.79	0.07	0.62–0.96
3α ol 5β A 11,17 one	9	0.18	0.03	0.11–0.25	0.659	0.71	0.07	0.55–0.87
3β,17β ol Δ^4 A	14	0.17	0.02	0.13–0.21	0.689	0.74	0.07	0.60–0.88
3β,17β ol 5α A	16	0.16	0.02	0.12–0.20	0.720	0.71	0.02	0.67–0.75
3α ol 5α A 7,17 one	8	0.16	0.02	0.12–0.20	0.720	0.70	0.04	0.59–0.81
3α,11β ol 5β A 17 one	8	0.13	0.02	0.09–0.17	0.826	0.53	0.05	0.41–0.65
3β ol 5α A 7,17 one	8	0.13	0.02	0.09–0.17	0.826	0.57	0.05	0.45–0.69
3α,17β ol 5β A	8	0.13	0.02	0.09–0.17	0.826	0.55	0.05	0.43–0.67
3β,11β ol 5α A 17 one	9	0.13	0.02	0.08–0.18	0.826	0.53	0.06	0.39–0.67

* System N (benzene: 19, ethanol: 1).
** See footnote *** to Table II.

The situation is the same if the ring has only one ketonic group: 5α- and 5β-dihydrotestosterone may be separated using system C, while in systems L and N only a partial separation is obtained.

The introduction of an unsaturated bond altered the mobility of very few steroids. R_F values of Δ^1-dehydro-androstanedione, androstanedione and etiocholanedione are very similar; their complete separation is, however, accomplished in systems C and N, and is especially good in system O. The separation of androst-4-ene-3β,17β-diol from the different epimeric forms of the androstanediols is equally difficult. It is possible to separate it from the isomers with a 3-equatorial hydroxyl group (3α,5β and 3β,5α) in system K, but not from 5α-androstane-3α,17β-diol; this separation is partially achieved in system D (R_F values: 0.47 and 0.50 respectively).

The situation is changed when a conjugated double bond is introduced: testosterone and 5α- or 5β-dihydro-testosterone may be satisfactorily separated in nearly all

TABLE XI

R_F, AND R_S (S = ANDROSTANEDIONE) OF ELEVEN STEROIDS ON THE C_{19} AND ONE ON THE C_{18} (NOR-SERIES) IN SOLVENT SYSTEM O*, USING THIN-LAYER CHROMATOGRAPHY (COMPLETE SATURATION) ON SILICA GEL G (MERCK A.G., BATCH NO. 62631)

Steroid	*n***	R_F	*S.D.*	R_S	*S.D.*
5β A 11 one	5	0.64	0.02	3.81	0.25
5α A 11 one	5	0.64	0.02	3.78	0.25
5α A 17 one	9	0.55	0.02	3.20	0.22
17β ol 5α A	9	0.31	0.02	1.82	0.07
Δ^2 5α A 7,17 one	9	0.27	0.03	1.56	0.09
5α A 3,17 one	9	0.17	0.01	1.00	
5β A 3,17 one	9	0.14	0.01	0.82	0.02
Δ^1 5α A 3,17 one	9	0.13	0.01	0.75	0.02
Δ^4 A 3,17 one	6	0.08	0.008	0.44	0.04
19-nor Δ^4 A 3,17 one	6	0.06	0.006	0.32	0.02
5β A 3,11,17 one	8	0.04	0.006	0.23	0.03
17β ol Δ^4 A 3 one	9	0.04	0.006	0.24	0.04

* System O (*n*-hexane: 75, ethyl acetate: 25).
** See footnote*** to Table II.

the systems, especially in systems E, A, C, N, and M (Tables II, III, V, IX and X). Another example of this is the separation of androstenedione and androstanedione or etiocholanedione in system O (Table XI).

In the case of isomers containing a 3-hydroxyl group, the separation depends upon the spatial configuration, an equatorial group being easily separated from an axial one, while the separation of two axial or equatorial isomers is very difficult.

In nearly all of the proposed systems it is possible to separate androsterone and 5β-epiandrosterone from etiocholanolone and epiandrosterone, complete separation being possible in systems C, D, H, K and L. The separation of both pairs from each other can be achieved in solvent systems C, N and M (Tables III, IX and X).

The same observation holds true for 11β-hydroxy-etiocholanolone and 11β-hydroxy-epiandrosterone, both containing a 3-hydroxyl group with an equatorial configuration. Their separation is only partially achieved in solvent system M, (R_F values: 0.51 and 0.48 respectively). On the other hand, 7-oxo-androsterone (axial) and 7-oxo-epiandrosterone (equatorial) are separable in all the systems studied, with the exception of system O, in which they remain at the origin.

Of the androstanediols studied, the complete separation of the axial isomer 5α-androstane-3α,17β-diol, from 5α-androstane-3β,17β-diol and 5β-androstane-3α,17β-diol, both equatorials, is possible in systems K and D (Tables IV and VII). The separation of the latter two is also achieved in systems D, L, N and M (Tables IV, VIII, IX and X).

The calculated ΔR_{Mg} values for some groups, *viz.* 3α-(axial), 3β-hydroxy-(equatorial), 3-oxo-, 7-oxo-, 11β-hydroxy- and 11-oxo-, are summarised in Table XII.

It may be noted that the sequence of polarity for 3-oxygenate derivatives is 3-oxo- $<$ 3α-hydroxyl (axial) $<$ 3β-hydroxyl (equatorial) in the majority of systems while in a few a reversal of polarities occurs. An 11β-hydroxyl group with rings A/B in the *cis*-configuration is less polar than the isomer with rings A/B in the *trans*-configuration. An 11-oxo group is more polar than an 11-hydroxy group in systems containing ethyl acetate and less polar in systems where benzene or chloroform is the principal component. This fact is of great value in the selection of systems for two-dimensional chromatography, because systems may be selected, which produce this reversal of polarity in the groups to be separated. This reversal can also be useful in the separation and identification of many substances, *e.g.* 11-oxo-etiocholanolone and 11β-hydroxy-etiocholanolone.

The ΔR_{Mr} obtained for the conversion of the group 11-oxo- to 11β-hydroxy- were negative in systems containing ethyl acetate and cyclo- or *n*-hexane: —0.10 (system E), —0.10 (system A), and —0.14 (system C), while positive values were obtained in systems containing benzene or chloroform: +0.05, +0.08, +0.14, +0.17 and +0.17 respectively for systems H, K, D, N and L.

The calculated ΔR_{Ms} values of 26 steroids for the pairs of systems E and C (I), A and L (II) and L and K (III) have been collected in Table XIII.

It can be seen that the introduction of various groups into the steroid molecule causes changes in polarity (ΔR_{Ms} values) with respect to the three solvent systems described. One such example is androsterone, the ΔR_{Ms} being greater for the change from system E to D (I) (+0.22) than for the changes (II) and (III) (+ 0.09 and +0.12 respectively). The reduction of the 17-oxo group results in ΔR_{Ms} values which are greater than those above, but almost identical for both changes (I) and (II),

TABLE XII

ΔR_{Mg} VALUES OF SOME HYDROXYL AND KETONIC GROUPS IN THE ANDROSTANE SERIES, CALCULATED FOR THE EIGHT PRINCIPAL SYSTEMS. $\Delta R_{Mg} = R_{M1} - R_{M2}$, WHERE THE R_{M1} IS THE R_M OF THE SUBSTANCE WITH THE GROUP UNDER CONSIDERATION AND R_{M2} IS THAT OF THE SUBSTANCE WITHOUT THESE GROUPS. *a* AND *e* SIGNIFY AXIAL AND EQUATORIAL

Group	*Compound in which radical is substituted*	*Remarks*	*System*								
			A	*C*	*D*	*E*	*H*	*K*	*L*	*M*	*N*
3-oxo	17β ol 5α A	Ring A/B *trans*	0.13	0.34	0.06	0.10	0.09	0.26	0.14	0.22	0.34
3β-OH	17β ol 5α A	Ring A/B *trans* 3β (*e*)	0.28	0.55	0.30	0.22	0.23	0.60	0.51	0.36	0.65
3α-OH	17β ol 5α A	Ring A/B *trans* 3α (*a*)	0.25	0.55	0.25	0.18	0.25	0.45	0.45	0.33	0.59
11β-OH	5α A 3,17 one	Ring A/B *trans* 3-oxo	0.11	0.36	0.22	0.05	0.04	0.28	0.41	0.25	0.48
	3β ol 5α A 17 one	Ring A/B *trans* 3β (*e*)	0.14	0.42*	0.26	0.08	0.07	0.22	0.45	0.18	0.39
	3α ol 5β A 17 one	Ring A/B *cis* 3α (*e*)	0.10	0.36*	0.24	0.10	0.07	0.18	0.40	0.16	0.30
11-oxo	3α ol 5β A 17 one	Ring A/B *cis* 3α (*e*)	0.20	0.50*	0.10	0.20	0.02	0.10	0.23	0.24	0.13
	5β A 3,17 one	Ring A/B *cis* 3-oxo	0.22	0.36	0.12	0.18**	0.02	0.18	0.17	0.23	0.23
7-oxo	17β ol 5α A 3 one	Ring A/B *trans* 3-oxo	0.30	0.60*	0.09	0.33	0.14	0.25	0.23	0.35	0.31
	3α ol 5α A 17 one	Ring A/B *trans* 3α (*a*)	0.20	0.56*	0.18	0.38	0.19	0.31	0.33	0.39	0.33
	3β ol 5α A 17 one	Ring A/B *trans* 3β (*e*)	0.34	0.83*	0.14	0.39	0.17	0.33	0.32	0.48	0.39

* Value calculated with an R_F under 0.15.
** Value calculated with one or both R_F values over 0.75.

TABLE XIII

ΔR_{Ms} VALUES FOR CHANGES FROM SYSTEMS E TO D (I), A TO L (II) AND L TO K (III) FOR TWENTY-SIX C_{19}-STEROIDS

Steroid	ΔR_{Ms}: *(I)*	*(II)*	*(III)*
Steroids without hydroxy-groups			
5α A 17 one	+0.276	−0.024	−0.049
5α A 3,17 one	+0.074	−0.107	+0.107
5β A 3,17 one	+0.096	−0.058	+0.132
$\Delta^1$5α A 3,17 one	−0.024	−0.114	+0.168
Δ^4 A 3,17 one	−0.086	−0.217	+0.199
Δ^2 5α A 7,17 one	+0.110	−0.060	+0.174
5β A 3,11,17 one	+0.030	−0.105	+0.140
Monohydroxy-C_{19}-steroids with a 3- or 17-oxo-group			
17β ol 5α A 3 one	+0.260	+0.140	+0.122
17β ol 5β A 3 one	+0.274	+0.157	+0.070
17β ol Δ^4 A 3 one	+0.148	+0.087	+0.072
3α ol 5α A 17 one	+0.223	+0.087	+0.122
3β ol 5α A 17 one	+0.270	+0.140	+0.089
3α ol 5β A 17 one	+0.249	+0.104	+0.091
3β ol 5β A 17 one	+0.219	+0.122	+0.105
17-Hydroxy-steroids without oxo-groups			
17β ol 5α A	+0.300	+0.128	0.000
3α 17β ol 5α A	+0.368	+0.327	0.000
3β 17β ol 5α A	+0.379	+0.354	+0.088
3β 17β ol Δ^4 A	+0.420	+0.327	+0.041
3α 17β ol 5β A	+0.445	+0.420	0.000
C_{19}-steroids with an 11β- hydroxy-group			
11β ol 5α A 3,17 one	+0.246	+0.192	+0.035
3β11β ol 5α A 17 one	+0.449	+0.445	−0.140
3α11β ol 5β A 17 one	+0.393	+0.403	−0.136
3- or 17-hydroxy-C_{19}-steroids with a 7- or 11-oxo-group			
3α ol 5β A 11,17 one	+0.159	+0.135	−0.040
17β ol 5α A 3,7 one	+0.017	+0.074	+0.139
3α ol 5α A 7,17 one	+0.018	+0.151	+0.105
3β ol 5α A 7,17 one	−0.018	+0.124	+0.093

(+0.37 and +0.33), while no change at all is observed for the pair of solvent systems (III).

When a 7-oxo group is introduced into the molecule, a comparison of mobilities in the three pairs of systems considered above shows ΔR_{Ms} values characteristic for this group, *i.e.* a decrease in the change (I), +0.018, and almost no alteration for the changes (II) and (III), when these are compared with those given by androsterone, +0.15 and +0.11, respectively.

The ΔR_{Ms} values for the change from system E to A show that the introduction of a double bond increases these values, while for the introduction of a conjugated double bond the opposite occurs. One may also note that the introduction of an hydroxyl group (3α- 3β- 11β-), or a reduction of the 17-oxo group increases the ΔR_{Ms} in change (I), while the introduction of the groups 7- or 11-oxo- decreases the values.

Formation of acetate derivatives

0.5 ml of pyridine and 0.5 ml of acetic anhydride is added to 1–20 μg of steroid in a ground-glass tube. The tube is sealed under an atmosphere of N_2 and allowed to stand at room temperature overnight. Under these conditions a 3α-, 3β- and 17β-, but not 11β-hydroxyl groups, are completely acetylated.

The R_F, R_S (S = testosterone) and R_M values for solvent system C of the seventeen acetates obtained are shown in Table XIV. A partial separation is possible of the acetates of 5α- and 5β-dihydro-testosterone from those of the isomeric forms of

TABLE XIV

R_F, R_S (S = TESTOSTERONE) AND R_M VALUES FOR SEVENTEEN ACETATES OF STEROIDS OF THE ANDROSTANE SERIES IN SOLVENT SYSTEM C*, USING THIN-LAYER CHROMATOGRAPHY AT COMPLETE SATURATION, ON SILICA GEL G (MERCK A.G., BATCH NO. 62631)

Acetate of	*n***	R_F	*S.D.*	R_M	R_S	*S.D.*
17β ol 5α A	11	0.67	0.03	—0.308	3.19	0.22
3α 17β ol 5β A	11	0.65	0.03	—0.269	3.05	0.18
3β 17β ol Δ^4 A	11	0.63	0.03	—0.231	2.94	0.17
3α 17β ol 5α A	11	0.62	0.03	—0.213	2.95	0.22
3β 17β ol 5α A	11	0.60	0.04	—0.176	2.84	0.20
3α ol 5β A 17 one	11	0.56	0.03	—0.105	2.65	0.16
3β ol 5α A 17 one	11	0.55	0.03	—0.087	2.63	0.21
3α ol 5α A 17 one	11	0.55	0.03	—0.087	2.60	0.18
3β ol 5β A 17 one	11	0.55	0.02	—0.087	2.60	0.16
17β ol 5α A 3 one	11	0.52	0.03	—0.035	2.52	0.21
17β ol 5β A 3 one	11	0.52	0.03	—0.035	2.47	0.19
3α 11β ol 5β A 17 one	11	0.45	0.03	0.087	2.12	0.10
3α ol 5β A 11,17 one	11	0.44	0.02	0.105	2.10	0.12
3β 11β ol 5α A 17 one	11	0.41	0.03	0.158	2.00	0.16
3β ol 5α A 7,17 one	11	0.38	0.04	0.213	1.75	0.18
3α ol 5α A 7,17 one	11	0.35	0.02	0.269	1.65	0.12
17β ol 5α A 3,7 one	11	0.30	0.02	0.368	1.41	0.08

* System C (ethyl acetate: 50, cyclohexane: 50).
** See footnote *** to Table II.

3-hydroxy-androstan-17-one. However, no separation is obtained among the isomers of the individual groups. Similarly, the almost identical R_F values of the various acetates of androstanediol isomers do not allow satisfactory separation.

Other solvents[7] such as benzene, chloroform, 1,2-dichloromethane and tetrachloromethane, or mixtures of solvents[1] (benzene–ethyl acetate 5:1 and 3:1) have been employed for thin-layer chromatography of weakly polar steroids or their acetates. They did not satisfactorily separate the acetates considered in this paper.

DISCUSSION

Partition paper chromatography has been widely applied to the separation of steroids of the androstane series using formamide[2,21], propylene glycol[2,16,22], triethylene glycol[31], or ethylene glycol[30] as stationary phases. These systems permit separation of the axial and equatorial isomeric forms of the 3-hydroxy derivatives, but two-axial or two-equatorial isomers are not satisfactorily separated.

The extensive experiments of KNUPPEN[15] to achieve separation of oestrogens using these systems have shown that the R_F values and resolution obtained for many of the compounds were greatly dependent both upon the humidity of the atmosphere during impregnation, and upon the temperature during the development of the chromatogram. The impregnated paper usually interferes with several of the colour reactions employed. However, the sensitivity of the Zimmermann reaction appears to be increased when triethylene glycol impregnated paper is used[31].

When thin-layer chromatography is applied to the free steroids of the androstane series it results in a total or partial separation of several isomers with reproducibility of the R_F and consequently of the ΔR_M functions.

A quantitative elution (95 %) of added steroid, measured by the Zimmermann reaction and the possibility of the application of several colour reactions *in situ* are other important advantages.

With regard to the separation of acetates, however, the results obtained by PASQUALINI AND JAYLE[25] using paper adsorption chromatography are more satisfactory than those obtained with the systems developed for thin-layer chromatography.

In paper chromatography, the conditions necessary to obtain reliable values of R_F which will permit calculation of the comparable ΔR_M functions have been studied intensively by GREEN AND MARCINKIEWICZ[13]. There has been similar study for thin-layer chromatography (an adsorption chromatography of ascending type). The ΔR_M values calculated here with an R_F higher than 0.75 and lower than 0.15 are considered to be approximate.

The R_M values given in each Table permit the calculation of the different ΔR_{Mg}, ΔR_{Ms} and ΔR_{Mr} for several steroids and systems. The relation between these functions and the application of chromatography to the structural analyses of steroids[5,14,20] has been extensively treated by others.

While this paper was in preparation, an article[8] appeared considering the application of thin-layer chromatography to androstane steroids using silica gel C as an adsorbent under unsaturated conditions.

Whenever extremely volatile solvents are employed, the irregular migration of a substance, dependent on its position at the origin (the so-called "Randeffekt" or edge-phenomenon), may be observed during thin-layer chromatography under unsaturated conditions. R_F values are dependent upon the distance from the origin to the solvent front and are influenced by the kind and the size of tank employed[23]. The compression of less polar steroids depends upon the irregularity of saturation along the chromatoplate even in such instances where no "Randeffekt" is observed.

Under these conditions, the resulting variations in R_F values do not allow the accurate calculation of the function R_M.

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SUMMARY

The behaviour of twenty-nine steroids of the androstane series was studied by thin-layer chromatography on Silica gel G under saturated conditions. Besides the R_F and R_S values in ten systems, the chromatographic behaviour of the steroids in relationship to their structure was also studied. In a few cases, examples using ΔR_{Mg}, ΔR_{Ms} and ΔR_{Mr} are given.

Chromatography of acetate derivatives, oxidation *in situ* of hydroxy groups and a number of colour reactions employed for the detection of spots are also described.

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CHROMATOGRAPHY ON ECTEOLA-CELLULOSE AT NEUTRAL pH VALUES

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INTRODUCTION

In a series of articles[1-3] we described the chromatographic separation of fibrinogen and the anti-haemophilic factor (AHF) from human plasma. Most of the experiments were carried out on columns of ECTEOLA-cellulose, but DEAE-cellulose has also been used. The AHF was our main interest, and it was in order to obtain better yields and a purer product that we were obliged to investigate the chromatographic process in detail. This investigation revealed several peculiarities of ECTEOLA-cellulose (and to a certain degree also of DEAE-cellulose) with regard to properties and behaviour, and about the way to handle it. It is with these peculiarities that this article will deal.

MATERIALS AND METHODS

ECTEOLA-celluloses

ECTEOLA-celluloses of various capacities were prepared as described earlier[4]. The types, most commonly used, had capacities of 0.36–0.41 mequiv./g.

Buffer systems

The following buffers were used:

(a) Imidazole–chloride buffers: 0.02 *M* imidazole, containing NaCl in various concentrations, dependent on the type of experiments, neutralized to pH 6.9 with 10% HCl.

(b) Imidazole–bromide buffer: 0.02 *M* imidazole, containing 0.5 *M* NaBr, was neutralized to pH 6.9 with 10% HBr solution, freed from Br_2 with a trace of $Na_2S_2O_3$.

pH values

These were measured with a Radiometer pH-meter, model 22, with external meter.

Preparation of fraction I

Fraction I, containing fibrinogen, AHF, and a variety of globulins, was precipitated from $BaSO_4$-treated oxalated human plasma at 8% ethanol concentration, according to COHN[5]. It was the starting product for the chromatographic purification of AHF.

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For a better understanding of this process we will summarize here the principles along which these experiments were carried out. The precipitated fraction I was dissolved in $^3/_5$ the original plasma volume of imidazole–chloride buffer. This solution was chromatographed on a column of ECTEOLA-cellulose (Cl-cycle), the column was washed with the same buffer, and, after fibrinogen and the bulk of contaminating

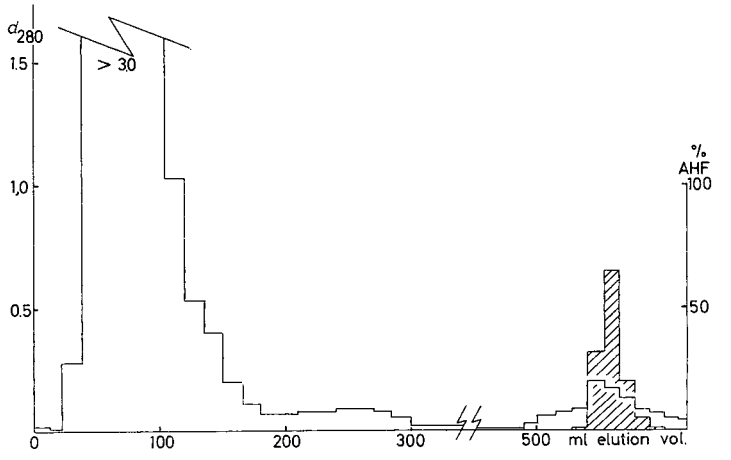

Fig. 1. Chromatography of plasma fraction I on ECTEOLA-cellulose. After 500 ml imidazole-chloride buffer had passed the column a change was made to imidazole-bromide buffer.

proteins had been washed out, the adsorbed AHF was eluted by displacement with imidazole–bromide buffer. Fig. 1 gives an example of a typical experiment. For further details see ref. 6.

EXPERIMENTAL AND RESULTS

(a) During the regeneration of the columns an important variable in the procedure, *i.e.* the reconditioning, was noted. After completion of a chromatographic run the column was washed with 0.5N NaOH until the effluent was strongly alkaline. In most cases recycling to the Cl-form was carried out by passing imidazole–chloride buffer through the column until the pH of the effluent equalled that of the buffer itself. The column was then considered ready for use. Columns thus treated gave yields varying from 10 to 50%. In some cases the regeneration was carried out with alkali followed by 0.5N HCl, and the column was subsequently equilibrated with imidazole–chloride buffer. This invariably resulted in a very low yield of AHF. We therefore decided to use only 0.5N NaOH for the regeneration and to wash the columns with water until the pH of the effluent was practically neutral before the equilibration was started.

(b) The equilibration proved to be one of the most important variables of the process. This is clear from the following experiments: regenerated and washed columns were equilibrated with increasing amounts of imidazole–chloride buffer and used in the chromatographic preparation of AHF. As will be seen from Table I, clear optima in the equilibration were found which gave maximum yields of AHF.

(c) From the foregoing, it can be concluded that the ECTEOLA-cellulose should not be converted completely into the Cl-form, and that some of the OH-ions should remain unsubstituted, in order to obtain satisfactory isolation of AHF. This could be verified by measuring the pH of all the effluent fractions during a chromatographic run. Fig. 2 shows the rise in pH following the shift from imidazole–chloride to –bromide buffer during a regular preparative run.

TABLE I

AMOUNT OF EQUILIBRATING BUFFER *vs.* YIELD

ECTEOLA-cellulose			
Capacity 0.40 mequiv./g		*Capacity 0.78 mequiv./g*	
Buffer ml	*AHF yield in %*	*Buffer ml*	*AHF yield in %*
125	20	125	11
250	50	250	20
500	70	500	30
1000	20	800	60
		1000	80
		1600	7

Statistically it was found that a quantitative yield of AHF was only obtained when the pH rose from 6.9 to about 7.35–7.40. By titration of all the effluent fractions, the amount of alkali liberated in this "pH-peak" was calculated as 6–7 % of the total capacity of the ECTEOLA-cellulose in the column.

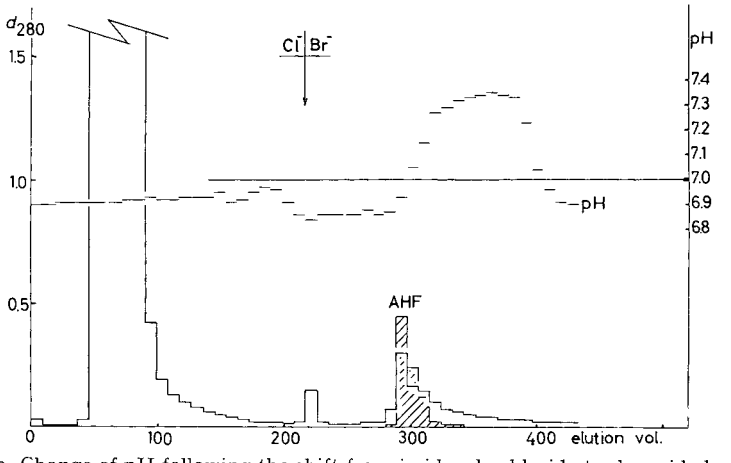

Fig. 2. Change of pH following the shift from imidazole-chloride to -bromide buffer.

The dimensions of this effect must in general be governed by:

(1) the amount of ECTEOLA-cellulose in g/column;
(2) the capacity of the ECTEOLA cellulose in mequiv./g;
(3) the total amount of buffer used for equilibration and for washing the preparation through the column;

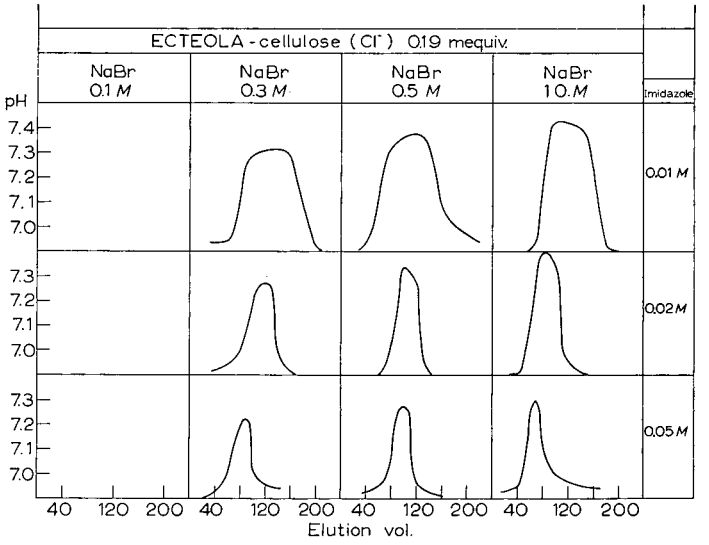

Fig. 3. Changes of pH following the shift from imidazole-chloride to -bromide buffer under various conditions of elution on ECTEOLA-cellulose of 0.19 mequiv./g.

(4) the anion concentration of this buffer;
(5) its affinity for the ion-exchanger[7];
(6) the concentration of the displacing anion;
(7) the affinity of the latter towards the ion-exchanger;
(8) the buffering capacity of the eluents;
(9) the very great affinity of OH-ions for ECTEOLA-cellulose[7].

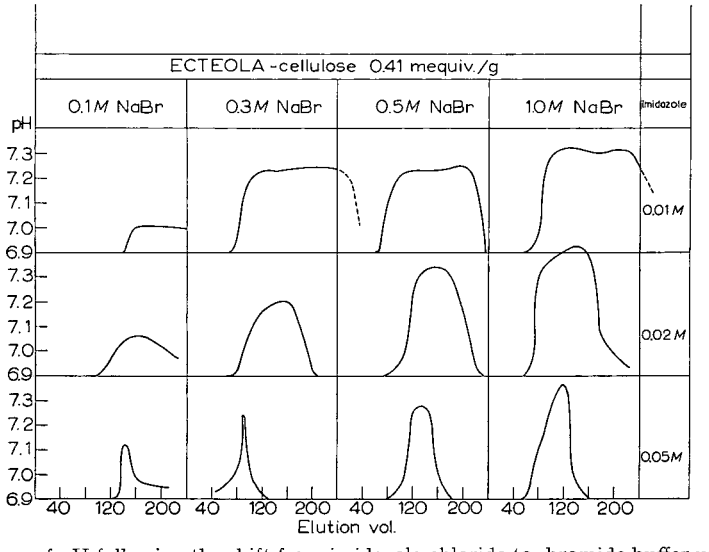

Fig. 4. Changes of pH following the shift from imidazole-chloride to -bromide buffer under various conditions of elution on ECTEOLA-cellulose of 0.41 mequiv./g.

This effect was investigated during regular chromatographic runs on two different types of ECTEOLA-cellulose for the shift of Cl- to Br-ions, at four different concentrations of the latter, and at three different buffering capacities (see Figs. 3 and 4).

(d) That some of the OH-ions of the ECTEOLA-cellulose remain in the column is due to the fact, already indicated earlier[4], that the rate of ion-exchange by ECTEOLA-cellulose in the range of pH 5–8 is very slow. This accounts for the fact that relatively large volumes of buffer of sufficient concentration must be used to convert the ion-exchanger completely from the OH-form into the desired form at neutral pH values*.

That OH-ions are tenaciously retained by the ECTEOLA-cellulose during the equilibration and chromatography was demonstrated in the following manner: a column was regenerated with alkali and washed with water until neutral in the usual way. It was then equilibrated with imidazole–chloride buffer to which phenol red was added. After the passage of 300 ml buffer solution of pH 6.91 the effluent was yellow, and had a pH of 6.92, whereas the column itself was red. After the passage of another 150 ml of buffer containing phenol red the colours were still the same. Since AHF determinations were not impaired by the dye, the column was used for the chromatography of fraction I, and washed with buffer containing phenol red; AHF was then eluted by displacement with imidazole–bromide buffer containing phenol red. Immediately after the shift to bromide, a dark red ring started passing through the column. In front of the ring the column remained red, behind it the column was yellow. Fig. 5 gives a picture of this experiment.

(e) Well-equilibrated columns could not be stored for longer periods than 1–2 days, because, even in the cold room, ion-exchange, although slow, continued to take place. In an aged column the optimum conditions for chromatography could not be attained because of liberation of the residual OH-ions, and the resulting high pH adversely affected the preparation to be chromatographed. In two experiments two columns were used, one freshly equilibrated and one one week old, but otherwise identical with regard to regeneration, washing, and equilibration. On the fresh column the yield of AHF was 85 %, on the old column 35 %. After the columns had been interchanged the experiment was repeated, and the highest yield was again obtained with the freshly prepared column.

(f) We also tried to reproduce the "pH-peak" by displacement with anions other than bromide. Most of these experiments were performed with sulphate, but a variety of organic anions have also been used. Although in most cases the affinity of the ECTEOLA-cellulose for the anion used was greater than for bromide, as determined in equilibrium experiments[7], the rise in pH was always smaller than was expected (see Table II).

(g) Efforts to elute AHF with "synthetic" pH-peaks, produced with dilute alkali or with buffers of higher pH, failed, because no sharp boundaries could be obtained.

(h) The pH-peaks could also be reproduced on columns of DEAE-cellulose, and by proper equilibration of the columns quantitative yields of AHF could be obtained with this material as well. Because of the greater protein-binding capacity of this anion-exchanger the ratio of biol. activity/d_{280} was, however, rather poor.

What has been said above about ECTEOLA-cellulose generally holds true also

* At low concentrations no complete conversion will occur, owing to hydrolysis of the ECTEOLA-cellulose-anion salt, *cf.* ref. 7.

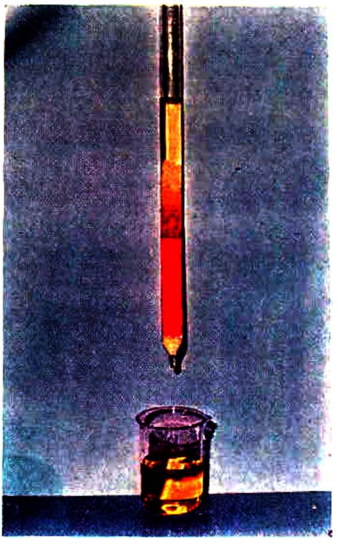

Fig. 5. Demonstration with phenol red of the increased OH-ion concentration in a column of ECTEOLA-cellulose.

TABLE II

EFFECT OF VARIOUS DISPLACING ANIONS ON pH-CHANGE

Column equilibrated with ml Cl-buffer	*Displacing anion*	*Mol. concn.*	K_c	*Rise of pH*	*ΔpH*
450	sulfate	0.25	12.1	6.91–7.08	0.17
200	sulfate	0.25		6.90–7.12	0.22
450	sulfate	0.50		6.85–7.13	0.28
500	succinate	0.10	9.8	6.92–7.06	0.16
500	succinate	0.20		6.89–7.00	0.11
480	formate	0.20	3.5	6.92–6.94	0.02
500	formate	0.50		6.93–7.07	0.14
500	glutamate	0.10	2.3	6.92–6.45	—0.47
500	glutamate	0.20		6.92–6.28	—0.64
500	propionate	0.20	1.2	6.84–6.90	0.06
500	pyruvate	0.20	1.1	6.90–6.96	0.06
500	chloride	0.50	1.0	6.88–7.16	0.28

for DEAE-cellulose except that the rate of exchange of the latter is greater, and hence fresh columns tend to deteriorate more rapidly than those of ECTEOLA-cellulose.

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SUMMARY

In the course of experiments on the chromatographic purification of the antihaemophilic factor, it was found that the cellulosic anion-exchangers, when regenerated

with alkali, tend to retain tenaciously a certain amount of hydroxyl-ions during their equilibration with neutral buffers. On shifting from one anion to another, *e.g.* in displacement chromatography, a rise in pH may result. The factors governing this phenomenon were investigated, and are discussed in the present article.

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LINEAR ELUTION ADSORPTION CHROMATOGRAPHY
VII. GRADIENT ELUTION THEORY

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INTRODUCTION

Gradient elution chromatography, first developed by ALM *et al.*[1], has found application to almost every form of elution chromatography which uses a liquid moving phase[2]. Conceptually, gradient elution bears a marked resemblance to temperature programming in gas chromatography[3], and offers the same experimental advantages. In the separation of broad-boiling mixtures by gas chromatography, temperature programming permits the separation of low-boiling sample components at low column temperatures with maximum resolution, while high-boiling components emerge from the column at higher temperatures within convenient analysis times. Similarly, gradient elution chromatography begins with a weakly displacing eluent that will separate the least strongly retained solutes, and ends with a sufficiently strong eluent for the convenient elution of the most strongly held substances. A further advantage in each of these two chromatographic techniques is that the last eluted sample bands have widths comparable to those of the first eluted bands. In conventional elution chromatography, band width increases with increasing band retention volume, and traces of strongly held substances frequently escape detection entirely because the very broad bands are lost within the noise or drift of the base line. Both temperature programming and gradient elution greatly increase sensitivity in the measurement of strongly held sample components. A final advantage of these two chromatographic techniques, which in fact supplied the major incentive for the original invention of gradient elution, is of importance primarily in adsorption chromatography. Excessive tailing of elution bands may occur either as the result of column overloading or solute chemisorption (Part VI)[4], and the use of gradient elution has been recommended for the reduction of such tailing.

The theory of gradient elution chromatography has been treated by several authors[5-7]. Previous theoretical work specific to adsorption chromatography has been of little practical value, since the required general relationships between eluent, solute structure, and retention volume have been unknown until recently. Preceding papers in the present series (Parts II[8], III[9], V[10] and VI[4]) have discussed the interacting roles of eluent and solute in determining retention volume for elution from alumina, silica and Florisil. Quantitative correlational equations have been derived for the prediction of retention volume in various linear elution adsorption chromatographic (LEAC) systems based on these three adsorbents. It is therefore appropriate to ex-

tend our preceding analysis of fixed eluent LEAC separations to include the case of gradient elution. This should serve to expand the usefulness of our original correlational equations, and perhaps clarify certain of the principles basic to the technique of gradient elution adsorption chromatography (GEAC).

The intention of the present communication is the development of a theory of GEAC, the exploration of some related theoretical problems, and the application of these results to a general discussion of the technique. A following paper will discuss some more practical considerations related to the use of GEAC, and provide experimental data on several GEAC systems for the confirmation of the theory given in the present paper.

OPTIMIZING SEPARATION IN GRADIENT ELUTION SYSTEMS

Following sections will explore in detail the theory of GEAC separation. The intention of the present section is a discussion of the results of this theoretical study and its application to some practical problems in the design of optimum GEAC separations. A GEAC system customarily involves the elements in the schematic diagram of Fig. 1. Prior to the beginning of separation, a weak eluent A and a strong eluent B

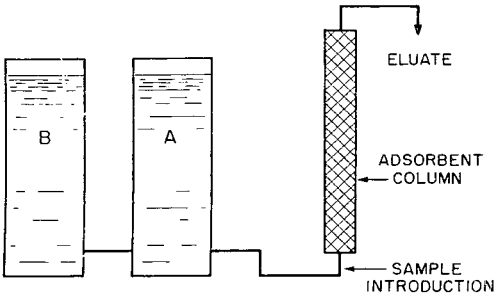

Fig. 1. Schematic diagram of GEAC separation.

are contained in separate units A and B. Unit A provides for the continuous mixing of its contents. Sample is introduced to the column, and elution begun. Eluent B flows into unit A, and the mixed eluent from A enters the column. At the beginning of separation, the eluent to the column is essentially pure A. As elution proceeds, the eluent becomes progressively richer in B and hence progressively stronger. The volume fraction of B in the combined eluent to the column (V_B) is determined by the geometry of units A and B, and their mode of connection. Exact relationships between V_B and V, the total eluate volume, have been derived for various gradient elution systems[5,11]. Gradient devices for the production of any desired eluent gradient (V_B *versus* V) have also been described[12].

As developed in preceding papers of the present series, the effectiveness of an eluent in the elution of a solute from an adsorbent column is measured by its eluent strength parameter ε°. Thus, if $\underline{R}_p$ is the linear equivalent retention volume (ml/g) of a solute eluted by pentane from a given adsorbent, its retention volume $\underline{R}^\circ$ for elution from the same adsorbent by an eluent of strength ε° is:

$$\underline{R}^\circ = \underline{R}_p \, 10^{-\alpha A_s \varepsilon^\circ} \qquad (1)$$

α is the adsorbent activity function[8], and A_s is the effective area of the solute[4]. For binary eluents, as occur in GEAC separation, the eluent strength is related to the strength of the two eluents, ε°_A and ε°_B, to the mole fraction of the stronger eluent X_B, to the adsorbent activity α, and to n_b, the value of A_s for the eluent B (considered as a solute)[9]:

$$\varepsilon^\circ = \varepsilon^\circ_A + \frac{\log (X_B \, 10^{\alpha n_b (\varepsilon^\circ_B - \varepsilon^\circ_A)} + 1 - X_B)}{\alpha n_b} \tag{2}$$

The eluate volume R_g (ml) required to elute a solute from a particular GEAC column can be calculated as discussed in the next section. For this calculation, we must know the dependence of ε° on V, as well as the values of certain solute parameters ($\underline{R}_p$, A_s) and of the adsorbent activity (α). Values of the fundamental parameters α, ε°_A, ε°_B, and n_b have been tabulated elsewhere[4] for a number of solvents and adsorbents, and $\underline{R}_p$ and A_s are calculable for many solutes from data summarized previously[4]. For eluent strength gradients of the so-called linear form,

$$\alpha\varepsilon^\circ = a + bV, \tag{3}$$

where a and b are constant throughout a GEAC separation, calculation of R_g takes a relatively simple form:

$$R_g = \frac{\log (2.31 \, A_s b W R_p \, 10^{-a A_s} + 1)}{b A_s} \tag{4}$$

W is the total adsorbent weight, and R_g assumes sample charged to dry column. An example of the calculation of R_g in a GEAC system is offered at the close of this section.

Qualitatively, it would appear desirable to have ε° increase linearly with V. Thus, eluent strength gradients (ε° *versus* V) severely concave to the V axis will show ε° increasing very rapidly with V at high values of V, and all strongly adsorbing solutes will be eluted as a single band. Alternately, convex gradients tend to level off to a constant value of ε° at large V, and strongly adsorbing solutes are then eluted very slowly. By calculating R_g for different solutes in GEAC systems employing one or more of these three gradient types, it should be possible to establish unequivocally which gradient type is indeed optimum. Prior to such a calculation, however, it is necessary to distinguish between the various sample types which it may be desired to separate.

A major application of gradient elution chromatography is in the initial study of mixtures of unknown composition and complexity. Thus, in a short time such sample types may be completely eluted from a column with near optimum resolution at each point in the separation. If we lump *all* the solute types thus far studied together and arrange them in order of increasing adsorption energy S° (from pentane), it is found that S° increases continuously while A_s tends to vary more or less randomly. *Alternately*, within individual sample groups (*e.g.*, the hydrocarbons) there is the tendency for A_s to increase with S° as additional adsorbing groups are added to the solute molecule. As a general rule, simple mixtures of known compositional range (*e.g.*, the product of nitrating a single parent molecule, mixtures of aromatic hydro-

carbons, etc.) will involve one or two individual sample types, and A_s for the solutes in such a sample will *increase* with $S°$. It is appropriate to distinguish between what we will call *simple* (known) and *complex* (unknown) sample types, A_s increasing with $S°$ for solutes in the former sample type and independent of $S°$ for solutes in the latter sample type. Obviously, some samples of known compositional range will prove to be "complex" with respect to the dependence of A_s on $S°$, and *vice versa*, but the coupling of "simple" with "known" samples and "complex" with "unknown" is a useful generalization.

The solute retention volume for elution by pentane $\underline{R}_p$ is related to $S°$ by

$$\underline{R}_p = V_a \, 10^{\alpha S°} \tag{5}$$

where V_a is the surface volume of the adsorbent[8,9]. For *complex* sample types, A_s may be treated as approximately constant, and for linear eluent strength gradients at *large values* of V and of R_g eqn. (4) simplifies to

$$\begin{aligned} R_g &\approx \log (2.31 \, A_s b W \, 10^{-a A_s} V_a) + (\alpha/bA_s)S° \\ &\approx C + DS° \end{aligned} \tag{6}$$

after substituting eqn. (5) for $\underline{R}_p$ into eqn. (4) and ignoring the unity term. The coefficients C and D are constant for various solutes where A_s is constant. Equation (6) states that after the beginning of elution of a *complex* sample from a linear eluent strength GEAC system, the R_g values for various solutes vary linearly with their adsorption energies. *This spacing of solute retention volumes is a generally desirable one.* In contrast, fixed eluent separations show solute retention volumes varying exponentially as in eqn. (5), leading to the difficulties gradient elution is designed to overcome.

Table I presents some calculated R_g values for a series of solutes in a *complex* sample (A_s constant, $S°$ varying). In the first column of R_g values, normal gradient elution using a linear eluent strength gradient is assumed, with the separation parameters at the top of Table I. Both R_g values and the spacing between adjacent solute peaks (ΔR_g) are shown. As predicted above, the spacing of solute peaks of regularly increasing adsorption energy $S°$ becomes constant after R_g becomes reasonably large (391). There is some compression of the peaks at low R_g values, but this is basic to all elution chromatographic systems, as illustrated by data (second column of R_g values) in Table I for elution of the same sample by pentane, a very weak eluent ($\varepsilon° = 0.00$). Obviously, *no* gradient shape could improve the spacing of the first two solutes of Table I. Alternately, initial elution by pentane (200 ml) followed by normal gradient elution (column 3) provides all the advantages of both fixed weak eluent and GEAC separation. Finally, in the last column of Table I normal gradient elution with V_a increased tenfold shows improved resolution of the lesser adsorbing solutes. Increasing adsorbent area is obviously the best solution to band compression in the initial part of either GEAC or fixed eluent separation.

Examination of eqn. (6) for the case of A_s increasing with $S°$ (*simple* samples) suggests that band spacing (R_g values) will tend to be slightly compressed at high values of R_g (as well as at low). This is confirmed in Table II for a model calculation

TABLE I

CALCULATED GRADIENT ELUTION SEPARATION OF A *complex* SAMPLE
(a = 0.0, b = 0.0003, (WV_a) = 0.20, A_s assumed equal 10.0)

$\alpha S°$	*R_g (ml) and band spacing (ΔR_g)*							
	Normal gradient elution		*Pentane elution*		*Initial pentane elution, followed by gradient elution*		*Normal gradient elution (WV_a) equal 2.0*	
	R_g	ΔR_g	R_g	ΔR_g	R_g	ΔR_g	R_g	ΔR_g
1.0	2.0		2.0		2.0		19	
		17		18		18		107
2.0	19		20		20		126	
		107		180		180		265
3.0	126		200		200*		391	
		265		1800		377		324
4.0	391		2000		577		715	
		324		18000				335
5.0	715		20000		908	335	1048	
		335		180000				335
6.0	1048		200000		1248	340	1383	

* Gradient elution begun.

based on a *simple* sample type. The R_g values are seen to be compressed with both low and high eluate volumes, with the band spacing ΔR_g a maximum at intermediate values of V. The compression of solutes bands at low V values cannot be avoided by changing the gradient shape (just as in the case for *complex* samples). The compression of the more strongly adsorbing solute bands, although relatively modest, can be avoided by using a mildly convex gradient. This is illustrated in the second case of Table II for a convex gradient approximated by two linear gradients: b = 0.0003 for $V \leqslant 347$ ml; b = 0.0002 for $V \geqslant 347$ ml.

TABLE II

CALCULATED GRADIENT ELUTION SEPARATION OF A *simple* SAMPLE
(a = 0.0, (WV_a) = 0.20)

$\alpha S°$	A_s	*R_g (ml) and band spacing (ΔR_g)*			
		Linear gradient, b = 0.0003 throughout		*Convex gradient**	
		R_g	ΔR_g	R_g	ΔR_g
1.0	6	2		2	
			17		17
2.0	8	19			19
			109		109
3.0	10	126		126	
			221		221
4.0	12	347		347	
			198		243
5.0	14	545		590	
			152		230
6.0	16	697		820	

* $V \leqslant 347$, b = 0.0003; $V \geqslant 347$, b = 0.0002.

It is desirable to keep solute band width w_g in GEAC separation relatively constant throughout the separation. As developed in a following section, w_g depends upon two independent aspects of the separation: the plate number p or separation efficiency of the column, and the instantaneous retention volume R_t of a solute at the time it leaves a GEAC column. R_t is the value of the retention volume ($\underline{R}°W$) for the solute assuming fixed eluent elution from the same column by eluent of that composition passing through the GEAC column at the time when $V = R_g$. Column efficiency in normal LEAC systems has not yet been investigated in detail. It is known that band width (inverse of efficiency) increases with increasing eluent viscosity[9] and flow rate[13,14]. If these latter two aspects of the separation can be maintained reasonably constant throughout a GEAC separation, column efficiency may be approximately constant and w_g values will be proportional to R_t values, at least at large values of R_g. Table III compares calculated R_t values for the separation systems of Tables I and II. It is seen that R_t becomes constant in the linear gradient

TABLE III

CALCULATED BAND WIDTH IN GEAC SEPARATION

(a = 0.0, (WV_a) = 0.20)

$\alpha S°$	A_s	$\log(Rt/Va)$*	
		Linear gradient b = 0.0003 throughout	*Convex gradient***
Complex			
1.0	10	0.99	—
2.0	10	1.94	—
3.0	10	2.62	—
4.0	10	2.83	—
5.0	10	2.85	—
6.0	10	2.86	—
Simple			
1.0	6	1.00	1.00
2.0	8	1.95	1.95
3.0	10	2.62	2.62
4.0	12	2.75	2.75
5.0	14	2.70	2.86
6.0	16	2.64	2.82

* (R_t/V_a) is proportional to band width.

** $V \leqslant 347$, b = 0.0003; $V \geqslant 347$, b = 0.0002.

separation of *complex* samples at large values of R_g, and this can be shown to follow from the fundamental GEAC equations (see a following section). At low R_g values, w_g tends to become constant and independent of R_t, so that the GEAC separation of *complex* samples appears to give optimum band widths with a linear gradient. The corresponding GEAC separation of *simple* samples shows a pattern reminiscent of the R_g data. Band width goes through a maximum (maximum R_t) at intermediate values of R_g. Use of convex gradients tends to correct for this effect as seen. Again, moderately convex gradients appear best in the separation of *simple* samples with respect to band width as well as band spacing.

Another aspect of GEAC separation which deserves comment is the phenomenon of band splitting[2,15]. If, after elution of a band maximum in a GEAC system, the eluent strength increases sufficiently rapidly, a portion of the original band tail may be *displaced* from the column to give an apparently new band. A similar phenomenon is encountered in the stepwise elution of a sample by a series of progressively stronger eluents[16]; after elution of the major part of a band by one eluent, changing to a stronger eluent elutes the band tail sufficiently rapidly to develop a new band maximum and give the appearance of a second solute band. Intuitively, it would seem unlikely that band splitting would ever occur with convex or linear gradients, and this is shown in a later section to be true for normal bands of the theoretical Gaussian shape. In the case of chemisorbed solutes[4], where a non-Gaussian tail is developed, it is possible to get band splitting with all three gradient types.

A final consideration in designing optimum GEAC separations is the phenomenon of *displacement*. By displacement is meant the tendency, in fixed binary eluent systems involving small concentrations of a strong eluent B, of the strong solvent to selectively adsorb at the beginning of the column and leave essentially pure A to elute the balance of the column[17]. In the case of solutes readily eluted by pure eluent B but not by A, the solute may advance along the column in front of the advancing zone of adsorbed B until displaced from the column. Table IV shows some experimental data illustrating this effect for a *fixed eluent* system. In the first column, experimental $\underline{R}^\circ$ values are reported for elution of several solutes from a particular LEAC

TABLE IV

DISPLACEMENT IN THE CHROMATOGRAPHIC SYSTEM 4.6% H_2O–SiO_2 (DAVISON CODE 12), 2% V ETHYL ETHER–PENTANE

Solute	$\underline{R}^\circ$ *(ml/g)*	
	*No equilibration**	*Equilibrated (true)*
Naphthalene	5.4	0.4
Phenanthrene	5.9	1.4
Triphenylene	5.8	2.5
Phenetole	5.8	1.2
Nitrobenzene	8.9	7.0

* Column pre-wet with one column volume prior to sample introduction.

system, without first passing sufficient eluent through the column to equilibrate the adsorbent and eluent. In the second column, the *true* experimental $\underline{R}^\circ$ values for *equilibrated* columns (initial passage of a large eluent volume through the column) are given.

A theoretical analysis of displacement in GEAC systems is offered in a later section. Displacement, or column non-equilibrium must always occur in the first stages of a GEAC separation. The volume of eluent required to bring the column to initial equilibrium may be defined as V_s. If V_0 is the total eluate passed through the column during separation (equal $\alpha(\varepsilon^\circ_B - \varepsilon^\circ_A)/b$ for total elution of contents of units A and B), then V_s/V_0 represents the fraction of the separation during which column non-equilibrium exists. Solutes normally eluted (assuming instantaneous

column equilibrium and no displacement) within the eluate volume V_s may be poorly separated in practice so that this fraction V_s/V_0 of the separation can be considered as possibly wasted or ineffective. The ratio V_s/V_0 obviously increases with the difference in eluent strengths ($\varepsilon^\circ_B - \varepsilon^\circ_A$) and the size of the strong eluent n_b, since these two factors promote selective adsorption of the strong eluent. Similarly, V_s/V_0 will increase with the capacity of the column (WV_a), since the more B that can be adsorbed, the longer non-equilibrium will persist. Fig. 2 summarizes the calculation of V_s/V_0 as a function of the separation conditions. The ratio $d_B M_A/d_A M_B$ is a mole correction factor converting percent volume of B to X_B; d_B and d_A are the densities of B and of A, M_B and M_A are the molecular weights of B and A. Normally, the ratio WV_a/V_0 will vary from about 0.001 to 0.03 in GEAC separation, and the mole correction factor will lie between 0.8 and 2.0. V_s/V_0 is therefore usually unimportant (less than 0.1 from Fig. 2) as long as αn_b ($\varepsilon^\circ_B - \varepsilon^\circ_A$) is less than 1.0. For GEAC eluent combinations where this latter term is greater than 1.0, W/V_0 or V_a may be varied so as to control the maximum value of V_s/V_0 by means of Fig. 2.

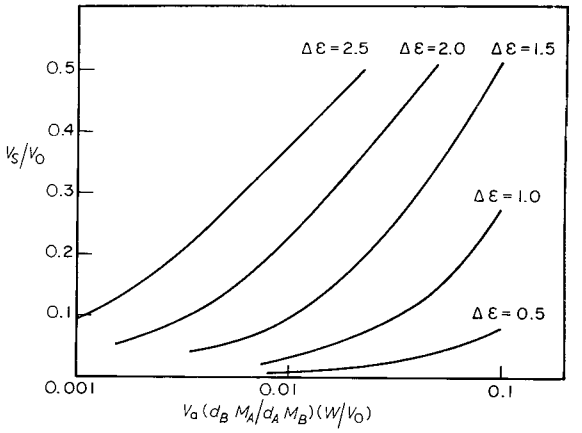

Fig. 2. Dependence of displacement or column non-equilibrium linear strength in GEAC systems on separation variables: $\Delta\varepsilon = n_b\,\alpha(\varepsilon^\circ_B - \varepsilon^\circ_A)$.

An alternate means of reducing the importance of displacement in GEAC separation, particularly where a wide range of ε° values is desired during elution, is through the use of more than two eluents. Thus, elution may be begun with a weak eluent A and an eluent B of intermediate strength. At the conclusion of the first elution, the eluent combination B (intermediate strength)–C (strong) may be used in tandem to achieve in the overall GEAC separation the ε° range between eluents A and C, but without the corresponding displacement. Frequently, for practical reasons, it will prove convenient to switch eluents prior to the end of the first elution, so that the tandem eluent system will be A–B (weak), C (strong). In this case, it is necessary to relate the strength ε° of a ternary eluent A–B–C to its composition, analogously to eqn. (2) for binary eluents. A later section provides a derivation of this relationship, eqn. (7), between ε° and the strength of the constituent solvents, ε°_A, ε°_B, and ε°_C ($\varepsilon^\circ_A < \varepsilon^\circ_B < \varepsilon^\circ_C$), the mole fractions of B and C, X_B and X_C, and the value of A_s for C, n_c:

$$\varepsilon^\circ = \varepsilon^\circ_B + \frac{\log [X_C \, 10^{\alpha n_c (\varepsilon^\circ_C - \varepsilon^\circ_B)} + X_B]}{\alpha n_c} \quad (7)$$

Equation (7) will prove useful in other applications, as discussed in the following paper of this series.

To summarize, linear eluent strength gradients provide the best separation of so-called *complex* sample types, with respect to both band width and band spacing. For *simple* samples, where A_s increases with S°, a moderately convex gradient is preferred, although it is not markedly superior to the linear gradient. The linear gradient case has the additional advantage that solute R_g values can be easily predicted. Linear or convex gradients are also preferred from the standpoint of band splitting and the development of spurious bands. Only concave gradients are normally capable of band splitting. Displacement in GEAC separation can be a problem whenever the strengths of the eluents A and B differ widely and/or n_b is large. Fig. 2 permits the problem to be anticipated in a given separation system and avoided through control of W or V_a, or by changing to a ternary eluent system.

As one example of the application of eqn. (4), consider the elution of the solute triphenylene from a GEAC system where $a = 0.03$, $b = 0.00187$, W equal 10 g, and the adsorbent is 3.7 % H_2O–Al_2O_3. R_p for this system can be calculated from eqn. (5), providing S° is first calculated as described previously[4].

$$\begin{aligned} S^\circ &= \sum^{i} Q^\circ_i + \sum^{j} q^\circ_j - f(Q^\circ_k) \sum^{i \neq j} Q^\circ_i \\ &= 18 \times 0.31 + 0 - 0 \\ &= 5.58 \end{aligned}$$

Substituting the above value of S° into eqn. (5), with $\alpha = 0.63$ and $V_a = 0.016$[8] gives $R_p = 51$. Finally, A_s for triphenylene may be calculated[9] as 12, and substituting into eqn. (4):

$$\begin{aligned} R_g &= \frac{\log (2.31 \times 12 \times 0.00187 \times 10 \times 51 \times 10^{-0.03 \times 12} + 1)}{0.00187 \times 12} \\ &= 52.2 \; ml \end{aligned}$$

An experimental value of 51 ml was observed with a GEAC system based on isooctane (A)–ethyl ether (B) and characterized by the above separation parameters.

SOLUTE RETENTION VOLUME

Solute retention volume plays a key role in determining the separation capabilities of any elution chromatographic system, and it is of interest to relate the retention volume of a solute in a GEAC separation to its molecular structure and to the conditions of separation. At some time t after the introduction of a solute to a GEAC column as in Fig. 1 and the beginning of elution, the solute will have traversed some fractional distance x along the column longitudinal axis. To a first approximation, the composition of the eluent both entering and within the column at time t can be assumed

identical (if displacement is unimportant), and the instantaneous retention volume R_t can be defined as previously. At any time during the separation, a differential flow of eluate dV will induce a corresponding differential flow of the solute band dx, equal to dV/R_t. The retention volume of the solute is then given by:

$$\int_0^{R_g} dx = 1$$

and

$$\int_0^{R_g} dV/R_t = 1 \qquad (8)$$

That is, the total length of column is traversed ($\Sigma\, dx = 1$) when the eluate volume V equals R_g (by definition). FREILING[6] has provided a similar derivation of eqn. (8) in his treatment of gradient elution in ion exchange systems. The quantity R_t is equal to $\underline{R}^\circ\, W$, and $\underline{R}^\circ$ is related to the eluent strength ε° through eqn. (1). ε° can in turn be expressed as a function of eluent composition through eqn. (2) or by experimental determination[9]. Finally, the arrangement and geometry of the gradient elution system permits eluent composition and hence R_t to be known as a function of V. Substitution of this function of V into eqn. (8) permits in principle the integration of eqn. (8) and the evaluation of R_g. In actual fact, however, the resulting expression under the integral sign of eqn. (8) is so complex in the general case as not permit an explicit algebraic solution.

It will be seen profitable to sidestep this difficulty in the general integration of eqn. (8) by restricting our attention to the special case of linear eluent strength systems, as defined by eqn. (3). Substitution of eqn. (3) into eqn. (1) gives:

$$\underline{R}^\circ = \underline{R}_p\, 10^{-A_s\,(a\,+\,bV)} \qquad (9)$$

and substitution of $\underline{R}^\circ$ from eqn. (9) into eqn. (8), with R_t equal $\underline{R}^\circ\, W$, provides:

$$\int_0^{R_g} \frac{10^{A_s(a\,+\,bV)}\, dV}{W\underline{R}_p} = 1 \qquad (10)$$

which upon integration gives eqn. (4). Equation (4) is the fundamental equation of linear strength GEAC separation.

The calculation of R_g for non-linear strength gradients (those not given by eqn. (3)) can be done using numerical integration of eqn. (8) with trial and error estimates of R_g. A similar, approximate, procedure is worth mentioning. A general non-linear $\varepsilon^\circ - V$ relationship as illustrated in Fig. 3 can be approximated by several line segments (dashed lines in Fig. 3) of the form:

$$\text{(segment 1)}\ \alpha\varepsilon^\circ = a + b_1 V$$

$$\text{(segment 2)}\ \alpha\varepsilon^\circ = a + b_1 V_1 + b_2\,(V - V_1),\ \text{etc.}$$

V_1, V_2, etc., are the eluate volumes V at which the approximating line segments intersect. Now define the function T:

$$T = \int_0^V \mathrm{d}V/R_t$$

$$= \int_0^{V_1} \mathrm{d}V/R_t + \int_{V_1}^{V_2} \mathrm{d}V/R_t + \cdots + \int_{V_i}^{V} \mathrm{d}V/R_t$$

Each of the integrals above can be evaluated as in the development of eqn. (4):

$$\int_0^V \mathrm{d}V/R_t = \frac{10^{a A_s}}{2.31\, R_p W A_s b_1} (10^{A_s b_1 V} - 1) \tag{11a}$$

$$\int_{V_1}^V \mathrm{d}V/R_t = \frac{10^{A_s(a + b_1 V_1 - b_2 V_1)} (10^{A_s b_2 V} - 10^{A_s b_2 V_1})}{2.31\, R_p W A_s b_2} \tag{11b}$$

and so forth.

For $V < V_1$, the resulting calculation of R_g is exactly equivalent to the linear case, with R_g given by eqn. (4). For $V_1 < V < V_2$:

$$T = \int_0^{V_1} \mathrm{d}V/R_t + \int_{V_1}^V \mathrm{d}V/R_t$$

$$= \frac{10^{a A_s}}{2.31\, R_p W A_s b_1} (10^{A_s b_1 V_1} - 1)$$

$$+ \frac{10^{A_s(a + b_1 V_1 - b_2 V_1)} (10^{A_s b_2 V} - 10^{A_s b_2 V_1})}{2.31\, R_p W A_s b_2}$$

T is obtained for trial values of V and the solution of $V = R_g$ obtained at $T = 1$. It should be noted that T is not linear in V so that interpolation between values of T greater and less than 1 requires care. The procedure for calculating T when $V > V_2$ is essentially similar.

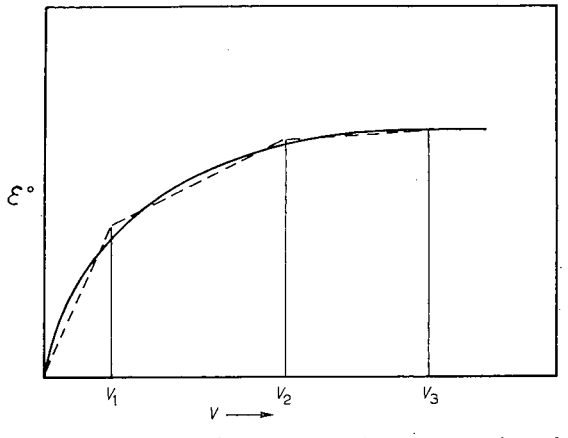

Fig. 3. Approximation of a non-linear ε°–V curve by line segments for calculation of R_g values.

SOLUTE BAND WIDTH

The equivalent plate model of elution chromatography[18] predicts that, in a given chromatographic system which may be approximated by p equivalent equilibrium plates, band width w (measured in the column eluate) is proportional to solute retention volume R'. In fact, p is customarily calculated from the ratio R'/w. As discussed previously, a major advantage of GEAC is its tendency to reduce this normal increase of solute band width with increasing retention volume. Ideally, all solute bands would have comparable widths in GEAC separation, and it is of interest to investigate the theoretical effect of the conditions of separation on solute band width.

Band width as measured in the eluate is determined both by the width of the *adsorbed* solute band immediately prior to elution from the column, and by the retention volume R'. Fig. 4 illustrates this relationship, an adsorbed band at the end of a long column being shown just prior to elution from the column, along with the resulting eluate band. By the time the band has arrived at the end of a *long* column,

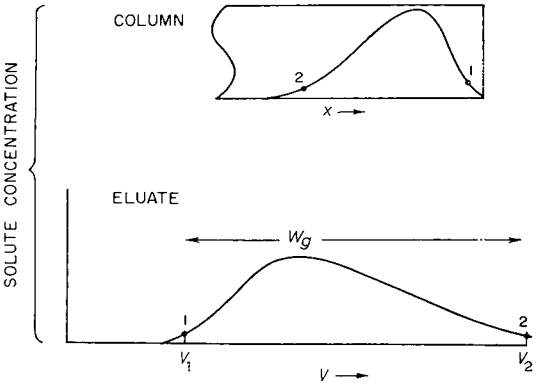

Fig. 4. Dependence of band width in eluate on adsorbed band width.

band shape has to a first approximation been determined, and the band appearing in the eluate may be regarded as arising from the simple elution of each element of the adsorbed band x by a corresponding eluent volume V. x in Fig. 4 represents the fractional distance along the column length, with a value of 1.0 to the column end. The eluent volume required to elute the band element at x is approximately $R'(1 - x)$ when $R' \gg 1$. If $(x_1 - x_2)$ represents the width of the adsorbed band as measured between the arbitrary points 1 and 2, then the volumes required to elute these two corresponding points are v_1 and v_2, equal $R'(1 - x_1)$ and $R'(1 - x_2)$, respectively. The width of the eluted band is $(v_2 - v_1)$, equal $R(x_1 - x_2)$. That is, the eluted band width is R' times the adsorbed band width. Since in the simple plate model theory R'/w is constant, the adsorbed band width of all solutes immediately prior to elution must also be constant (independent of R').

In the case of GEAC separation, the above considerations similarly apply, as long as the plate number p of the column is constant throughout separation. That is, adsorbed band widths of all solutes are comparable, and eluate band width w_g is proportional to R_t, the instantaneous retention volume at the time the band is eluted

from the column ($V = R_g$). For large values of R_g, R_t may be calculated as follows. First, eqn. (1) reduces to:

$$R_g \approx (\log 2.31\, A_s b\, WR_p\, 10^{-a A_s})/bA_s$$

and substitution of eqn. (5) for $\underline{R}_p$ gives:

$$R_g \approx (\log 2.31\, A_s b\, WV_a)/bA_s - (a/b) + \alpha S^\circ b/A_s$$

At the time of band elution, $V = R_g$, and from eqn. (3):

$$\alpha\varepsilon^\circ = a + bR_g$$
$$\alpha\varepsilon^\circ \approx a + (\log 2.31\, A_s b\, WV_a)/A_s - a + S^\circ/A_s$$

R_t equal $W\,\underline{R}^\circ$ is now obtained from eqns. (1) and (5) with substitution of the above expression for $\alpha\varepsilon^\circ$:

$$R_t \approx WV_a\, 10^{\alpha S^\circ - A_s(\log 2.31\, A_s\, bWV_a)/A_s - \alpha S^\circ} \approx \frac{1}{2.31\, A_s b} \qquad (12)$$

For solutes of constant or approximately constant A_s values, eqn. (12) predicts constant band widths at large R_g values for linear strength gradient separations.

BAND SPLITTING

Fig. 5(a) provides an illustration of what is meant by band splitting. Toward the end of elution, an initially normal elution band appears suddenly to develop a second maximum, giving the appearance of two rather than one eluted bands. The origin of band splitting is easily understood in terms of our previous discussion of band width. For fixed eluent elution, as in Fig. 4, or a band that has reached the end of a column, it was assumed that R_t was approximately constant during the course of final elution, and than $R_t x$ ml of eluent were required to elute the solute from a

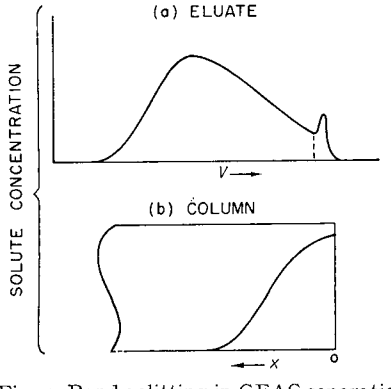

Fig. 5. Band splitting in GEAC separation.

column element at x. This process assumes transfer of all solute from a given column element into a corresponding volume element dV. Since V equals $R_t x$, dV/dx equals R_t, and the ratio of solute concentrations in the eluent element relative to the original column element is $1/R_t$. The solute concentration in the eluate is then related to the concentration in the original adsorbed band C_x by:

$$C_v = C_x/R_t \tag{13}$$

In GEAC separation, R_t actually is assumed to decrease during the final elution of the band, although this normally insignificant effect was ignored in our preceding discussion of band width. C_v must therefore increase up to the point x_m corresponding to the original adsorbed band maximum. Past the elution of the band element originally at x_m, C_x decreases while $1/R_t$ continuous to increase. If the decrease in C_x is large relative to the increase in $1/R_t$, C_v will begin to decrease and the eluted band will have developed a band maximum. If at some later time, $1/R_t$ suddenly increases more rapidly than C_x is decreasing, C_v will then increase, and a second band maximum in the eluate will have developed. It is of interest to establish under what experimental conditions band splitting can occur.

Fig. 5(b) shows the adsorbed solute band eluted to the original point x_m; *i.e.*, with the adsorbed band maximum at the column end ($x_m = 0$). The x axis is now reversed for convenience in Fig. 5(b), the column end corresponding to $x = 0$, and the front end of the column having $x = 1.0$. The preceding discussion indicates that no band maximum will have developed in the eluate prior to the arrival of the adsorbed band maximum at the column end ($x = 0$), so that the shape of the eluate band past this point ($V = R_g$) will determine the occurrence of band splitting. Theoretically, the adsorbed elution band in Fig. 5(b) should be a Gaussian curve[18], and:

$$C_x = Ce^{-Dx^2} \tag{14}$$

where C and D are constants for a given column of p equivalent plates. Analogously to eqn. (8), we can write for the elution of the band in Fig. 5(b):

$$\int_0^x dx = \int_{R_g}^{V_x} dV/R_t$$

where V_x is the eluate volume V at which the column element dx is eluted. In terms of eqns. (1) and (3):

$$R_t = R_g \, 10^{-b(V_x - R_g)} \tag{15}$$

for a linear eluent strength gradient, and substitution of this into the previous integral with integration gives:

$$x = \frac{10^{-bR_g}}{2.31b\,R_g}\left(10^{bV_x} - 10^{bR_g}\right)$$

or:

$$x = G\left(10^{bV_x} - 10^{bR_g}\right) \tag{16}$$

Substitution of eqns. (14) and (15) into eqn. (13) gives:

$$C_v = \frac{Ce^{-Dx^2} 10^{b(V_x - R_g)}}{R_g} \tag{17}$$

The conditions for C_v being a maximum at some eluate volume V is $\partial C_v/\partial V = 0$, or differentiating eqn. (17) and rearranging gives:

$$x\partial x/\partial V = b/2\ CD \tag{18}$$

Substituting eqn. (16) and its derivative with respect to V into eqn. (18) then gives:

$$10^{bV_x}\ (10^{bV_x} - 10^{bR_g}) = 1/2\ CDG^2 \tag{19}$$

The term within the brackets of eqn. (19) is zero when $V = R_g$. For $V_x > R_g$, both 10^{bV_x} and the bracketed factor in eqn. (19) increase continuously with V_x, as must their product. Therefore, at one and only one value of V_x, the product on the left must equal the positive constant on the right, or there is only one band maximum in the eluate. *Consequently, it has been proved that linear strength gradients do not permit band splitting.*

An exception to the above conclusion may result whenever the adsorbed band is non-Gaussian, as in the case of solute chemisorption[4]. Chemisorbed solutes show pronounced, very flat tailing of the elution band with fixed eluent elution, and it seems quite likely that GEAC separation under such conditions will lead to band splitting even with linear eluent strength gradients.

DISPLACEMENT

The phenomenon of displacement is illustrated in Fig. 6. A fixed eluent chromatographic system is assumed, with elution by a dilute solution of a strongly adsorbing eluent B in a weak adsorbing eluent A. The eluent issuing from the column will be initially depleted of B, because of mass transfer to the adsorbed phase. At some time

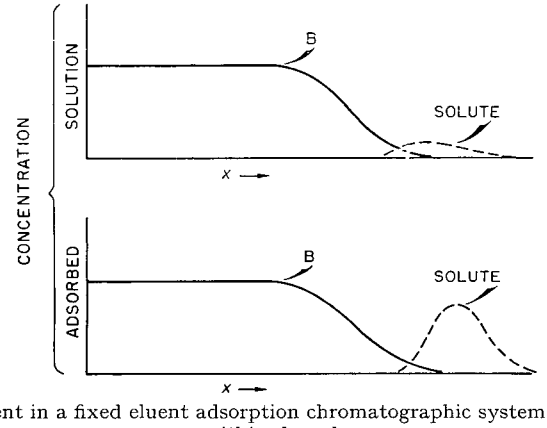

Fig. 6. Displacement in a fixed eluent adsorption chromatographic system. Concentration profiles within the column.

during elution, the concentration profile of B in adsorbed and solution phases within the column will be as represented by Fig. 6. Solutes that are readily eluted (small $\underline{R}^\circ$) by the given binary eluent from *equilibrated* columns (entering eluent in equilibrium with leaving eluent) will tend to move down the column ahead of the adsorbed B zone by displacement, as illustrated in Fig. 6 (dashed solute band). The data of Table IV for that fixed eluent chromatographic system suggest, after taking column void volume into account, that 6.8 ml/g of eluent must flow through this system before the adsorbent is saturated with ethyl ether, since all weakly adsorbed solutes (true $\underline{R}^\circ < 3$) have identical retention volumes in the non-equilibrated column. The adverse effect of displacement on separation is also illustrated in the data of Table IV. Whereas reasonable separation occurs between phenanthrene and triphenylene in the equilibrium column, no separation exists in the non-equilibrium column.

The mathematically precise formulation of the displacement effect in GEAC separation is obviously a formidable task. The reasonably complex equilibria associated with analogous fixed binary eluent systems are further complicated in GEAC by the rapidly changing composition of eluent entering the column. Consequently, it is necessary to seek a less exact, mathematically tractable, approximation of displacement in GEAC systems.

Because displacement will be most serious for strongly adsorbing eluents B, a reasonable initial assumption is that the adsorbent surface will remove all B from the eluent, up to the point where the adsorbent surface is completely covered by adsorbed B. After deriving the relevant equations for displacement based on this approximation, we will show how to correct for the case where the adsorbent surface is *not* largely covered by B after equilibrium is reached within the column. With the assumption of complete surface coverage by B, the eluate volume V_s required to saturate the adsorbent surface (whose total volume capacity is WV_a) is defined by:

$$WV_a = \int_0^{V_s} V_B dV \tag{20}$$

where V_B refers to the column fraction of B in the entering eluent at a given time. For small values of V_B, where displacement is of most importance, the mole and volume fractions of B in the entering eluent are related by:

$$X_B \approx (d_B M_A / d_A M_B) V_B \tag{21}$$

where d and M refer to the density and molecular weight, respectively, of the subscript species. Now, for convenience, define the symbols: $\Delta\varepsilon \equiv n_b\,\alpha(\varepsilon^\circ_B - \varepsilon^\circ_A)$ and $\varepsilon \equiv n_b\,\alpha(\varepsilon^\circ - \varepsilon^\circ_A)$, which may be regarded, respectively, as the effective eluent strength *range* of the separation, and the relative eluent strength at a given time. For a linear strength gradient:

$$\varepsilon = \Delta\varepsilon\, V/V_0 \tag{22}$$

where V_0 is the total eluate volume associated with the GEAC separation (equal V when ε° equal ε°_B). From eqn. (2):

$$\begin{aligned} X_B &= (10^{\varepsilon} - 1)/(10^{\Delta\varepsilon} - 1) \\ &= (10^{\Delta\varepsilon V/V_0} - 1)/(10^{\Delta\varepsilon} - 1) \end{aligned} \tag{23}$$

Solution for V_B by eliminating X_B from eqns. (21) and (23), substituting into eqn. (20), and integrating gives:

$$(WV_a d_B M_A \Delta\varepsilon / d_A M_B V_0)\ (10^{\Delta\varepsilon} - 1) = 10^{\Delta\varepsilon V_s/V_0} - 1 - \Delta\varepsilon V_s/V_0 \qquad (24)$$

Tables of the function $(10^x - 1 - x)$ permit the solution of V_s/V_0 as a function of $WV_a d_B M_A \Delta\varepsilon / d_A M_B V_0$ and $\Delta\varepsilon$. At the time V equal V_s, the adsorbent surface may be only fractionally covered by solute, so that the above expression (24) generally overestimates the magnitude of V_s/V_0. If the fraction of the surface covered by B is S_B, this is equivalent, in terms of the amount of B required for surface saturation, to a lower value of W or V_a. Consequently, tables of V_s/V_0 can be prepared as a function of $WV_a d_B M_A \Delta\varepsilon / d_A M_B V_0$ for various values of $\Delta\varepsilon$, assuming $S_B = 1.0$, S_B actually calculated for each value of V_s/V_0 obtained from the table assuming an equivalent reduction in the (WV_a, etc.) term by a factor of S_B. A new value of S_B can be calculated, a further reduction in WV_a estimated, and further correction of V_s/V_0 obtained. By this iterative procedure, final tabulated values of V_s/V_0 can be obtained corrected for the actual value of S_B. This is the basis of the calculated values of Fig. 2. The required function S_B has been derived previously in connection with the derivation of eqn. (2), and is [eqn. (6), Ref. (9)]:

$$S_B = \frac{X_B\, 10^{\Delta\varepsilon}}{X_B\, 10^{\Delta\varepsilon} + 1 - X_B}$$

Substitution of X_B from eqn. (23) and simplification gives finally:

$$S_B = \frac{10^{\Delta\varepsilon(1 + V_s/V_0)}}{10^{\Delta\varepsilon(1 + V_s/V_0)} + 10^{\Delta\varepsilon} + 1 - 10^{\Delta\varepsilon V_s/V_0}}$$

THE ELUENT STRENGTH OF TERNARY SOLVENTS

It is desired to calculate ε° for ternary solvents A–B–C as a function of the ternary compositions, X_A, X_B, X_C, and the eluent strengths of the pure constituents, $\varepsilon^\circ_A, \varepsilon^\circ_B, \varepsilon^\circ_C (\varepsilon^\circ_A < \varepsilon^\circ_B < \varepsilon^\circ_C)$. For the usual case of interest to GEAC separation, it may be assumed that $\varepsilon^\circ_C \gg \varepsilon^\circ_A$ and X_A does not approximate unity (the eluent has an appreciable concentration of B and/or C). Under these conditions, the adsorbent surface may be considered to be covered almost exclusively by B and C. Referring back to the original derivation of eqn. (2) in Ref. 9, it is seen that the ternary case under the above conditions almost exactly duplicates the binary case: the mole fraction of adsorbed B and C for the ternary is the same as A and B for the binary, and the solution mole fractions of B and C differ from those of A and B for the binary only in that the sum of X_B and X_C are not unity in the ternary case, as X_A and X_B were for the binary. The derivation of the ternary ε° relationship now follows that of the previous binary derivation exactly, differing only in that the term X_B (instead of $1 - X_C$) is retained in the final expression (7).

The accuracy of eqn. (7) is checked in Table V for a series of ternary eluents

TABLE V

COMPARISON OF EXPERIMENTAL ELUENT STRENGTHS OF TERNARY SOLVENTS WITH VALUES CALCULATED FROM EQUATION (7); 3.7% H_2O–Al_2O_3

Solvent composition % (vol.)				*log R°**	*αε° (expt.)***	*ε°*	
Pentane	*Benzene*	*Methylene chloride*	*Dioxan*			*(Expt.)***	*(Calc.)****
0	100	0	0	1.06	—	—	0.32
0	0	100	0	0.39	—	—	0.42
60	30	10	0	1.72	0.141	0.22	0.20
30	30	40	0	1.09	0.204	0.32	0.35
60	38	0	2	0.90	0.223	0.35	0.31
60	35	0	5	0.61	0.252	0.40	0.37
60	30	0	10	0.36	0.277	0.44	0.43
60	0	38	2	0.66	0.247	0.39	0.38
60	0	35	5	0.42	0.271	0.43	0.40
60	0	30	10	0.16	0.297	0.47	0.44
60	0	20	20	−0.12	0.325	0.52	0.50
20	0	75	5	0.08	0.305	0.48	0.44
20	0	70	10	−0.10	0.323	0.51	0.47
20	0	60	20	−0.35	0.348	0.55	0.50

* For 6-methoxyquinoline.
** From $\underline{R}^\circ$ and eqn. (1), assuming $A_s = 10$ and log $\underline{R}_p = 3.11$.
*** From eqn. (7), assuming ε° dioxan = 0.65; a previously reported value[9] (0.63) was in error.

using pentane, benzene, methylene chloride, and/or dioxan. Measurement of the retention volume $\underline{R}^\circ$ for a solute of known A_s value in each of these ternary solvents and in two pure solvents of known ε° value permits the derivation of experimental ε° values, which are seen in Table V to agree with the calculated values with an average precision of $\pm$ 0.03 unit. Equation (7) is not expected to be a good approximation when the various constituent eluents are of roughly comparable strength, or when the mole fraction of A is close to one. Neither of these cases, however, is of practical interest in the use of ternary solvent systems in GEAC separation.

ACKNOWLEDGEMENT

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GLOSSARY OF TERMS

A_s	Solute effective area.
A, B, C	Refer to solvents A, B, C.
a, b	Coefficients of eqn. (3).
b_1, b_2	Values of b occurring in various parts of a GEAC separation.
C, D, G	Constants.
C_v, C_x	Concentration of solute in eluate at volume V, and on column at point x.
d_A, d_B	Density of solvents A, B.

M_A, M_B	Molecular weight of A, B.
n_b, n_c	Value of A_s for solvents B, C.
p	Number of theoretical equivalent plates in a column.
$\underline{R}^\circ$	Solute linear equivalent retention volume (ml/g).
$\underline{R}_p$	Value of $\underline{R}^\circ$ for elution by pentane (ml/g).
R_g	Retention volume in a GEAC separation (ml), corrected for column volume.
ΔR_g	Difference in R_g values for adjacent solutes.
R_t	Instantaneous retention volume; see text (ml).
R'	Uncorrected solute retention volume (ml).
S°	Solute adsorption energy; pentane solvent.
S_B	Mole fraction in adsorbed phase of B.
T	Fractional movement of a solute along a GEAC column; see equations 11a, 11b
v_1, v_2, v_x	Eluate volume required to elute point 1, 2, x on adsorbed solute band.
V_a	Adsorbent surface volume (ml/g); proportional to surface area.
V	Eluate volume.
V_e	Total volume of eluate for a given GEAC separation.
V_s	Eluent volume required to bring GEAC column into equilibrium with entering eluate; volume at which displacement effect ends.
V_B	Volume fraction of B in eluent.
V_1, V_2	Eluate volume at which eluent strength gradient b changes; see treatment of non-linear eluent strength GEAC separation.
W	Weight of adsorbent in column.
w	Band width in eluate (ml).
w_g	w in GEAC separation.
x, x_1, x_2	Fractional distance along the column, with origin ($x = 0$) taken variously at either column end.
x_m	Value of x at band maximum.
X_B, X_C	Mole fraction of B, C in eluent.
α	Adsorbent activity function.
$\Delta\varepsilon$	$n_b\,\alpha(\varepsilon^\circ_B - \varepsilon^\circ_A)$.
ε°	Eluent strength; particularly the eluent strength in a GEAC separation at time t.
ε	$n_b\,\alpha(\varepsilon^\circ - \varepsilon^\circ_A)$.

SUMMARY

The fundamental correlational equations previously developed for normal, linear elution from alumina, silica, and Florisil have been extended to include the technique of gradient elution. It has been shown that in general the eluent gradient should be of the *linear strength* form. The theoretical advantages of linear strength gradient elution include: (1) approximately equal spacing of solute peaks according to increasing solute adsorption energy, providing maximum resolution between both weakly and strongly adsorbing solutes with minimum separation time; (2) approximately equal band widths for maximum detection sensitivity; (3) no band splitting with development of spurious double peaks; (4) easily calculable retention volumes for solutes whose adsorption parameters have been measured or can be estimated. For some sample types a mildly convex eluent strength gradient is predicted to give

a better separation system, although the advantages over the corresponding linear strength case will normally be small. The use in gradient elution of solvents of widely differing strengths can lead to displacement, rather than elution, with resulting loss of resolution for weakly adsorbing solutes. The experimental conditions required for displacement to occur are discussed, as is the avoidance of this phenomenon through various means including the use of ternary solvent mixtures. The theoretical strength of such ternary eluents has been derived as a function of the eluent composition and the strengths of the pure constituent solvents.

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CHROMATOGRAPHIC STUDIES ON ISONICOTINIC ACID HYDRAZIDE AND ITS METABOLIC DERIVATIVES

IV. NEW TECHNIQUES OF ISOLATION AND IDENTIFICATION

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INTRODUCTION

Various techniques for the isolation of isonicotinic acid hydrazide (INH) and its metabolic derivatives from samples of biological material have already been developed in our laboratory, such as centrifugal ultrafiltration combined with descending paper chromatography[1], paper electrophoresis[2,3] and direct chromatographic separation of the metabolites from the wet sample[4].

The application of paper electrophoresis is limited to the isolation of acid metabolites and, as in the case of direct chromatographic separation from wet samples, can only be used when large amounts of the metabolic derivatives are present. Centrifugal ultrafiltration combined with descending paper chromatography allows the concentration of the metabolic derivatives but is, however, a laborious and time-consuming technique.

We have now developed a technique for the isolation of INH-derivatives from biological samples, such as serum and plasma, based on its dehydration in columns of anhydrous sodium sulphate and subsequent elution of the said derivatives with a mixture of chloroform and diethylamine. Fairly large samples can thus be used, the eluted material being easily concentrated.

The good results obtained with diethylamine for the elution of INH-derivatives led us to study this reagent in solvent mixtures for the paper chromatography of the above compounds. Furthermore, new reagents have been investigated for the localization and identification of INH-derivatives on paper chromatograms.

MATERIALS AND METHODS

Isolation of INH-derivatives by column chromatography

Various materials were tested for the chromatographic isolation of INH-derivatives, such as silica gel, anhydrous copper sulphate and anhydrous sodium sulphate, but the best results were obtained with the last-mentioned material.

A chromatographic column, 20 × 1 cm, was packed with 4 g of anhydrous sodium sulphate (Merck, Darmstadt), forming a column 5 cm high. The sample to be

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examined was then added dropwise on to the top of the column, by means of a capillary pipette; air pressure was used to force the sample into the column. Sample volumes up to 1 ml can be treated in this way.

The water in the sample is absorbed by the anhydrous sodium sulphate and the proteins, salts and metabolic intermediates, including INH and its metabolic derivatives, are precipitated inside the column.

The INH and its derivatives can then be eluted from the column with a mixture of chloroform–diethylamine (90:10) (flow-rate = 2 ml/min). The eluate was evaporated to dryness on the water-bath, and the residue was taken up in a few microlitres of diethylamine.

Paper chromatography

Paper chromatography was carried out by the ascending technique, using Macherey-Nagel No. 261 filter paper. Control chromatograms were run with *n*-butanol saturated with 1 % ammonium hydroxide[1].

New solvent mixtures containing varying amounts of diethylamine, water and an organic solvent were also studied. The following solvents were tested: *n*-butanol, *n*-amyl alcohol, isoamyl alcohol, *n*-propyl alcohol, isopropyl alcohol, benzyl alcohol, ethyl alcohol, acetone and chloroform.

Complete sets of R_F values were determined for the solvent mixtures yielding the best separations for a sample containing INH and the isonicotinyl hydrazones of pyruvic acid and of acetaldehyde.

The sensitivity of a few more reagents towards INH and its derivatives was determined as described previously[5] by means of spot tests on filter paper. The following reagents were tested:

I. *Sodium nitroprusside.* 5 g of the salt are dissolved in a 10 % solution (v/v) of acetaldehyde in water; before using an equal volume of 2 % (w/v) sodium carbonate is added; after spraying the sample is heated at 120° for 10 min.

II. *Wachsmuth reagent*[6]. 2 g of quinhydrone are dissolved in 95 ml of ethanol plus 5 ml of pyridine; after spraying the sample is heated at 100° for 2 min.

III. *Ninhydrin.* 0.2 % (w/v) solution in acetone[7]; after spraying the sample is heated at 120° for 15 min.

IV. *Percheron reagent*[8]. 0.5 g barbituric acid dissolved in 100 ml ethanol containing 2 ml of 85 % phosphoric acid; after spraying and heating at 120° for 5 min the sample is detected by observing under a U.V. lamp.

V. Isatin. 1 g of the reagent is dissolved in 100 ml isopropanol containing 1 ml pyridine and 1.5 g zinc acetate; the sample is dried in an oven at 110°C and examined under a U.V. lamp.

VI. *Pyridine–acetaldehyde*[9]. The sampled is sprayed with a mixture containing equal parts of the reagents, dried at 110° and observed under a U.V. lamp.

RESULTS AND DISCUSSION

The above technique for the isolation of INH-derivatives by column chromatography was tested with mixtures containing INH, acetyl-INH and the INH hydrazones of pyruvic acid and acetaldehyde, dissolved in blood serum (1 % w/v concentrations of each component).

Elution was checked by paper chromatography, and it was found that 50 ml of chloroform–diethylamine were sufficient to completely elute the INH-derivatives, as shown in Table I.

TABLE I

ELUTION RATE OF VARIOUS INH-DERIVATIVES PURIFIED BY MEANS OF COLUMN CHROMATOGRAPHY

Fraction (10 ml)	*INH-derivatives present*			
	INH	*acetyl-INH*	*INHzone acet.*	*INHzone pyr.*
I	+	+	—	—
II	+	+	+	+
III	+	+	+	+
IV	—	—	+	+
V	—	—	+	+
VI	—	—	—	—

Blood samples, collected from mice 30 min after the intraperitoneal injection of INH (5 μg/g), were purified as described, and the derivatives eluted from the column were separated by two-dimensional paper chromatography[4]. Free INH, acetyl-INH and the hydrazones of pyruvic acid and acetaldehyde were identified by this method, confirming earlier results[1], together with isonicotinamide and di-isonicotinyl hydrazide.

Among the new diethylamine solvent mixtures tested, the following gave the best results: (I) *n*-butanol–diethylamine–water (40:10:satd.); (II) isopropyl alcohol–diethylamine–water (60:20:10); (III) benzyl alcohol–diethylamine–water (40:10:satd.); (IV) chloroform–diethylamine–water (40:20:satd.); (V) *n*-amyl alcohol–diethylamine–water (40:10:satd.). The R_F values found for the INH-derivatives are shown in Table II.

TABLE II

R_F VALUES OF INH-DERIVATIVES IN SOLVENT MIXTURES CONTAINING DIETHYLAMINE

Compound	*Solvent mixture*				
	I	*II*	*III*	*IV*	*V*
INH	0.53	1	0.53	0.38	0.22
Acetyl-INH	0.47	1	0.47	0.02	0.13
Pyruvic acid INHzone	0.42–0.56	1	0.41–0.53	0.09	0.08–0.19
Acetaldehyde INHzone	0.55	1	0.61	0.03	0.20
Isonicotinamide	0.76	1	0.67	0.58	0.52
Isonicotinic acid	0.54	1	0.49	0.14	0.18
Di-INH	0.55	1	0.60	0.03	0.20

As can be seen, solvent II (isopropyl alcohol–diethylamine–water) is particularly useful for the direct chromatographic separation of INH derivatives from wet samples[4]; solvent IV (chloroform–diethylamine–water) is suitable for separating INH, isonicotinic acid and isonicotinamide from the remaining derivatives.

The results for the sensitivity of the spray reagents described under "materials

and methods" are shown in Table III, together with the colours given by the various derivatives.

Reagent I (sodium nitroprusside) was found to have good sensitivity to all derivatives, giving characteristic colours with INH, the pyruvic hydrazone and isonicotinic acid. Reagent IV (barbituric acid) was shown to be specific for the hydrazones,

TABLE III

SENSITIVITY OF VARIOUS REAGENTS TOWARDS INH AND ITS METABOLIC DERIVATIVES

INH-derivative	*Reagent*					
	I	*II*	*III*	*IV*	*V*	*VI*
			Sensitivity			
INH	2	2	2	—	4	30
Acetyl-INH	2	5	20	—	4	—
Pyruvic acid INHzone	2	2	2	0.5	8	20
Acetaldehyde INHzone	2	2	2	20	4	10
Isonicotinamide	2	10	20	—	—	—
Isonicotinic acid	2	2	1	—	—	—
Di-INH	2	2	2	—	4	50
			Colour			
INH	Orange	Brownish	Yellow	—	Brown	Yellow
Acetyl-INH	Brownish	Brownish	Yellow	—	Yellow	—
Pyruvic acid INHzone	Bordeaux	Bordeaux	Brown	Brown	Brown	Blue
Acetaldehyde INHzone	Brownish	Brownish	Orange	Brown	Yellow	Yellow
Isonicotinamide	Brownish	Bordeaux	Yellow	—	—	—
Isonicotinic acid	Yellow	Bordeaux	Yellow	—	—	—
Di-INH	Brownish	Yellow	Orange	—	—	—

having a high sensitivity to the pyruvic derivatives. Reagent VI (pyridine–acetaldehyde) although being specific for INH, di-INH and the hydrazones, had lower sensitivity. Reagents II (quinhydrone) and III (ninhydrin) showed good sensitivity towards most IHN-derivatives, except acetyl-INH and isonicotinamide. Reagent V (isatin) was found to be specific for the derivatives containing the hydrazine moiety; sensitivity, however, was not very high.

ACKNOWLEDGEMENTS

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SUMMARY

The authors studied the isolation of INH-derivatives from samples of biological material, such as plasma and serum, by means of chromatography on columns of anhydrous sodium sulphate, followed by elution with chloroform–diethylamine

(90:10). Fairly large volumes of material can be treated, the eluted derivatives being easily concentrated by evaporating to dryness.

Solvent mixtures for paper chromatography containing diethylamine were also studied. The solvent mixture chloroform–diethylamine–water (40:20:satd.) was found to give a good separation of INH, isonicotinic acid and isonicotinamide from the remaining derivatives.

Among the new reagents tested for INH-derivatives, good results were obtained with sodium nitroprusside, quinhydrone and ninhydrin. Specific reactions were obtained with barbituric acid (hydrazones), pyridine–acetaldehyde (INH, di-INH and the hydrazones) and isatin (derivatives containing the hydrazine moiety).

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ÉLECTROPHORÈSE SUR PAPIER D'ACÉTATE DE CELLULOSE DANS DES ÉLECTROLYTES FORTS ET CONCENTRÉS

APPAREILLAGE ET TECHNIQUE DÉTERMINATION DES MOBILITÉS ÉLECTROPHORÉTIQUES ET SÉPARATION DE QUELQUES ÉLÉMENTS TRANSURANIENS EN MILIEU NITRIQUE

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L'électrophorèse sur papier permet l'étude des ions complexes: détermination du signe de leur charge et calcul de leur constante de stabilité[1]. Applicable aisément lorsqu'il s'agit de faire migrer des ions dans des électrolytes faibles, peu conducteurs, cette technique ne peut être utilisée avec des électrolytes forts et concentrés qu'en employant un appareillage convenable susceptible de produire un refroidissement efficace au niveau du papier imbibé d'électrolyte, soumis au champ électrique. Ces milieux étant, en effet, très conducteurs, l'intensité du courant est élevée, ce qui entraîne un dégagement de chaleur important par effet Joule, au sein de la bande de papier. Il est donc nécessaire de la refroidir pour maintenir sa température constante, pour réduire l'évaporation du liquide et, par suite, le flux hydrodynamique, et pour éviter sa destruction.

La plupart des migrations électrophorétiques dans des électrolytes forts, ont été réalisées selon la technique de KUNKEL ET TISELIUS[2] ou de LEDERER ET WARD[3]. Récemment, SHUKLA ET ADLOFF[4], utilisant cette dernière méthode, ont fait migrer, pendant 45 min avec une tension d'alimentation de 300 V, RaD, RaE et RaF dans HCl de 0.0025 N à 8 N, dans HNO_3 de 0.0005 N à 3.6 N, dans $HClO_4$ 1 N et dans des mélanges d'acide nitrique et de nitrate de potassium. Comme le remarquent ces auteurs, l'intérêt de leurs résultats est purement qualitatif, car ils ne contrôlent pas la température au niveau du papier.

Ayant entrepris l'étude des complexes d'éléments cis et transuraniens dans différents milieux acides minéraux, nous avons dû réaliser un appareil permettant la détermination correcte des mobilités ioniques électrophorétiques dans des électrolytes très conducteurs et très acides tout en ayant un champ électrique suffisamment intense pour que les migrations s'effectuent en un temps assez court.

L'utilisation du papier filtre de cellulose comme support est limitée à une certaine acidité du milieu dépendant de la nature même de l'acide. SHUKLA ET ADLOFF[4] n'ont pu utiliser le papier d'Arches No. 302 au-dessus d'une concentration 3 N en HCl et 1 N en $HClO_4$. Pour des concentrations en HCl allant de 3 N à 8 N, ils l'ont remplacé

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par du papier filtre en fibre de verre Whatman GF/A qui résiste bien aux acides mais qui présente les inconvénients suivants: porosité irrégulière et adsorption de nombreux ions. Nous avons choisi comme support des feuilles d'acétate de cellulose. Déjà utilisées dans l'électrophorèse des protéines[5–7], les membranes d'acétate de cellulose ont une porosité parfaitement définie et une faible épaisseur, ce qui permet d'obtenir une imbibition très reproductible et d'avoir une intensité de courant beaucoup plus basse qu'avec le papier filtre pour un électrolyte et une tension électrique donnés. Les essais de résistance aux agents chimiques que nous avons effectués montrent que ces membranes sont utilisables avec des électrolytes très acides. Elles présentent également l'avantage de ne pas adsorber la plupart des ions.

Dans cet article, nous exposons les caractéristiques de l'appareil utilisé, notre technique, et nous donnons quelques résultats obtenus en milieu nitrique avec des éléments transuraniens.

CARACTÉRISTIQUES DE L'APPAREILLAGE ET TECHNIQUE

L'appareil que nous avons réalisé est inspiré de celui que GROSS[8] a décrit pour l'électrophorèse à haute tension. Les problèmes posés par cette technique sont, en effet, les mêmes que ceux de l'électrophorèse dans les électrolytes forts. L'appareil doit permettre une dissipation efficace de la chaleur dégagée par effet Joule dans le support:

$$Q = \frac{RI^2}{J} = \frac{VI}{J}$$

où Q = chaleur dégagée par seconde,
R = résistance électrique du support,
I = intensité du courant,
J = équivalent mécanique de la calorie (4.185 joule/cal).

(1) *Description de l'appareil*

Etant donné la forte acidité des milieux étudiés (acides concentrés), l'appareil est entièrement construit en matière plastique ("lucoflex"). Les réservoirs d'électrolyte où plongent les électrodes (cathode en charbon; anode en platine) ont une capacité d'un litre chacun et sont divisés en deux compartiments communiquant par un orifice garni de fibre de verre pour éviter la diffusion des produits de décomposition formés aux électrodes vers le support où s'effectuent les électromigrations. Ces réservoirs peuvent être mis en communication pour réaliser l'équilibre des niveaux d'électrolyte.

Les plaques réfrigérantes entre lesquelles est incluse la membrane d'acétate de cellulose servant de support mesurent 32 cm × 9 cm et sont munies, à chaque extrémité, d'une petite encoche permettant d'encastrer un morceau de papier filtre de fibre de verre (Whatman GF/B) qui relie la membrane d'acétate de cellulose aux réservoirs d'électrolyte.

Chaque plaque est parcourue par un courant d'eau en circuit fermé (débit = 1 l/min) refroidi par un groupe frigorifique (puissance de dissipation calorifique = 1000 kcal/h). Nous maintenons la température de l'eau de réfrigération à l'entrée des plaques à 4°, à l'aide de thermomètres à contact de platine contrôlant le fonctionne-

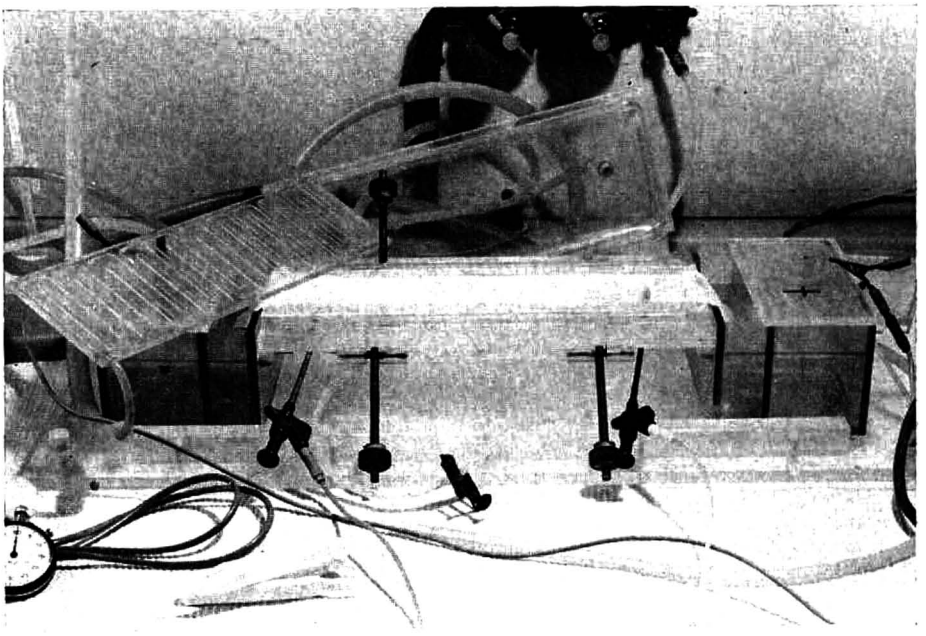

Fig. 1. Vue détaillée de l'appareil d'électrophorèse.

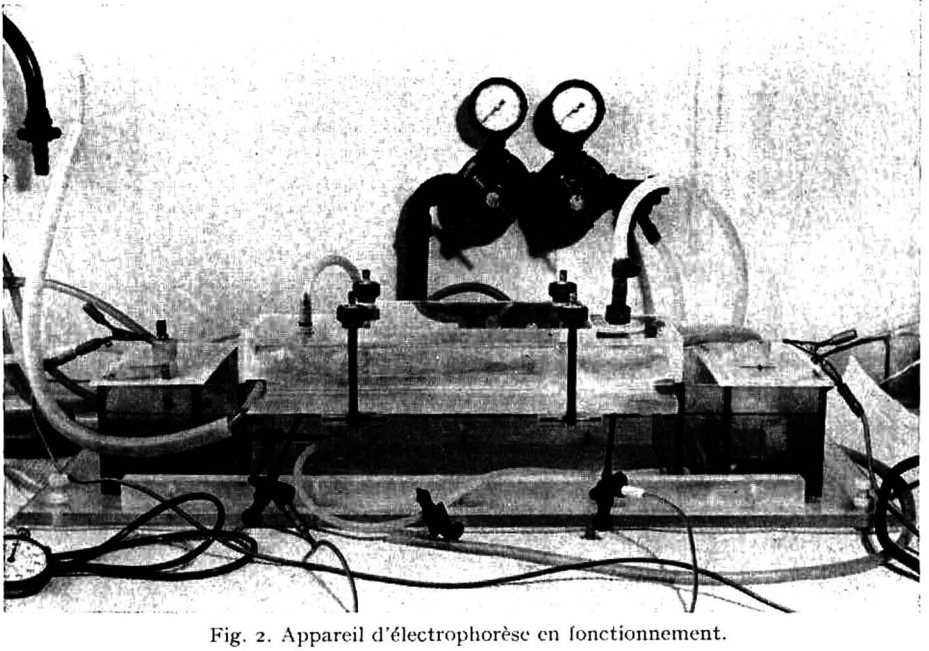

Fig. 2. Appareil d'électrophorèse en fonctionnement.

ment des groupes frigorifiques: dans ces conditions, la température au niveau du support est de 5°.

Sur la plaque inférieure sont prévus des petits trous espacés de 15 mm, permettant le passage d'électrodes en platine servant à mesurer le champ électrique dans le support.

Une pression uniforme est appliquée sur les plaques par l'intermédiaire d'un coussin pneumatique en feuille de polyvinyle et d'un dispositif de serrage mécanique approprié. Les Figs. 1 et 2 représentent l'appareil démonté et en fonctionnement.

Cet appareil est associé d'une part à une alimentation en courant continu stabilisé de 5 kVA et d'autre part à un voltmètre à lampes (résistance d'entrée = $10^8 \Omega$) relié à un enregistreur permettant de mesurer et d'enregistrer la tension aux extrémités du support en cours d'expérience.

(2) *Étude du support d'acétate de cellulose*

Nous utilisons le papier "Millipore" HAWP. C'est une membrane d'acétate de cellulose dont les pores ont un diamètre de 0.45 μ. Nous avons effectué sur ce papier les essais suivants.

(a) *Résistance aux agents chimiques.* Des morceaux de papier "Millipore" sont imbibés des acides suivants, à différentes concentrations: HCl, HNO_3, $HClO_4$, H_2SO_4, HBr; on essore entre quelques feuilles de papier Joseph. Chaque échantillon est placé entre deux verres de montre constituant une petite enceinte étanche. On maintient à 5° pendant 6 h. Les résultats sont donnés au Tableau I.

TABLEAU I

RÉSISTANCE DU PAPIER "MILLIPORE" HAWP AUX AGENTS CHIMIQUES

Acide étudié	*Résultats*
$HClO_4$ 5 N	Le papier devient élastique
HBr (environ 7 N)	Bonne résistance
HNO_3 14 N	Le papier devient très élastique
H_2SO_4 12 N	Aucune attaque
HCl 12 N	Le papier devient légèrement élastique

(b) *Adsorption des ions sur le papier "Millipore".* Nous avons étudié le comportement des ions suivants:

Th(IV): solution HNO_3 1 N
^{233}Pa(V): solution HCl 1 N
U(VI): solution HNO_3 1 N
^{237}Np(IV): solution HCl 0.5 N–$N_2H_4 \cdot$HCl 0.05 M
^{237}Np(V): solution HNO_3 1 N
^{237}Np(VI): solution $HClO_4$ 1 N
^{239}Pu(III): solution HCl 0.5 N–$N_2H_4 \cdot$HCl 0.05 M
^{239}Pu(IV): solution HNO_3 6 N
^{239}Pu(VI): solution HNO_3 0.5 N
^{241}Am(III): solution HNO_3 1 N
^{242}Cm(III): solution HNO_3 1 N

Des chromatogrammes sur bandes de papier "Millipore" ont été réalisés avec ces diverses solutions, selon la technique de chromatographie descendante. Le développement est fait avec les solvants suivants: HCl de 1 N à 12 N; HNO_3 de 1 N à 12 N; $HClO_4$ de 1 N à 5 N; H_2SO_4 de 1 N à 12 N; HBr de 1 N à 4 N et H_2SO_4 1 N-$K_2Cr_2O_7$ 1 N. Les solutions d'ions ont été déposées au front du solvant qu'on a laissé migrer seul sur une petite distance et on développe, le temps nécessaire pour un déplacement du front d'environ 10 cm. Les ions radioactifs sont révélés par autoradiographie sur film Structurix D 10 Gevaert; Th(IV) et U(VI) sont révélés par pulvérisation d'une solution aqueuse de thorin (0.2%) sur les chromatogrammes.

Résultats: Tous les ions examinés ont un $R_F = 1$ sur le papier "Millipore" dans les milieux étudiés, mais pour ^{237}Np et ^{233}Pa, on a noté une tache principale à $R_F = 1$, suivie d'une traînée allant jusqu'à l'origine. Cependant, un comptage proportionnel alpha des chromatogrammes de ^{237}Np localise cet élément à $R_F = 1$ et une analyse spectrométrique gamma indique que la traînée observée sur les autoradiogrammes correspondants est constituée de ^{233}Pa, descendant de ^{237}Np.

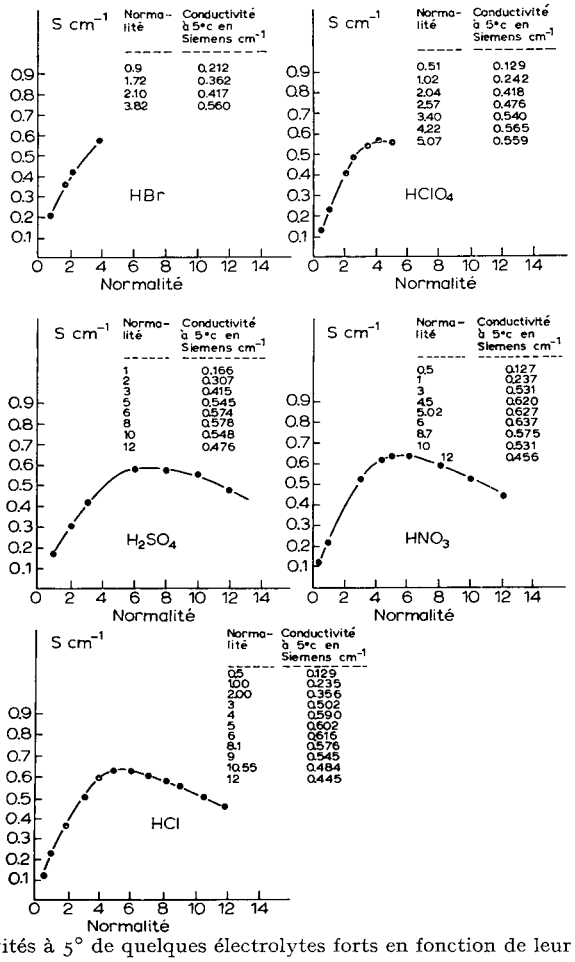

Fig. 3. Conductivités à 5° de quelques électrolytes forts en fonction de leur concentration.

Le papier "Millipore" HAWP constitue donc un support convenable pour réaliser des électromigrations d'ions dans des électrolytes forts très acides.

(*c*) *Utilisation du papier "Millipore" sur l'appareil d'électrophorèse.* Nous utilisons des bandes de papier de 32 cm × 5 cm qui permettent de faire migrer simultanément plusieurs taches des solutions à examiner. On imbibe la bande d'électrolyte; on l'essore en la laissant reposer quelques minutes sur une feuille de papier Joseph. On la place alors sur la plaque inférieure de l'appareil recouverte d'une mince feuille de téflon (épaisseur = 0.05 mm); elle repose à chaque extrémité sur le bord d'un morceau de papier filtre de fibre de verre (Whatman GF/B) trempant dans l'électrolyte. La membrane d'acétate de cellulose est alors recouverte d'une autre feuille de téflon et on réalise l'étanchéité sur les bords par de la graisse de silicone. On recouvre avec la plaque réfrigérante supérieure et le dispositif de fixation. On gonfle le coussin pneumatique à une pression de 50 mm Hg/cm^2.

(*3*) *Examen des conditions de fonctionnement de l'appareil*

Malgré ses nombreux avantages, l'électrophorèse sur papier présente l'inconvénient de faire intervenir de nombreux facteurs difficiles à contrôler qui influencent les valeurs des mobilités électrophorétiques[3,9]. Il est indispensable d'étudier ces facteurs pour un appareil donné afin de se rendre compte de leur importance et de faire intervenir les corrections nécessaires aux valeurs expérimentales des mobilités.

Nous avons mesuré les conductivités à 5° des électrolytes que nous nous proposons d'employer dans nos expériences d'électromigration d'éléments cis et transuraniens (Fig. 3). C'est HNO_3 6 *N* qui est le plus conducteur; HNO_3 est aussi un oxydant; nous l'utiliserons donc pour déterminer le fonctionnement de l'appareil dans ses plus mauvaises conditions d'utilisation.

(*a*) *Limite maximale d'utilisation de l'appareil.* Utilisant HNO_3 6 *N*, nous avons augmenté la tension électrique d'alimentation jusqu'à rupture du support d'acétate de cellulose par calcination; chaque tension examinée est appliquée pendant une heure et l'on mesure la différence de potentiel entre deux points A et B du support distants de 24 cm avec un voltmètre à lampes (résistance d'entrée = $10^8 \Omega$) qui permet, en pratique, de réaliser des mesures en circuit ouvert. Des expériences identiques ont été faites aussi avec HNO_3 1 *N*. Les résultats sont donnés au Tableau II où sont indiquées les puissances électriques mises en jeu pour une surface de bande de 24 cm × 5 cm = 120 cm^2. Dans les deux cas, le papier "Millipore" se rompt lorsque la puissance électrique dépasse 1.4 W/cm^2. Cette valeur, indépendante de la conductivité de l'électrolyte est une constante caractéristique de l'appareil, dépendant de sa puissance de dissipation calorifibue.

(*b*) *Etude du champ électrique dans le support d'acétate de cellulose.* La détermination des mobilités électrophorétiques nécessite une répartition uniforme du champ électrique dans le support.

Utilisant des petites électrodes de platine distantes les unes des autres de 15 mm, traversant la plaque réfrigérante inférieure de l'appareil et venant au contact de la bande de papier "Millipore", nous avons mesuré la tension électrique le long du support. Nous avons obtenu les résultats suivants:

Pour une tension donnée, le champ électrique est stabilisé après 10 min de passage du courant (Fig. 4).

Le champ est régulier jusqu'à une tension voisine de la tension électrique de

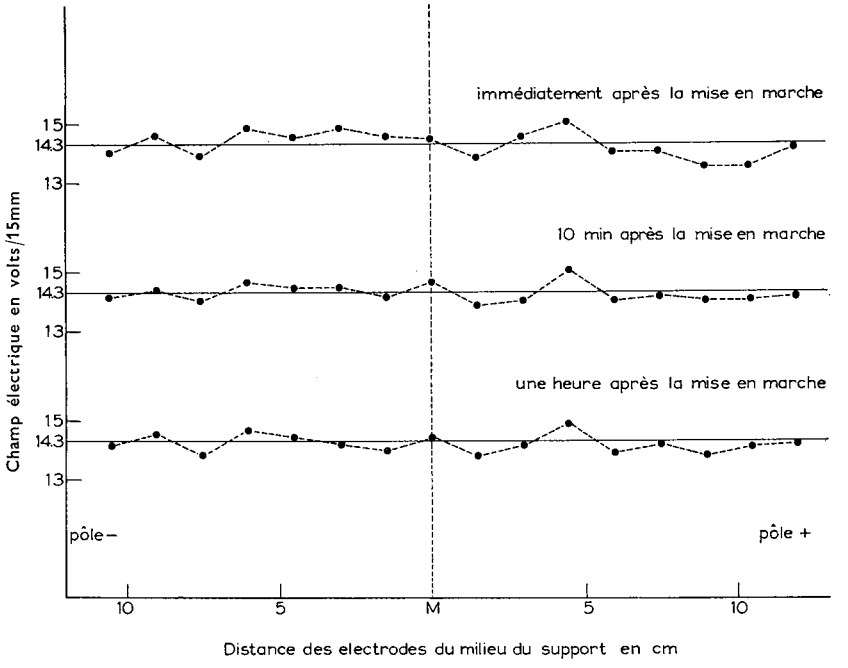

Fig. 4. Variation du champ électrique dans le support en fonction du temps. Électrolyte: HNO_3 6 *N*. Support: papier "Millipore" HAWP. Tension d'alimentation: 300 volts.

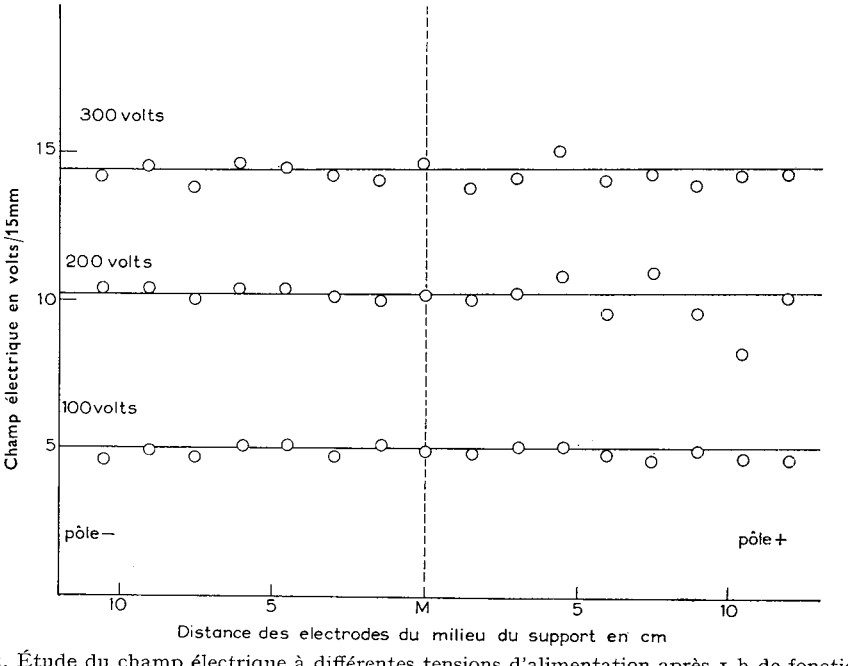

Fig. 5. Étude du champ électrique à différentes tensions d'alimentation après 1 h de fonctionnement. Électrolyte: HNO_3 6 *N*. Support: papier "Millipore" HAWP. Tension électrique de rupture > 350 V (HNO_3 6 *N*).

TABLEAU II

Electrolyte	*Tension de l'alimentation (V)*	V_{A-B}	*Intensité (mA)*	$V_{A-B} \times I$ *(W)*	*Puissance électrique (W/cm²)*
HNO_3 1 *N*	100	75	42	3.15	0.026
	150	115	64	7.36	0.06
	200	155	92	14.3	0.12
	250	194	122	23.6	0.20
	300	230	150	34.5	0.29
	350	268	200	53.6	0.45
	400	310	255	79	0.66
	450	342	340	116	0.96
	500*	380	440	167	1.39
HNO_3 6 *N*	100	74	102	7.55	0.063
	150	115	165	19.0	0.16
	200	150	250	37.5	0.31
	250	185	365	67.5	0.56
	300	230	435	100	0.83
	325	245	560	137	1.14
	350**	262	640	168	1.40

* Tension d'alimentation > 500 V: rupture du support.
** Tension d'alimentation > 350 V: rupture du support.

rupture du papier (Fig. 5) mais il est préférable pour des mesures de mobilité d'utiliser une tension plus basse n'amenant aucun échauffement du support. La migration des ions devra aussi se faire dans la région où le champ est le plus régulier. La vitesse de déplacement électrophorétique, corrigée de l'électroosmose et du flux hydrodynamique est proportionnelle à la tension électrique jusqu'à une certaine valeur de celle-ci, puis elle s'accélère pour des tensions plus élevées, un échauffement du support se produisant alors (Fig. 6). Lorsque le champ électrique est régulier, les déplacements des ions sont proportionnels au temps (Fig. 7).

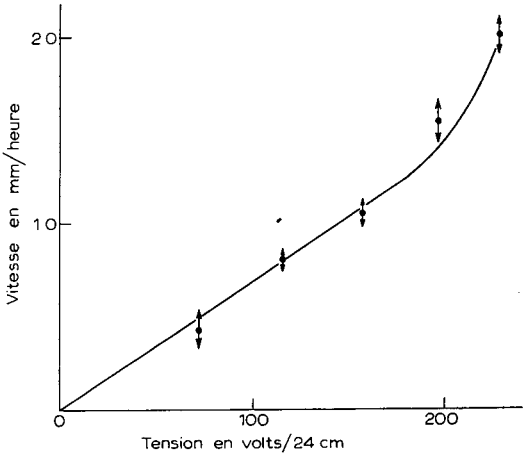

Fig. 6. Vitesse moyenne de U(VI) en fonction de la tension électrique. Électrolyte: HNO_3 6 *N*. Support: papier "Millipore" HAWP. Temps: 1 h 30 min.

(*c*) *Etude du flux hydrodynamique et de l'électroosmose.* Pour cette étude, on dépose des gouttes de H_2O_2 à différents endroits sur la bande d'acétate de cellulose imbibée de l'électrolyte considéré et on mesure leurs déplacements après passage du courant électrique. Il reste suffisamment de H_2O_2, non décomposé, pour pouvoir révéler les taches par pulvérisation d'une solution de KI (formation de taches d'iode). H_2O_2

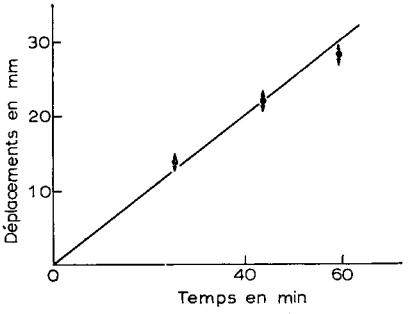

Fig. 7. Déplacements de U(VI) en fonction du temps. Électrolyte: HNO_3 6 *N*. Support: papier "Millipore" HAWP. Tension d'alimentation: 300 V.

étant une molécule électriquement neutre se déplace uniquement sous l'effet du flux hydrodynamique et de l'électroosmose. Sa vitesse de migration est la somme algébrique de la vitesse du courant électroosmotique et du flux hydrodynamique.

$$v_{H_2O_2} = v_{os} + v_{hydro}$$

où $v_{H_2O_2}$ = vitesse expérimentale de H_2O_2,

v_{os} = vitesse électroosmotique, indépendante du point de départ des ions sur le support,

v_{hydro} = vitesse du flux hydrodynamique; elle dépend de la position de départ des ions sur le support.

Dans la Fig. 8 nous avons représenté les courbes de variation de la vitesse moyenne du flux hydrodynamique en fonction de la position de départ des ions. Cette vitesse est obtenue en retranchant de la vitesse expérimentale de H_2O_2, sa vitesse vraie déterminée selon DE WAEL[10].

DÉTERMINATION DES MOBILITÉS ÉLECTROPHORÉTIQUES ET SÉPARATION DE QUELQUES ÉLÉMENTS TRANSURANIENS EN MILIEU NITRIQUE CONCENTRÉ

Utilisant la méthode expérimentale graphique de DE WAEL[10], nous avons déterminé les mobilités de U(VI), Pu(IV), Am(III) et Cm(III) dans HNO_3 6 *N*, HNO_3 10 *N* et HNO_3 12 *N*. La feuille de papier "Millipore" est imbibée d'électrolyte et placée sur l'appareil comme il a déjà été indiqué. Tenant compte de notre étude du champ électrique dans le support, on fait passer le courant pendant 10 min avant de déposer les solutions d'ions à étudier; comme nous l'avons montré, le champ électrique est alors stabilisé. On démonte l'appareil et on dépose des gouttes de la solution de l'ion dont on veut déterminer la mobilité, en les plaçant de telle sorte que les déplacements se fassent sur des pistes différentes. On remonte l'appareil et on fait passer le courant le temps nécessaire pour pouvoir observer des déplacements suffisants en fonction de la

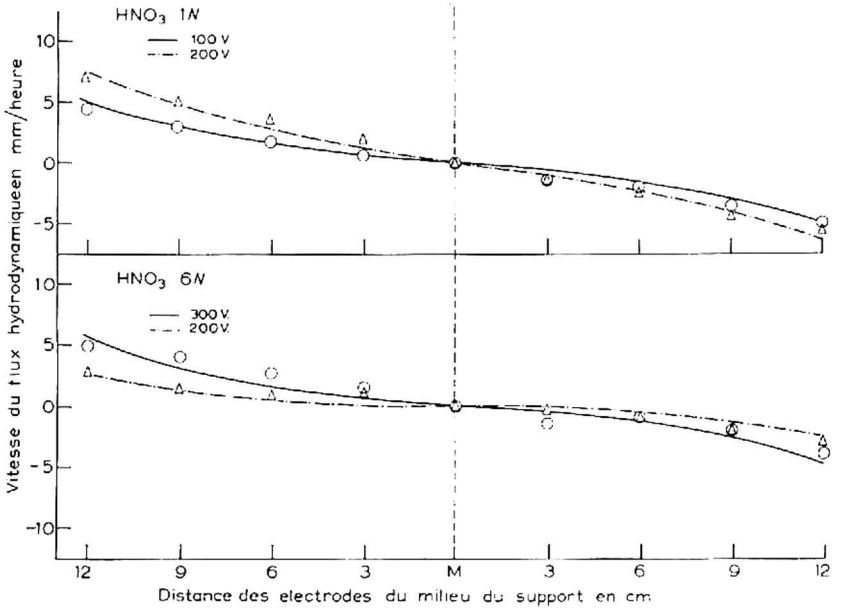

Fig. 8. Variation de la vitesse moyenne du flux hydrodynamique en fonction du point de départ. Migration de H_2O_2. Électrolytes: HNO_3 1 *N* et HNO_3 6 *N*. Support: papier "Millipore" HAWP. Temps: 2 h.

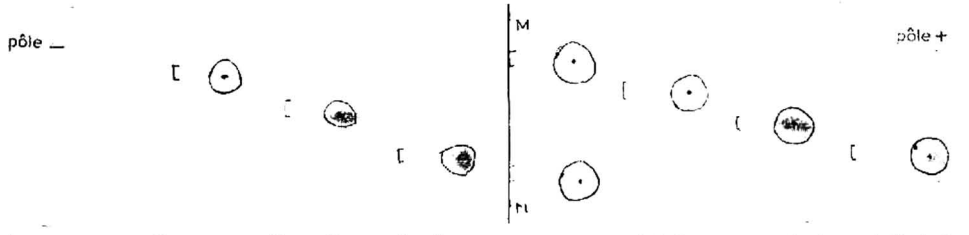

Fig. 9. Autoradiogramme d'un électrophorégramme ayant servi à la mesure de la mobilité de Pu(IV) dans HNO_3 10 *N*. Support: papier "Millipore" HAWP. Tension d'alimentation: 250 V. Temps: 2 h

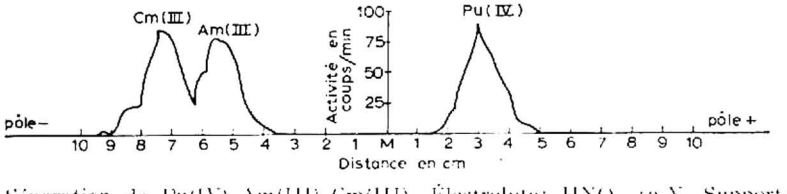

Fig. 10. Séparation de Pu(IV) Am(III) Cm(III). Électrolyte: HNO_3 10 *N*. Support: papier "Millipore" HAWP. Tension d'alimentation: 250 V. Temps: 6 h.

tension choisie. Nous utilisons une tension d'alimentation de 250 V. Pendant toute la durée de l'expérience, on enregistre la tension entre deux points du support distants de 24 cm, les migrations s'effectuant dans cette zone. Après séchage de la feuille de papier "Millipore" tendue sur une plaque de verre, on a révélé U(VI) par pulvérisation d'une solution aqueuse de thorin; les éléments radioactifs sont révélés par autoradiographie sur film Structurix D 10 Gevaert (Fig. 9).

Toutes nos déterminations de mobilités sont faites à 5° ± 1°. Les résultats sont donnés au Tableau III. Les valeurs de mobilités ont été corrigées de l'électroosmose, mesurée par la mobilité de H_2O_2 dans les électrolytes correspondants.

TABLEAU III

MOBILITÉS À 5° DE U(VI), Pu(IV), Am(III) ET Cm(III) EN MILIEU HNO_3 CONCENTRÉ

	Mobilités (cm²/V⁻¹/sec⁻¹)		
Elément	*HNO_3 6 N*	*HNO_3 10 N*	*HNO_3 12 N*
U(VI)	$+4.0\cdot10^{-5}$	$-1.7\cdot10^{-5}$	$-2.0\cdot10^{-5}$
^{239}Pu(IV)	$+1.9\cdot10^{-5}$	$5.0\cdot10^{-5}$	$-5.5\cdot10^{-5}$
^{241}Am(III)	$+3.5\cdot10^{-5}$	$-0.3\cdot10^{-5}$	$-0.9\cdot10^{-5}$
^{242}Cm(III)	$+5.5\cdot10^{-5}$	$+1.3\cdot10^{-5}$	$+0.2\cdot10^{-5}$

On note des différences de complexation importantes par NO_3^- pour les ions examinés, permettant des séparations analytiques (Fig. 10).

Des travaux sont en cours pour étudier par électrophorèse les complexes d'éléments cis et transuraniens dans des électrolytes forts.

RÉSUMÉ

Un appareil pour électrophorèse des ions dans les électrolytes forts a été mis au point. Les membranes d'acétate de cellulose ont été choisies comme support. Les mobilités électrophorétiques de U(VI), Pu(IV), Am(III) et Cm(III) ont été déterminées dans HNO_3 6 *N*, HNO_3 10 *N* et HNO_3 12 *N*. Une séparation est indiquée.

SUMMARY

An apparatus for the electrophoresis of ions in strong electrolytes is described. Cellulose acetate membranes were used as the support. The electrophoretic mobilities of U(VI), Pu(IV), Am(III) and Cm(III) were determined in 6 *N* HNO_3, 10 *N* HNO_3 and 12 *N* HNO_3. A separation was obtained.

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STUDIUM DER KOMPLEXVERBINDUNGEN IN LÖSUNG MITTELS PAPIERELEKTROPHORESE*

I. ELEKTROPHORETISCHE BEWEGLICHKEIT UND ZUSAMMENSETZUNG DER KOMPLEXE

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Professor A. Okáč zum 60. Lebensjahre gewidmet

ALLGEMEINES

Die Papierelektrophorese der niedermolekularen Verbindungen bietet eine unmittelbare Information über das Ladungszeichen der wandernden Ionen. Deshalb wurde die Papierelektrophorese in mehreren Fällen von physikalisch-chemischem Studium der Komplexverbindungen in Lösung mit der Absicht benutzt, das Ladungszeichen des Komplexions zu bestimmen oder zu bestätigen, bzw. auch die Ladungsgrösse abzuschätzen. Es handelte sich z.B. um Quecksilber (II)–Chlorokomplexe[1], Nickel (IV)–dimethylglyoximat[2], Eisen (III)–Antipyrin-Komplex[3], Titankomplexe der Chromotropsäure[4], Komplexe des Formaldoxims[5], Eisen (III)–Salicylatokomplexe[6], Komplexe der Äthylendiaminotetraessigsäure[7], Fluorozirkonate[8] u.a. In meisten Fällen handelt es sich um eine isolierte Bestimmung der elektrophoretischen Beweglichkeit unter angegebenen Bedingungen. Die so gewonnene Auskunft hat ziemlich begrenzte Bedeutung und kann leicht zu irrigen Resultaten führen. Wir haben uns überzeugt, dass man bei Verfolgung vom elektrophoretischen Verhalten der Komplexe unter möglichst breitem Umfang der Versuchsbedingungen wesentlich verlässlichere und ausführlichere Informationen über ihre Zusammensetzung und Stabilität bekommen kann. Die Papierelektrophorese kann auf diese Weise als eine weitere Methode für physikalisch-chemische Analyse der Komplexe in Lösung dienen. Das gilt allerdings nur für lösliche Komplexe mit negativ geladenen Liganden, wo sich mit der Komplexbildung die Ladung des Teilchens ändert.

Am zweckmässigsten zeigt sich die Verfolgung der Beweglichkeit des Zentralions im breiten Konzentrationsbereich des Komplexbildners im Grundelektrolyt. Graphische Darstellung dieser Abhängigkeit ist die "elektrophoretische Beweglichkeitskurve" (Fig. 1). An dieser sind die waagerechten Plateaus und die sinkenden Äste sichtbar. Die Plateaus beschränken das Konzentrationsgebiet, wo im Gleichgewichtsgemisch eine bestimmte Komplexstufe (oder auch "freies", nur hydratisiertes Zentralion) bei weitem überwiegt. Die Werte der Beweglichkeit in diesen Gebieten weisen auf Zusammensetzung und Ladung des betreffenden Komplexes hin. Dieser Zusammenhang wird in dieser Mitteilung behandelt. Die sinkenden Äste der Beweglich-

* Vorläufige Mitteilung: *Česk. Farm.*, 12 (1963) 44.

keitskurve entsprechen der Koexistenz von vergleichbaren Konzentrationen von zwei oder mehreren nachfolgenden Komplexstufen; die Beweglichkeitswerte in diesem Gebiet geben Auskunft über Stabilität der sich bildenden Komplexe. Dieses Problem wird in der weiteren Mitteilung dieser Reihe besprochen.

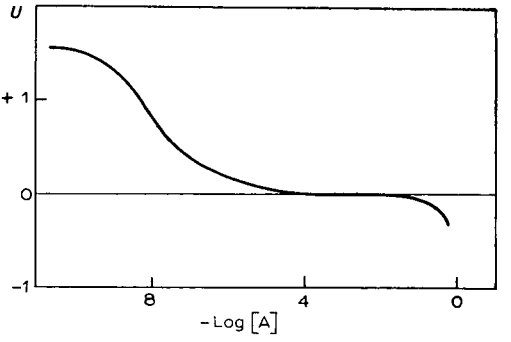

Fig. 1. Die experimentelle Beweglichkeitskurve vom Glycin–Kupfer (II)-Komplex. $c_{Cu} = 5 \cdot 10^{-2}$, $c_{Glycin} = 5 \cdot 10^{-2}$, resp. 1.0 für $[A] > 10^{-2}$; T = 20°; $\mu = 0.1$ (KNO_3); [A] = Konzentration des Glycinations; U = Beweglichkeit relativ zu $(C_2H_5)_4N^+$.

EINLEITUNG

Die Werte der elektrophoretischen Beweglichkeiten zeigen deutlich, dass sie mit der Zusammensetzung der wandernden Ionen in einer gesätzmässigen Beziehung stehen. Die Erwägungen über deren Charakter müssen unter Voraussetzung der geraden Proportionalität zwischen der Beweglichkeit in freier wässeriger Lösung u_* und bei Papierelektrophorese u gemacht werden: $u = \rho \cdot u_*$ (wo ρ den Tortuositätskoeffizienten des Papiers bedeutet). Die meisten Betrachtungen gehen aus dem Gesätz von Stokes aus. Danach ergibt sich für die elektrophoretische Beweglichkeit bei einem Spannungsgefälle von 1 V/cm der Wert:

$$u = \frac{z \cdot e \cdot \rho}{6\pi\eta r \cdot 300} = k \cdot \frac{z}{r} \qquad (1)$$

wo z die Ladung des Ions und r seinen Halbmesser bedeutet. Die Anwendung der Gleichung stösst auf grössere Hindernisse. Es ist schwierig eindeutig und allgemein den effektiven Halbmesser von mehratomigen Ionen aus ihrer Zusammensetzung zu bestimmen. Die unregelmässige Form nimmt EDWARD[9] in Betracht, indem er die Gleichung (1) korrigiert:

$$u = k \cdot \frac{z}{r_w} \cdot \frac{f_0}{f}$$

wo r_w den van der Waals-Radius und f/f_0 das Reibungsverhältnis für eine andere als Kugelförmige Partikel bedeutet. Für Komplexionen in Lösung ist es kaum möglich diese Grössen zu ermitteln. Die umgekehrte Proportionalität zwischen der äquivalenten Beweglichkeit u/z und der dritten Wurzel aus dem Ionenvolumen von Nucleotiden setzen MARKHAM UND SMITH[10] voraus. GONICK[11] führt den Begriff des „effektiven Kugelvolumens" als exponentieller Funktion der Anzahl empirisch bestimmter

Volumelemente für einzelne Bauteile der organischen Ionen ein, um ihre Beweglichkeiten (in freier Lösung!) berechnen zu können. Die Grössen dieser Volumelemente müssen durch empirische Konstanten korrigiert werden, welche nur für einzelne homologische Serien von Ionen gültig sind. Neuerdings korrigiert ELWORTHY[12] die an Hand von Modellen berechneten echten Ionenhalbmesser auf die Stokes-halbmesser mittels einer empirisch festgestellten Korrektion.

Die Benutzung von eindeutigeren Zahlendaten für Berechnung von Beweglichkeiten könnte der von LORENZ[13] gefundene lineare Zusammenhang zwischen dem effektiven Halbmesser und der Anzahl der Atome des Ions ermöglichen, welcher für organische Ionen mit mehr als 5 Atomen gültig ist:

$$r = a + b \cdot N^*$$

wo N^* die Anzahl der Atome ist. Eine andere Möglichkeit hat HAIS[14] angedeutet, welcher die umgekehrte Proportionalität zwischen der Beweglichkeit und dem Molekulargewicht m bei einer Reihe von Derivaten des Hydroxycumarins festgestellt hat:

$$\frac{u}{z} = k \cdot \frac{1}{m} \qquad (2)$$

Diese Beziehung wurde in einigen Fällen von Komplexverbindungen benutzt[2,4–6].

Um grössere Anzahl von Versuchsdaten zu gewinnen, haben wir die oben angeführten Voraussetzungen bei einer Reihe von grösseren organischen und komplexen Ionen verfolgt, deren physikalische Hydratation gering ist. Wir haben keine befriedigende Korrelation zwischen der äquivalenten Beweglichkeit und der reziproken dritten Wurzel aus dem Ionenvolumen (aus krystallographischen Daten berechnet) gefunden. Eine annehmbare Korrelation gab es zwischen der Beweglichkeit und der reziproken Anzahl der Atome des Ions[13] (die Wasserstoffatome ungeachtet), siehe Tabelle I. Bei der Verifikation der Beziehung von HAIS [14] wurde festgestellt, dass sie in einem breiteren Umfang von Molekulargewichten nicht mehr den linearen Verlauf aufweist. Eine befriedigende lineare Korrelation haben wir zwischen der äquivalenten Beweglichkeit und der umgekehrten Wurzel aus dem Molekulargewicht des Ions gefunden (Tabelle I und Fig. 2).

EXPERIMENTELLES

Materiale

Die Substanzen (ausser die in Tabelle I mit[a] und[b] bezeichneten) wurden als 0.05 M wässerige Lösungen an befeuchtetes Papier aufgetragen (1–2 μl). Die Grundelektrolyte wurden so gewählt, dass im Falle von organischen Säuren und Basen ihre vollständige Ionisation in die betreffende Stufe gesichert wurde. Im Falle von Komplexverbindungen garantierten die Konzentration des Komplexbildners und der pH-Wert der Lösung die Existenz der betreffenden Komplexstufe. Die Ionenstärke von Grundelektrolyten (ausser[a] und[b]) wurde durch Zusatz von KNO_3 auf den Wert $\mu = 0.1$ aufgestellt.

Arbeitsmethodik

Es wurde eine modifizierte Arbeitsweise nach PRUSÍK UND KEIL[15] benutzt mit der Kühlung des Papiers durch eine isolierte Kühlplatte; die Stromzuleitungsbrücken

sind vom Elektrophoregramm durch eine Cellophanmembrane abgetrennt, um die Dochtwirkung zu vermeiden. Die Kühlplatte wurde mittels Wasser vom Ultrathermostaten auf 20° ± 0.1° temperiert. Die Elektrophorese verlief bei einem Spannungsgefälle von 15 V/cm etwa 1 Stunde.

Bei allen Versuchen wurde das Papier Whatman Nr. 1 benutzt, dessen Benetzungsgrad 1.2 war und sich während der Versuche nicht änderte.

Auswertung der Ergebnisse

Die Detektion wurde auf übliche Weise ausgeführt. Die Beweglichkeiten wurden als relative Werte gemessen, wobei das Tetraäthylammonium $(C_2H_5)_4N^+$ als Bezugsion diente[16]; seine Vorteile sind die volle Ionisation im ganzen pH-Bereich, keine Tendenz zur Komplexbildung, minimale Hydratation und keine Adsorption an das Papier. Seine Beweglichkeit wurde gleich $u = +1.00$ gestellt. Die Elektroosmose wurde mittels Antipyrin verfolgt[16], in Lösungen mit pH < 3 mittels Glukose[1]. Für die Auswertung wurde bei symmetrischen Flecken die Mitte, bei schwanzbildenden die Stelle der maximalen Intensität in Betracht genommen.

Die Beweglichkeitswerte[a] (Tabelle I) wurden aus der Literatur[17] durch Umrechnung auf relative Werte gewonnen. Als Grundwert der Umrechnung wurde die versuchsmässig ermittelte relative Beweglichkeit vom 3-Nitrobenzoat genommen.

Die Werte[b] wurden auf dieselbe Weise nach der Abhandlung[18] gewonnen (Vergleichssubstanz Piperidin).

ERGEBNISSE UND DISKUSSION

Von den Beweglichkeitswerten in den Mitteilungen von FRANC UND WURST[17] und BLAU[18] wurden in die Tabelle I nur diejenigen aufgenommen, bei welchen das Migrationsmedium und ihre Ionisationskonstanten eine vollständige Ionisation sicherten. Darüber hinaus wurden die Werte der aromatischen Polynitro- und Polysulfo-verbin-

TABELLE I

DIE ÄQUIVALENTEN ELEKTROPHORETISCHEN BEWEGLICHKEITEN DER IONEN

Ion	*Mol. Gew.*	*N**	*u/z*		
			exptl.	*berechnet nach Gleichung*	
				(3)	*(4)*
3-Nitrophthalat	209.13	15	0.78[a]	0.73	0.68
3-Nitrobenzoat	166.12	12	0.88	0.85	0.78
3,5-Dinitrobenzoat	211.12	15	0.84[a]	0.72	0.68
2-Aminobenzoat	136.13	10	0.93[a]	0.97	0.88
3-Aminobenzoat	136.13	10	0.87[a]	0.97	0.88
4-Aminobenzoat	136.13	10	0.83[a]	0.97	0.88
3-Hydroxy-4-methoxybenzaldehyd	151.14	11	0.89[a]	0.91	0.82
1-Naphthylamin-4-sulfonat	222.24	15	0.70[a]	0.70	0.68
1-Naphthylamin-5-sulfonat	222.24	15	0.72[a]	0.70	0.68
1-Naphthylamin-6-sulfonat	222.24	15	0.67[a]	0.70	0.68
1-Naphthylamin-7-sulfonat	222.24	15	0.67[a]	0.70	0.68
1-Naphthylamin-8-sulfonat	222.24	15	0.72[a]	0.70	0.68
2-Naphthylamin-1-sulfonat	222.24	15	0.73[a]	0.70	0.68

(Fortsetzung S. 455)

TABELLE I (*Fortsetzung*)

Ion	*Mol. Gew.*	*N**	*u/z*		
			exptl.	*berechnet nach Gleichung*	
				(3)	*(4)*
2-Naphthylamin-5-sulfonat	222.24	15	0.70[a]	0.70	0.68
2-Naphthylamin-6-sulfonat	222.24	15	0.70[a]	0.70	0.68
2-Naphthylamin-7-sulfonat	222.24	15	0.70[a]	0.70	0.68
1-Naphthol-4-sulfonat	222.23	15	0.76[a]	0.70	0.68
1-Naphthol-5-sulfonat	222.23	15	0.76[a]	0.70	0.68
2-Naphthol-6-sulfonat	222.23	15	0.74[a]	0.70	0.68
2-Naphthol-8-sulfonat	222.23	15	0.77[a]	0.70	0.68
2-Nitrophenolat	138.11	10	0.95[a]	0.96	0.88
3-Nitrophenolat	138.11	10	0.86[a]	0.96	0.88
4-Nitrophenolat	138.11	10	0.85[a]	0.96	0.88
Benzoat	121.1	9	1.03	1.05	0.94
Salicylat	137.1	10	1.00	0.97	0.88
Phthalat	164.1	12	0.82	0.86	0.78
4-Sulfosalicylat	216.2	14	0.76	0.71	0.70
Tetramethylammonium	74.1	5	1.39	1.42	1.48
Tetraäthylammonium	130.3	9	1.00	1.00	0.94
Trimethyl-benzyl-ammonium	150.2	11	0.96	0.91	0.82
Arginin ($C_6H_{15}O_2N_4^+$)	175.20	12	0,90[b]	0.82	0.78
n-Butylammonium	74.14	5	1.28[b]	1.42	1.48
Diäthylammonium	74.14	5	1.40[b]	1.42	1.48
Dimethylammonium	46.08	3	2.07[b]	1.87	(2.28)
Äthanolamin ($C_2H_8ON^+$)	62,08	4	1.51[b]	1.58	(1.78)
Äthylammonium	46.08	3	2.02[b]	1.87	(2.28)
Piperidin ($C_5H_{12}N^+$)	86.15	6	1.17	1.30	1.28
n-Propylammonium	60.11	4	1.44[b]	1.61	(1.78)
Pyrrolidin ($C_4H_{10}N^+$)	72.12	5	1.67[b]	1.44	1.48
Methylammonium	32.07	2	2.38[b]	2.31	(3.28)
$[Cu(C_2O_4)_2]^{2-}$	239.6	13	0.71	0.66	0.74
$[Cu(Malon)_2]^{2-}$	267.6	15	0.56	0.61	0.68
$[Cu(Sulfosal)_2]^{4-}$	495.9	29	0.36	0.37	0.48
$[Co(OH_2)_6]^{2+}$	167.0	7	0.80	0.85	1.13
$[Ni(en)_3]^{2+}$	239.0	13	0.68	0.66	0.74
CoX^-	247.04	14	0.57	0.64	0.70
AlY^-	315.1	21	0.60	0.54	0.56
CoY^{2-}	347.04	21	0.49	0.50	0.56
Al-*meso*-DBTA$^-$	343.3	23	0.60	0.50	0.54
Co-*meso*-DBTA^{2-}	375.24	23	0.45	0.47	0.54
Co-DPTA^{2-}	377.24	23	0.47	0.47	0.54
Al-Chenta$^-$	369.3	25	0.60	0.48	0.52
Co-Chenta^{2-}	401.24	25	0.43	0.44	0.52
Al-DL-DBTA$^-$	343.3	23	0.60	0.50	0.54
Co-DL-DBTA^{2-}	375.24	23	0.43	0.47	0.54
$[Co(Glycin)_3]^-$	281.1	16	0.54	0.58	0.65
$[Co(Alanin)_3]^-$	323.1	19	0.47	0.53	0.59
$[Co(Leucin)_3]^-$	449.3	28	0.38	0.40	0.49
$[Co(Methionin)_3]^-$	503.6	28	0.34	0.37	0.49
$[Cu(Glutamat)_2]^{2-}$	353.7	31	0.46	0.49	0.47
Variationskoeffizient				± 8.6%	± 13.5%

[a] Werte nach Zit.17; [b] Werte nach Zit. 18.

en = Äthylendiamin, H_4Y = Äthylendiamino-tetraessigsäure, H_3X = Nitrilotriessigsäure, *meso*-DBTA = *meso*-2,3-Diaminobutan-tetraessigsäure, DL-DBTA = DL-2,3-Diaminobutan-tetraessigsäure, DPTA = 2-Hydroxy-1,3-diaminopropan-tetraessigsäure, Chenta = 1,2-Diaminocyclohexan-tetraessigsäure.

dungen ausgelassen; bei diesen führt die erhöhte Hydratation durch Wasserstoffbindungen nach GONICK[11] zur Verminderung des effektiven Halbmessers und zu anomalen Werten von Leitvermögen (und damit auch elektrophoretischer Beweglichkeit).

Die numerischen Data der Tabelle I wurden mit der Methode der kleinsten Quadrate verarbeitet und führten zu empirischen Gleichungen:

$$\frac{u}{z} = 14.7 \frac{1}{\sqrt{m}} - 0.29 \tag{3}$$

berechnet auf Grund der Molekulargewichte m, oder:

$$\frac{u}{z} = 6.00 \cdot \frac{1}{N^*} + 0.28 \tag{4}$$

berechnet auf Grund der Anzahl der Atome N^* ausgenommen die Wasserstoffatome.

Bei Berechnung von Konstanten der Gleichung (4) wurden Werte der Ionen mit weniger als 5 Atomen unterlassen, aber auch so weisen die berechneten Beweglichkeiten einen ziemlich grossen Variationskoeffizienten.

Wesentlich bessere Resultate gewinnt man nach der Gleichung (3), siehe Fig. 2. Die Anwendung der Korrelation zwischen der Beweglichkeit und der umgekehrten Wurzel aus dem Molekulargewicht scheint gerechtfertigt zu sein, wenn man die

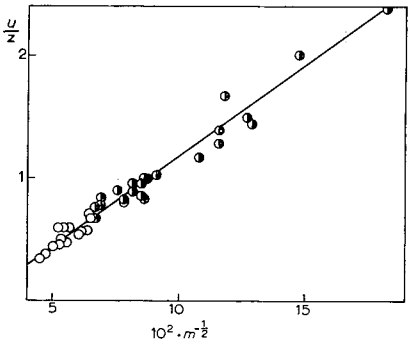

Fig. 2. Die Abhängigkeit der äquivalenten elektrophoretischen Beweglichkeit u/z von der umgekehrten Wurzel aus dem Molekulargewicht m. ◑ Werte der organischen Ionen; ○ Werte der komplexen Ionen; ——— die Gerade nach der Gleichung (3).

Elektromigration (mit Hinsicht auf das Verhältnis von Grössenordnungen der Ionen und der Assoziate von Wassermolekeln) als eine Bewegung im diskontinuierlichen Medium ansieht. Dann handelt es sich bei einzelnen Teilchen um gleichmässig beschleunigte Bewegung, deren Geschwindigkeit zwischen einzelnen Zusammenstössen unter Zurücklegung der Strecke s von 0 auf v wächst, und die durch konstante elektrische Anziehungskraft P bewirkt ist; ihre Beschleunigung ist gleich $a = v^2/2s$. Für die durchschnittliche Geschwindigkeit $\bar{v}$ (die der Beweglichkeit proportional ist) gilt dann:

$$\bar{v} = \frac{v}{2} = \sqrt{\frac{P \cdot s}{2}} \cdot \frac{1}{\sqrt{m}} \tag{5}$$

Die Strecke s kann man in erster Annäherung ebenfalls als konstant betrachten.

Diese Erklärung ist auch von anderer Seite her unterstützt. Der Diffusionskoeffizient ist gleich:

$$D = \frac{RT}{N \cdot 6\pi\,\eta r}$$

Nach Einsetzen in Gleichung (1) bekommt man (vergl. Zit. 19):

$$\frac{u}{z} = \frac{e \cdot \rho \cdot N}{300 \cdot R.T.} \cdot D = k' \cdot D \qquad (6)$$

Der Diffusionskoeffizient ist unter Voraussetzung von minimaler Hydratation der Wurzel vom Molekulargewicht umgekehrt proportional[20], so dass man zu derselben Gleichung kommt:

$$\frac{u}{z} = k \cdot \frac{1}{\sqrt{m}}$$

Die Bestimmung der Ionengewichte nach der Dialysenmethode[21] hat analogische Züge: umgekehrte Proportionalität der dialytischen Koeffizienten und der Wurzeln aus den Ionengewichten.

Die empirischen Konstanten der Korrelationsgleichungen (3) und (4) gelten unter benutzten Bedingungen und bei der Ionenstärke des Grundelektrolytes von $\mu = 0.1$. In mehreren Arbeiten haben wir beim Studium von Komplexen die Gleichung (3) benutzt. Die Schätzung des Molekulargewichtes ist zwar nur annähernd mit einer ziemlich grossen Streuung, sie gestattet aber in meisten Fällen unter einigen gegebenen Möglichkeiten eindeutige Entscheidung. Grosse Abweichungen kommen vor, wenn die elektrophoretische Beweglichkeit in der freien Lösung nicht proportional ist, vor allem im Falle einer stärkeren Adsorption an das Papier; dann müssen die Ergebnisse mit grösster Vorsicht beurteilt werden. Es ist weiter begreiflich, dass die grobe Korrelation den Einfluss der Struktur (Isomerie) auf die Beweglichkeiten nicht erfassen kann. Bei Komplexen mit zwar gleicher Bauart, aber verschieden schweren Zentralatomen, tritt der Unterschied der Molekulargewichte nicht deutlich auf, die Beweglichkeiten können fast die gleichen sein. Auch bei diesen Vorbehalten ist die empirische Gleichung (3) von praktischer Nützlichkeit und hat sich in unseren Arbeiten beim Studium von Komplexen bewährt. Sie ermöglicht aus der gemessenen Beweglichkeit an die Zusammensetzung des komplexen Ions zu schliessen; bei bekannter Zusammensetzung kann man die Oxydationsstufe des Zentralatoms beurteilen. Der besondere Vorteil der elektrophoretischen Methode ist ihr Separationsvermögen und die einfache Versuchsanordnung.

ZUSAMMENFASSUNG

Es wurde die Abhängigkeit der relativen elektrophoretischen Beweglichkeit von einigen Parametern der organischen und komplexen Ionen untersucht. Die Korrelation zwischen der äquivalenten Beweglichkeit und der Wurzel aus dem Molekulargewicht der Ionen erwies sich als verhältnismässig befriedigend mit einem Streuungskoeffizienten von $\pm$ 8.6%. Sie kann bei der Interpretation von elektrophoretischen Beweglichkeitskurven zu Voraussetzungen über die Zusammensetzung von Kom-

plexionen, oder (bei bekannter Zusammensetzung) zur Bestimmung der Oxydationsstufe des Zentralatoms benutzt werden.

SUMMARY

The dependence of the relative electrophoretic mobility of organic and complex ions on some parameters was studied. The correlation between the equivalent mobility and the square root of the molecular weight of the ions was found to be valid with an accuracy of $\pm$ 8.6%. This correlation can be used in the interpretation of electrophoretic mobility curves for estimating the composition of complex ions or (if the composition is known) for determining the oxidation state of the central atom.

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IDENTIFIZIERUNG ORGANISCHER VERBINDUNGEN*

LV. PAPIERCHROMATOGRAPHISCHE TRENNUNG UND IDENTIFIZIERUNG VON NITRO- UND NITROSOPHENOLEN

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Im Zusammenhang mit dem ausführlichen Studium der Farbreaktion der Phenole mit salpetriger Säure[1] waren wir vor die Aufgabe gestellt, verschiedenste Nitro- und Nitrosophenole in komplizierten Gemischen zu identifizieren. Wir versuchten das Problem durch Anwendung der bisher in der Literatur beschriebenen papierchromatographischen Methoden zu lösen, fanden jedoch, dass das Verhalten dieser Klasse von Verbindungen komplizierter ist, als es auf den ersten Blick zu sein scheint und dass es grössere Aufmerksamkeit erfordern wird.

In den bisherigen Arbeiten[2-22] wurde das papierchromatographische Verhalten nur einiger einfacher Nitrophenole beschrieben, grösstenteils im Zusammenhang mit Verbindungen von anderem Charakter. Es wurde meist auf unvorbehandeltem Papier unter Anwendung von sauren (Benzol–Essigsäure–Wasser, Petroläther–Benzol–Ameisensäure, Butanol–Essigsäure–Wasser, usw.) oder basischen Lösungsmittelsystemen (Butanol–Ammoniak, Butanol–Ammoniumcarbonat–Ammoniak, Äthanol–Ammoniak–Wasser, usw.) oder Salzlösungen chromatographiert, oder man hat von umgekehrten Phasen Gebrauch gemacht, indem man das Lösungsmittelsystem Petroleum/Äthanol–Wasser, Diäthylanilin/Wasser, acetyliertes Papier/Butanol–Methanol–Ameisensäure oder Olivenöl/Pufferlösungen angewendet hat; weiter wurde auch auf mit Formamid imprägnierten Papieren chromatographiert.

In dieser Mitteilung sollen die Resultate und Erfahrungen zusammengefasst werden, die wir bei der Reproduktion der bisherigen Methoden und beim Ausarbeiten unserer neuen Methode, bei der das Lösungsmittelsystem 1-Bromnaphthalin/80 % Essigsäure angewendet wird, sammeln konnten.

EXPERIMENTELLER TEIL

Die Nitrophenole wurden auf die Chromatogramme in Form ihrer benzolischer oder alkoholischer Lösungen aufgetragen. Es wurden Mengen bis 50 μg chromatographiert, bei Anwendung des Lösungsmittelsystems 1-Bromnaphthalin/80 % Essigsäure können verlässlich auch viel grössere Mengen aufgetragen werden, ohne dass es auf die Qualität der Chromatogramme oder die R_F-Werte einen Einfluss hat.

Bei Anwendung der mit 1-Bromnaphthalin imprägnierten Papiere wurde das

* LIV. Mitteilung: *J. Chromatog.*, 12 (1963) 385.

TABELLE I

R_F-WERTE DER NITRO- UND NITROSOPHENOLE IM LÖSUNGSMITTELSYSTEM 1-BROMNAPHTHALIN/80% ESSIGSÄURE

Verbindung	*Förbung*	R_F-*Wert*	R_M-*Wert*
2-Nitrophenol	ockergelb	0.49	+0.017
2-Nitro-6-methylphenol	ockergelb	0.31	+0.347
2-Nitro-5-methylphenol	ockergelb	0.36	+0.250
2-Nitro-4-methylphenol	ockergelb	0.34	+0.288
2-Nitro-4,6-dimethylphenol	orange	0.22	+0.545
2-Nitro-4-*tert.*-butylphenol	ockergelb	0.26	+0.454
2-Nitro-4-methyl-6-*tert.*-butylphenol	gelb	0.11	+0.908
2-Nitro-4-*tert.*-butyl-6-methylphenol	orange	0.14	+0.788
2-Nitro-4-chlorphenol	ockergelb	0.34	+0.288
2-Nitro-4-bromphenol	ockergelb	0.27	+0.432
2-Nitro-4-jodphenol	ockergelb	0.20	+0.602
2-Nitro-4,6-dichlorphenol	orangegelb	0.18	+0.659
2-Nitro-4-chlor-6-bromphenol	orangegelb	0.14	+0.788
2-Nitro-4,6-dibromphenol	orange	0.09	+1.005
2-Nitro-4,6-dijodphenol	rotbraun	0.04	+1.380
2-Nitro-4-chlor-5-methylphenol	ockergelb	0.24	+0.501
2-Nitro-4-brom-6-methylphenol	orange	0.13	+0.825
2-Nitro-4-methyl-6-bromphenol	orange	0.16	+0.720
3-Nitrophenol	ockergelb	0.77	
4-Nitrophenol	gelb	0.76	
4-Nitro-3-methylphenol	gelb	0.76	
4-Nitro-2,5-dimethylphenol	gelb	0.76	
4-Nitro-2-chlorphenol	gelb	0.73	
4-Nitro-2,6-dichlorphenol	gelb	0.60	
4-Nitro-2-chlor-6-bromphenol	gelb	0.53	
4-Nitro-2,6-dibromphenol	gelb	0.45	
4-Nitro-2,6-dijodphenol	gelb	0.21	
2,4-Dinitrophenol	gelb	0.39	+0.194
2,4-Dinitro-6-methylphenol	gelb	0.20	+0.602
2,4-Dinitro-5-methylphenol	gelb	0.28	+0.410
2,4-Dinitro-6-*tert.*-butylphenol	gelb	0.10	+0.954
2,4-Dinitro-6-chlorphenol	gelb	0.22	+0.545
2,4-Dinitro-6-bromphenol	gelb	0.16	+0.720
2,4-Dinitro-6-jodphenol	gelb	0.09	+1.005
2,5-Dinitrophenol	orange	0.39	+0.194
2,6-Dinitrophenol	ockergelb	0.48	+0.035
2,6-Dinitro-4-methylphenol	orange	0.32	+0.327
2,6-Dinitro-3,4-dimethylphenol	orangegelb	0.22	+0.545
2,6-Dinitro-4-*tert.*-butylphenol	orange	0.25	+0.477
2,6-Dinitro-4-chlorphenol	orange	0.27	+0.432
2,6-Dinitro-4-bromphenol	rotbraun	0.22	+0.545
2,6-Dinitro-4-jodphenol	rotbraun	0.15	+0.753
2,6-Dinitro-3-methyl-4-chlorphenol	gelborange	0.19	+0.630
2,4,6-Trinitrophenol	ockergelb	0.55	−0.087
4-Nitrosophenol	grüngelb	0.76	
4-Nitroso-3-methylphenol	grüngelb	0.76	
4-Nitroso-2,5-dimethylphenol	gelb	0.76	
2,4-Dinitroresorzin	gelb	0.73	
2,4,6-Trinitroresorzin	gelb	0.75	

Whatmanpapier Nr. 3 durch eine 10 % 1-Bromnaphthalinlösung in Chloroform durchgezogen und 15 Min. an der Luft im Digestorium hängen gelassen. Nach dem Aufbringen der chromatographierten Verbindungen wurden die Chromatogramme mit 80 % Essigsäure absteigend entwickelt. Diese mobile Phase musste stets mit 1-Bromnaphtalin sorgfältig gesättigt werden, um das Auswaschen der stationären Phase während der Chromatographie zu vermeiden. Nachdem die Lösungsmittelfront die nötige Entfernung vom Start (30–35 cm) erreicht hat, wurden die Chromatogramme der Kammer entnommen und im Digestorium bei Raumtemperatur getrocknet.

Bei Verwendung der mit Formamid vorbehandelten Papiere wurde das Whatmanpapier Nr. 3 durch eine 20 % alkoholische Formamidlösung durchgezogen und auf der Luft zum Abdunsten des Alkohols hängen gelassen. Nach dem Auftragen der Probelösungen wurden die Chromatogramme absteigend entwickelt. Als bewegliche Phase wurde Hexan, Benzol oder Chloroform, oder Gemische dieser Lösungsmittel mit Essigsäure im Verhältniss 95:5 angewendet. Das Trocknen der Chromatogramme geschah wieder im Digestorium bei Zimmertemperatur.

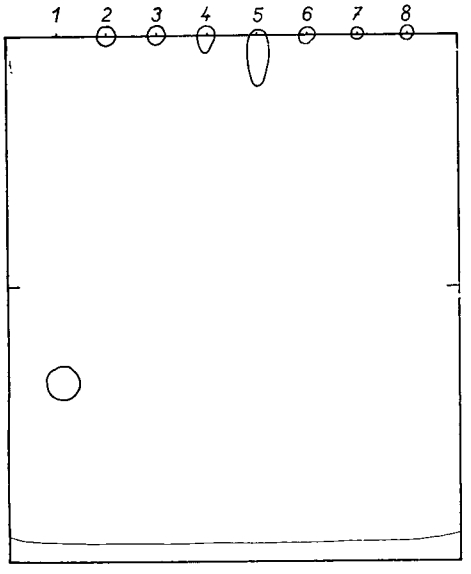
Die Sichtbarmachung der Flecken geschah durch Besprühen mit einer 1 % wässrig-alkoholischen (1:1) Kalilaugelösung, wobei die Nitro- sowie Nitrosoverbindungen als zitronengelbe bis rotorangefarbige Flecken erschienen. Ihre Fleckenfarbe, sowie die R_F-Werte im System 1-Bromnapthalin/80 % Essigsäure sind in der Tabelle I angeführt, die Trennmöglichkeiten auf mit Formamid imprägnierten Papieren sind den Fig. 1(a–f) zu entnehmen. Ein spezifischer Nachweis der Nitrosoverbindungen wurde durch Besprühen mit einer 0.5–1 % wässrigen Lösung von Natriumpentacyanoaminoferrat(II) durchgeführt[23]. Die Nitrosophenole erschienen als blaue Flecken.

RESULTATE UND DISKUSSION

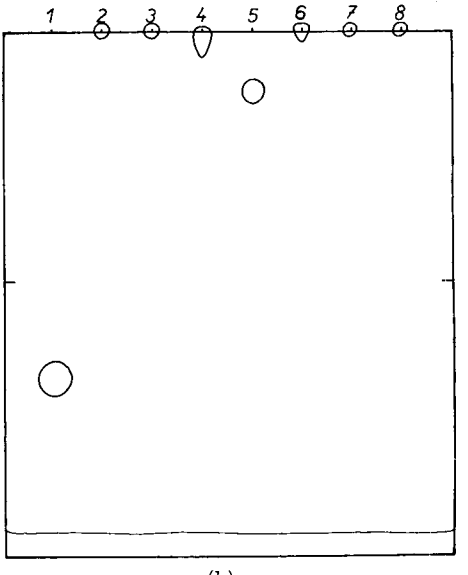
Wahl der Lösungsmittelsysteme

Wenn wir die Löslichkeitsverhältnisse der Nitrophenole in Betracht nehmen[24] ist es verständlich, dass auf unvorbehandelten Papieren unter Anwendung saurer Lösungsmittelsysteme die Nitrophenole sehr hohe R_F-Werte aufweisen werden, da sie in Wasser wenig löslich sind. Wird auf unvorbehandelten Papieren ammoniakalisches Medium angewendet, so liegen die Nitrophenole in Form ihrer intensiv gefärbten Nitrophenolate vor, deren Wasserlöslichkeit schon grösser ist; auch hier liegt jedoch bei den einwertigen Phenolen die Verteilung noch zu Gunsten der organischen Phase und die Nitrophenole weisen auch hier höhere R_F-Werte auf; diese Lösungsmittelsysteme, z.B. *n*-Propanol–Ammoniak (2:1) können zur Auftrennung der Nitroderivate der zweiwertigen Phenole Anwendung finden, wie aus dem Beispiel der Nitroderivate des Resorzins ersichtlich ist (Whatmanpapier Nr. 3, T = 20°): 2,4-Dinitroresorzin R_F = 0.26; 2,4,6-Trinitroresorzin R_F = 0.38.

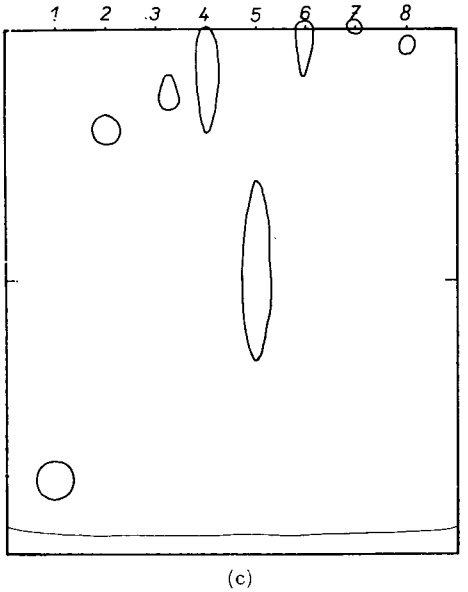
Die Lösungsmittelsysteme mit wenig polaren stationären Phasen weisen bessere Trennfähigkeit einzelner Phenole auf, es hat sich jedoch gezeigt, dass das Verhalten der Nitrophenole von dem pH-Wert der mobilen Phase abhängig ist. Wie schon erwähnt wurde, sind die Nitrophenole Verbindungen von saurem Charakter, die im bestimmten pH-Bereich, das für die einzelnen Typen der Nitrophenole charakteristisch ist, in Nitrophenolate übergeführt werden. Mann kan also durch eine richtige Wahl des geeignetsten pH-Wertes der mobilen Phase das erzielen, dass die stärker sauren Nitrophenole als tieffarbige Anionen wandern, die schwach sauren Nitro-



(a)



(b)



(c)

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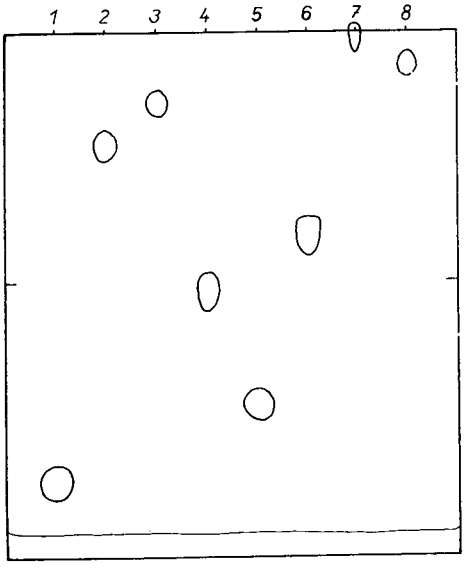
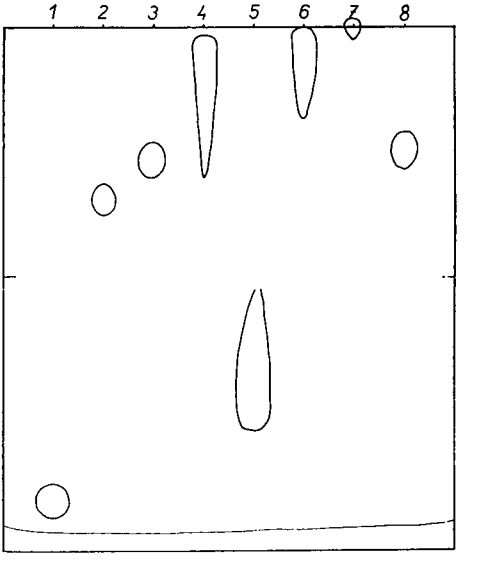
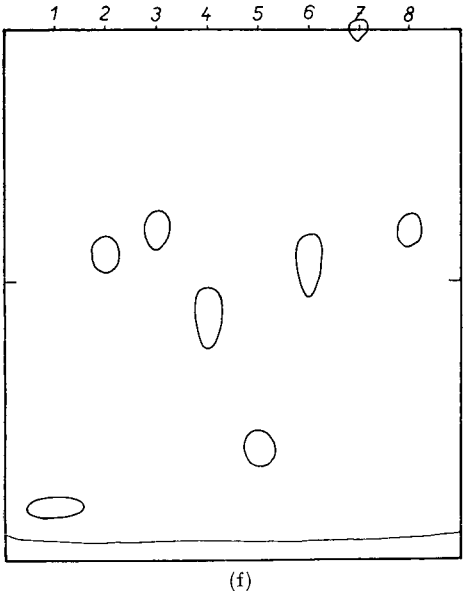


(d)



(e)



(f)

Fig. 1. Schema der Chromatogramme der Nitrophenole in Lösungsmittelsystemen: (a) Formamid/Hexan; (b) Formamid/Hexan–Essigsäure (95:5); (c) Formamid/Benzol; (d) Formamid/Benzol–Essigsäure (95:5); (e) Formamid/Chloroform; (f) Formamid/Chloroform–Essigsäure (95:5). (1) 2-Nitrophenol; (2) 3-Nitrophenol; (3) 4-Nitrophenol; (4) 2,4-Dinitrophenol; (5) 2,5-Dinitrophenol; (6) 2.6-Dinitrophenol; (7) 2,4,6-Trinitrophenol; (8) 4-Nitrosophenol.

phenole dagegen in der undissoziierten Form vorliegen, was zu gewünschten Trennungen führen kann. In anderen Fällen kann aber die Dissoziation während der Chromatographie eintreten, was eine Streifenbildung zur Folge hat. Es ist also auch möglich die möglichst höchste Säurekonzentration in der mobilen Phase zu wählen, damit alle Nitrophenole in der undissoziierten Form vorliegen. Unter diesen Bedingungen ist es uns gelungen im Lösungsmittelsystem 1-Bromnaphthalin/80 % Essigsäure ein sehr geeignetes System zur Auftrennung aller von uns chromatographierten einwertigen Mono- bis Trinitrophenole zu finden. Alle Verbindungen bildeten sehr schöne runde Flecken und die Chromatogramme waren sehr gut reproduzierbar. Die einzigen Nachteile dieser Methode waren, dass sich das sehr flüchtige *o*-Nitrophenol und *o*-Chlor-*o'*-nitrophenol beim Trocknen der Chromatogramme grösstenteils verflüchtigte und dass die *p*-Nitrophenole von den entsprechenden *p*-Nitrosophenolen nicht getrennt wurden.

Sehr interessant ist das Verhalten der Nitrophenole auf den mit Formamid imprägnierten Papieren. Die Basizität des Formamids verursacht nämlich die Dissoziation einiger Nitrophenole, sodass diese gleich nach dem Auftragen auf das Chromatogramm als gefärbte Flecken erscheinen, und während der Chromatographie dann Streifen bilden. Fügt man aber zu der mobilen Phase Essigsäure zu, so wird die Dissoziation aufgehoben und alle Nitrophenole erscheinen dann nach dem Chromatographieren als runde Flecken (Fig. 1(a–f)). Die Trennfähigkeit dieser Lösungsmittelsysteme ist sehr gross, und da auch die Durchflussgeschwindigkeit sehr gross ist, bringt diese Methode gewisse Vorteile mit sich. Wir konnten zum Beispiel durch Anwendung des Systems Formamid/Hexan sehr schnell und bequem die Nitrierung des *p*-Kresols zu dem Mono- und Dinitroderivat verfolgen. Diese zwei Verbindungen werden nämlich sehr gut aufgetrennt: das *o*-Nitroderivat wandert in der undissoziierten Form fast mit der Lösungsmittelfront mit, das *o,o'*-Dinitroderivat bleibt in der farbigen, dissoziierten Form am Start zurück. Ein weiterer Vorteil der Lösungsmittelsysteme mit Formamid als stationäre Phase ist, dass durch Anwendung des Systems Formamid/Benzol das *p*-Nitro- und *p*-Nitrosophenol getrennt werden können.

Chromatographisches Verhalten und Konstitution

Die Beziehungen zwischen dem chromatographischen Verhalten und der Struktur der Nitrophenole sind zwar interessant, aber nicht einfach. Der Einfluss der Anzahl der Hydroxylgruppen wurde schon im Kapitel über die Wahl der Lösungsmittelsysteme erläutert. In Weiterem werden die Beziehungen im Lösungsmittelsystem 1-Bromnaphthalin/80 % Essigsäure diskutiert. Hier kommt die Einführung der zweiten Hydroxylgruppe durch Vergrösserung der R_F-Werte zum Ausdruck. Bei den einwertigen Phenolen hat den grössten Einfluss auf das chromatographische Verhalten die Stellung und Anzahl der Nitrogruppen. Die *o*-Nitrophenole haben stets kleinere R_F-Werte als die übrigen Isomere, da zwischen der Nitrogruppe in der *o*-Stellung und der Hydroxylgruppe eine Wasserstoffbrückenbindung gebildet wird, die die Löslichkeit des *o*-Nitrophenols zu Gunsten der weniger polaren stationären Phase verschiebt. Die *m*- und *p*-Nitrophenole weisen demzufolge höhere R_F-Werte auf und der Beitrag der Nitrogruppe in der *m*- und *p*-Stellung ist fast der gleiche, was übrigens der Fall auch bei anderen aromatischen Verbindungen immer ist. In der Reihe der Dinitrophenole weisen die *o,o'*-Dinitrophenole nur etwas geringere R_F-Werte auf als die *o*-Mononitrophenole, dagegen haben die *o,p*-Dinitrophenole sehr kleine R_F-Werte,

da die Einführung der zweiten Nitrogruppe in das Molekül des *o*-Nitrophenols durch eine wesentliche Verkleinerung des R_F-Wertes zum Ausdruck kam, sodass die *o,p*-Dinitrophenole die niedrigsten R_F-Werte von allen Nitrophenolen haben. Dasselbe gilt auch für die *o,m*-Dinitrophenole. Bei der Pikrinsäure verursacht dann die Einführung der dritten Nitrogruppe eine Vergrösserung des R_F-Wertes gegenüber den Dinitrophenolen. Die Alkylgruppen verursachen eine Verminderung der R_F-Werte, die grösser ist, wenn die Alkylgruppe sich in der *o*-Stellung befindet, und ähnliches Verhalten zeigen auch die Halogene. Die R_F-Werte werden in der Reihe Cl > Br > I verkleinert. Wir versuchten weiter in der Reihe der *o*-Nitrophenole die Beiträge einzelner Substituenten zu den R_F-Werten[25] auszurechnen (siehe Tabelle II) und aus

TABELLE II

DIE ERRECHNETEN ΔR_M-WERTE

Substituent	R_M-*Wert*
o-CH_3	+0.33
m-CH_3	+0.23
p-CH_3	+0.27
p-tert.-C_4H_9	+0.44
p-Cl	+0.27
p-Br	+0.42
p-J	+0.59
o-Cl	+0.37
o-Br	+0.62

diesen ΔR_M-Werten errechneten wir dann wieder die R_F-Werte der Nitrophenole mit mehreren Substituenten. In der Tabelle III sind die errechneten und die gefundenen R_F-Werte verglichen. Aus diesen Ergebnissen ist ersichtlich, dass mittels der ausgerechneten ΔR_M-Werte nicht nur die R_F-Werte einer Reihe von *o*-Nitrophenolen mit mehreren Substituenten errechnet werden können, dass aber diese auch für die Reihe der 2,6- und 2,4-Dinitrophenole anwendbar sind. Die einzige Ausnahme bilden

TABELLE III

DIE ERRECHNETEN UND GEFUNDENEN R_F-WERTE EINIGER NITROPHENOLE

Verbindung	R_F-*Wert*	
	Errechnet	*Gefunden*
2-Nitro-4,6-dimethylphenol	0.20	0.22
2-Nitro-4-*tert.*-butyl-6-methylphenol	0.14	0.14
2-Nitro-4-chlor-5-methylphenol	0.23	0.24
2-Nitro-4-brom-6-methylphenol	0.15	0.13
2-Nitro-4-methyl-6-bromphenol	0.14	0.16
2,4-Dinitro-6-methylphenol	0.23	0.20
2,4-Dinitro-5-methylphenol	0.27	0.28
2,4-Dinitro-6-chlorphenol	0.21	0.22
2,4-Dinitro-6-bromphenol	0.17	0.16
2,6-Dinitro-4-methylphenol	0.33	0.32
2,6-Dinitro-3,4-dimethylphenol	0.22	0.22
2,6-Dinitro-4-*tert.*-butylphenol	0.25	0.25
2,6-Dinitro-4-chlorphenol	0.33	0.27!
2,6-Dinitro-4-bromphenol	0.26	0.22!
2,6-Dinitro-4-jodphenol	0.19	0.15!
2,6-Dinitro-3-methyl-4-chlorphenol	0.22	0.19!

die halogensubstituierte 2,6-Dinitrophenole, bei denen die ausgerechneten Werte stets höher sind als die gefundenen. Wenn man aber den ΔR_M-Wert für *p*-Cl aus den R_M-Werten des 2,6-Dinitrophenols und des 2,6-Dinitro-4-chlorphenols ausrechnet und mit diesem dann den R_F-Wert von 2,6-Dinitro-3-methyl-4-chlorphenol bestimmt (0.18), stimmt dieser gut mit dem tatsächlich gefundenem (0.19) überein. Wahrscheinlich kommt hier eine neue Interaktion zwischen den *p*-Halogenen und den 2,6-Dinitrogruppen zum Ausdruck.

ZUSAMMENFASSUNG

Es wurde das chromatographische Verhalten der Nitro- und Nitrosophenole in verschiedenen Lösungsmittelsystemen studiert. Die besten Resultate wurden unter Anwendung des Systems 1-Bromnaphthalin/80 % Essigsäure und auf den mit Formamid imprägnierten Papieren mit Hexan, Benzol, Chloroform oder Gemischen dieser Lösungsmittel mit Essigsäure als mobile Phase erzielt. Für Nitroderivate der zweiwertigen Phenole wird *n*-Propanol–Ammoniak (2:1) empfohlen. Die Beziehungen zwischen dem chromatographischen Verhalten und der Struktur der Nitrophenole werden diskutiert.

SUMMARY

The chromatographic behaviour of nitro- and nitrosophenols in various solvent systems was studied. The best results were obtained in the solvent system 1-bromonaphthalene/80 % acetic acid and on papers impregnated with formamide, hexane, benzene, chloroform or their mixtures with acetic acid being the mobile phase. For nitro derivatives of dihydroxybenzenes the solvent system *n*-propanol–ammonia (2:1) is most suitable. The relations between chromatographic behaviour and structure of nitrophenols is discussed.

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CENTRIFUGAL PAPER CHROMATOGRAPHY AND SOME OF ITS APPLICATIONS

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Separations by paper chromatography frequently require many hours, yet this technique is very useful for the separation of many organic mixtures. Often a suitable developing system is not known, and appreciable time may be required in the evaluation of various solvents. Thus paper chromatography suffers from a time disadvantage when compared to thin layer and vapor phase chromatography.

To overcome these disadvantages centrifugal force[1] has been employed as a means of obtaining within minutes instead of hours paper chromatographic separation of mixtures. The advantages and disadvantages have been reported for several centrifugal paper chromatographic units[2–10].

The major limitation of commercial units is poor performance for separations requiring volatile solvents despite the use of a pre-equilibrated chamber. Other mechanical limitations are irregularities of flow of solvent from the feed systems and paper disintegration.

EXPERIMENTAL

Unit

A centrifugal unit (Fig. 1) was designed and built at the Pearl River Laboratories to eliminate these defects. The unit has no development chamber; instead, the paper is pressed between two metal plates. A small hole in the center of the upper plate allows the solvent to be fed to the paper. Four screws are used to attach the upper plate securely to the face plate.

The feed nozzle, which is positioned manually, supplies a continuous stream of solvent (0.8–1.5 ml/min). The nozzle, which is constructed of stainless steel, has an inner solvent chamber divided by a sintered stainless steel filter of 10 mμ porosity. A thin stream of liquid is delivered as a result of the solvent being forced through the orifice (0.008 in. diameter) by an air pressure mechanism. Both the filter and orifice disc are fitted securely using teflon gaskets to ensure tight seals and inertness to solvents.

Operational procedure

The sample is centrally spotted on a circular chromatographic paper, and the chromatogram developed with 4 ml of solvent using a pressure of 4 p.s.i. The air pressure is not turned on until a constant speed of 900 r.p.m. is obtained. The rotating head is stopped when all of the solvent has been delivered. The paper is hung in a ventilated hood to dry, and the zones of the chromatogram are then located with the appropriate detection reagents.

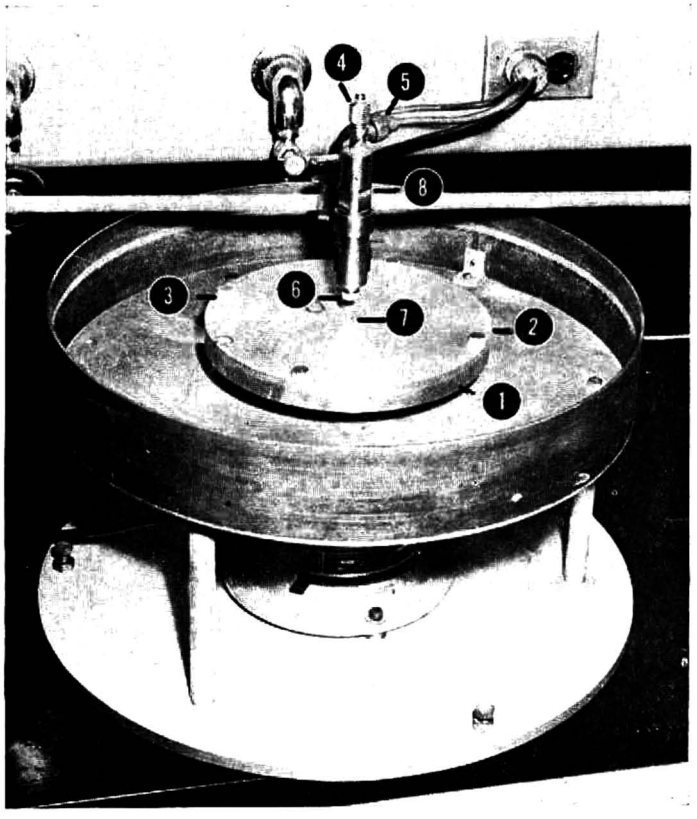

Fig. 1. 1 = Lower plate. 2 = Upper face plate. 3 = Peripheral bolt (4). 4 = Removable cap. 5 = Air line. 6 = Drilled disc. 7 = Solvent access hole. 8 = Solvent feed assembly.

Paper

The paper used was Schleicher & Schüll 470. Circles of 19 cm diameter were cut from sheets. Treated as well as untreated papers were used in this investigation.

Treatment consisted of dipping the circular paper in an appropriate coating solution and then drying. Aqueous buffers used were 5.0 % oxalic acid having a pH of 1.0, and 0.1 *M* sodium dihydrogen phosphate adjusted to a pH of 2.0 with phosphoric acid. For the Zaffaroni type development the paper was treated with a 5 % solution of acetamide in acetone; for inverse-phase separations the paper was impregnated with 5 % Dow Corning Silicone Fluid No. 550 in chloroform.

Data

Seven organic mixtures were selected to evaluate the unit as a means of obtaining rapid paper chromatographic separation using relatively volatile and very volatile solvent systems. Conventional paper chromatographic procedures has previously been developed for each mixture. A comparison of R_F values determined by conventional and centrifugal chromatography was thought to be a suitable method of evaluating this unit.

In most cases (Table I) the R_F values determined by centrifugal chromatography

TABLE I

COMPARISON OF R_F VALUES BY CENTRIFUGAL AND STANDARD CHROMATOGRAPHY

Compounds	*Solvent system*			
	R_F *Cent.**	R_F *St.*	R_F *Cent.**	R_F *St.*
	Methyl ethyl ketone–0.1 N NH_4OH			
1. Steroids				
Triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione)	0.93	0.83		
Triamcinolone 21-monohemisuccinate	0.32	0.17		
Triamcinolone 16,21-dihemisuccinate	0.13	0.02		
Development time	180 sec	4 h		
Detection: Blue tetrazolium[11], plain paper.				
	n-Butanol–ethanol–water (4:1:1)		*n-Butanol–acetic acid–pyridine–water (4:1:1:2)*	
2. Urea and related compounds				
Urea	0.53	0.30	0.60	0.43
Guanylurea hydrochloride	0.42	0.17	0.51	0.53
Cyanamide	0.93	0.69	0.95	0.70
Dicyandiamide	0.67	0.43	0.73	0.52
Development time	240 sec	16.5 h	240 sec	16.5 h
Detection: Nitroprusside[12], plain paper.				
	Acetone–acetonitrile–water (14:1:20)			
3. Nematocide				
O,O-Diethyl O-2-pyrazinyl phosphorothioate	0.63	0.33		
Sodium pyrazinol	with solvent front	0.82		
Development time	200 sec	4 h		
Detection: Iodine vapor; inverse phase, paper treated with silicone; chromatograms somewhat irregular.				
	Aq. ammonia–ethanol–n-propanol (3:6:1)		*Acetone–benzene–formic acid–water (10:10:2:6) (upper layer used)*	
4. Organic acids				
Malic acid	0.51	0.34	0.23	0.11
Maleic acid	0.51	0.37	too diffuse	0.41
Fumaric acid	0.69	0.44	0.85	0.75
Development time	240 sec	15.5 h	140 sec	4.5 h
Detection: 0.2% ethanol solution of bromophenol blue containing 3% $HgCl_2$, plain paper.				
	Benzene–hexane–methanol (10:10:1)			
5. Pesticide				
Dimethoate (O,O-dimethyl-S-methyl-carbamoylmethyl phosphorodithioate)	0.40	0.35		
O,O,S-Trimethyl phosphorodithioate	nothing	solvent front		
Development time	200 sec	4 h		
Detection: Iodine vapor and 2,6-dibromo-N-chloro-*p*-benzoquinone imine[13]; paper treated with acetamide; chromatograms somewhat irregular.				

(continued on p. 470)

TABLE I (*continued*)

Compounds	Solvent system			
	R_F Cent.*	R_F St.	R_F Cent.*	R_F St.
	Methyl ethyl ketone satd. with water			
6. *Tetracyclines*				
a. Anhydro-tetracycline	0.20	0.41		
Tetracycline	0.00	0.25		
Development time	210 sec	15 h		
Detection: Ultraviolet radiation and ammonia vapor; paper treated with oxalic acid.				
	n-Butanol satd. with water			
b. 6-Demethyl-tetracycline	0.73	0.52		
A Mannich type product of 6-demethyl-tetracycline and lysine	0.00	0.05		
Development time	185 sec	16 h		
Detection: Ultraviolet radiation and ammonium vapor; paper treated with phosphate buffer.				

* Average of several values.

were greater than those determined by the conventional method. However, results are comparable.

The best resolutions were obtained for steroids, tetracyclines, amino acids and urea derivatives. Separations were not as good for the remaining mixtures. The two bands for O,O-diethyl O-2-pyrazinyl phosphorothionate and sodium pyrazinol were not well defined; this phenomenon is probably due to the pretreatment of the paper with acetamide. The bands for the organic acids were diffuse and difficult to detect. Inasmuch as the detection reagent for these acids was an acid–base indicator, both development systems gave trouble since one contained formic acid and the other ammonia. Even though overnight drying prior to spraying did not correct this difficulty completely, the zones could still be detected against the background.

Detection was not difficult with a thinner paper such as Whatman No. 3; however, a continuous flow of solvent could not be maintained without flooding this paper. The high volatility of O,O,S-trimethyl phosphorodithioate resulted in it being removed completely from the paper even though the paper was pressed between two plates.

Because of these generally favorable results this unit was used to follow a Mannich base reaction. Lysine hydrochloride (183 mg) was mixed with 6-demethyltetracycline (430 mg) in methylcellosolve and the basicity of the mixture was adjusted with 0.1 *N* sodium hydroxide to effect solution. The rate of reaction was followed qualitatively by centrifugal chromatography. A nearly complete reaction was indicated at the end of 21 min (Table I, 6b).

Other organic mixtures that were separated were solutions of amino acids and FD & C standard dyes (Table II). For the separation of the amino acids the motor was run at $^1/_3$ speed and 6.5 ml of solvent was used instead of 4 ml. Good separations were obtained for both these mixtures.

TABLE II

SEPARATIONS BY CENTRIFUGAL CHROMATOGRAPHY

Amino acids	*R_F value*
Leucine	0.95
Proline	0.62
Histidine	0.42

Detection: ninhydrin, plain paper.
Developing solvent: *n*-butanol–acetic acid–water (50:12:50).
Developing time: 140 sec.

FD & Standard dyes	*R_F value*
FD & C Red No. 4	0.42
FD & C Green No. 1	0.62
FD & C Yellow No. 8	0.99

Detection: paper treated with phosphate buffer.
Developing solvent: *n*-butanol saturated with water.
Developing time: 185 sec.

DISCUSSION

The evaluation of this unit indicates the advantage of two contiguous plates. Because of this modification very regular solvent fronts are formed even when solvents such as acetone, benzene, or methyl ethyl ketone are used. Usable chromatograms can also be obtained with pretreated paper. Since there is no development chamber, no pre-equilibration with the solvent system is necessary. All separations reported in Tables I and II were obtained using dried paper. In a few cases the paper was equilibrated in a separate chamber with the solvent system before development. The resulting chromatograms showed more diffuse bands than those obtained with dry paper.

Even with a known developing system, separation by this technique is not always successful. However, a large number of chromatograms can be obtained in a short time making possible an evaluation of a series of solvent systems in order to determine the feasibility of separation by the standard paper chromatographic technique. Though the ratios of R_F values for centrifugal chromatography to those for the standard technique are not constant, results are comparable. In this investigation the R_F ratios (R_F Cent./R_F Std.) vary only from 1 to 2, with but one exception and that involved the use of methyl ethyl ketone as solvent. If a mixture of compounds tends to travel with the solvent by centrifugal chromatography and resulting R_F values are 0.8–0.9, separation might be achieved by employing the standard technique since R_F values will usually be lower.

Qualitative results are quickly obtained when complete separation is achieved by this accelerated technique. R_F values are reproducible if the same operational procedure is carefully followed. Even quantitative work should be possible using

this technique. By off center spotting and subsequent development, one half of the paper can be sprayed to locate the bands of the various components and with this pattern the other half marked, cut out, extracted and analyzed.

The unit reported in this investigation does have certain drawbacks. It can be improved by using a motor with a governor and a tachometer. The feed system occasionally shows stream irregularities and stoppages in spite of the built-in filter. This difficulty can usually be eliminated by cleaning the orifice disc periodically and filtering the solvent system through a very fine glass filter before transferring to the nozzle. The thicker chromatographic paper required takes longer to dry, and if an acid is used in the developing solvent, detection with an acid–base indicator is difficult. In spite of these difficulties, the unit has two distinctive advantages: a drastic reduction in the time required for paper chromatography and the ability to use volatile solvents for developing systems.

MODIFICATION OF A NEW COMMERCIAL UNIT

A new commercial centrifugal chromatographic unit was introduced about the time this investigation was completed. This unit has a tachometer, a governor controlled $^1/_8$ h.p. motor (300–700 r.p.m.), and a closed development chamber containing a rotating plate on which the chromatographic paper is placed. The important innovation of this unit is a new automatic feed system which replaces a constant flowing stream of earlier commercial units. The solvent is delivered automatically by an electronically activated solvent metering system consisting of a timed relay and a graduated buret equipped with a solenoid valve for intermittent rather than continuous flow.

This new unit was modified in the Stamford Laboratories by fitting a second plate over the one in the development chamber. Good separation of mixtures of dicyandiamide, guanidine, and biguanide and mixtures of malic, maleic, and fumaric acids (Table III) have been achieved with this modified unit. Separations of the nitrogen compounds can be obtained without the second plate, but they tend to be irregular and the bands more diffuse. Separation and detection of the organic acids was possible using the modified commercial unit whereas difficulties were encountered using the one built in the Pearl River Laboratories (Table I). Succeess was achieved by using (1) a slower feed, (2) reduction of motor speed, and (3) thinner paper. This second plate must be used with developing solvents such as benzene and acetone; otherwise no chromatogram is obtained even though the chamber is equilibrated with developing solvent beforehand. To insure uniform development of chromatograms with such solvents, great care must be taken to seat evenly the upper plate.

Two other problems were resolved using this modified unit: (1) mixture of trace amounts of cyanamide and dicyandiamide, and (2) reaction mixtures taken from an autoclave initially charged with dicyandiamide. Resolutions of both mixtures were successfully carried out.

Quantitative as well as qualitative results were requested for the second problem. This was readily accomplished by off center spotting of duplicate samples. One half of the developed chromatogram was used for band detection, and then using this as a pattern the bands of the other half were located and then extracted. The materials extracted were then analyzed by ultraviolet spectrophotometry. For

TABLE III

SEPARATIONS USING THE MODIFIED COMMERCIAL UNIT

Mixture of dicyandiamide, biguanide and guanidine (200 μg)

	R_F value	
	No upper plate, chamber saturated with solvent	*With upper plate*
Dicyandiamide	0.61	0.61
Guanidine	0.41	0.43
Biguanide	0.28 irregular development	0.28 regular development

Off center spotting, 700 r.p.m.; 6 ml added in 1400 sec.
Paper: Schleicher & Schüll 470.
Developing solvent: butanol–ethanol–water (4:1:1).
Detection: nitroprusside[12], off center spotting.

Mixture of malic, maleic and fumaric acids (250 μg of each)

	R_F value
	With upper plate
Malic acid	0.12
Maleic acid	0.61 very diffuse
Fumaric acid	0.97

Off center spotting, 600 r.p.m.; 2.5 ml added in 1900 sec.
Paper: Whatman No. 3.
Developing solvent: acetone–benzene–formic acid–water (10:10:2:6) (used upper layer).
No chromatographic development without upper plate.
Detection: bromophenol blue, off center spotting.

example, the recovery of 193 μg of pure guanyl-O-methyl isourea was found to be 190 μg by this technique. By conventional paper chromatography 20 h were required to obtain equivalent results, by this centrifugal technique only 2 h were required.

Reproducible chromatograms have consistently been obtained with this intermittent feed system and not once has stoppage or irregular delivery of solvent occurred. Modifying the unit by the addition of a second plate has made it very versatile and useful for chromatographic development with volatile solvents. Following the progress of microbiological reactions as well as organic reactions should be possible.

SUMMARY

Greater applicability of centrifugal chromatography can be obtained by placing the paper between two metal plates instead of in a development chamber. Several

examples of the usefulness of such an arrangement are reported. With this modification incorporated in the design of a commercial unit centrifugal chromatography should drastically reduce the time required to obtain separations on paper. As a consequence paper chromatography will no longer suffer from a time disadvantage when compared with thin layer and gas liquid chromatography.

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SECOND SUPPLEMENT FOR THE PAPER CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PHENOL DERIVATIVES AND RELATED COMPOUNDS OF BIOCHEMICAL INTEREST, USING A "REFERENCE SYSTEM"*

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INTRODUCTION

Further paper-chromatographic data, to those already presented[1,2] for approximately 700 compounds mainly belonging to the series of phenol derivatives, hydroxylated heterocyclic compounds, *e.g.* indole derivatives, metabolites of aromatic amino acids, products of vegetable and mould origin and some compounds of pharmacological interest, are presented in this paper.

As before, six solvent systems were used throughout, but instead of ten standard colour reagents for identification purposes, the number of colour reagents was increased to twelve, including two Ehrlich reagents, in order to cover the colour reactions of a number of amino derivatives of the above-mentioned compounds studied in the present investigation. The systematic presentation of compounds is somewhat more heterogeneous than previously, the main reason being that some of the available sources have been exhausted. The data were obtained and recorded under as uniform working conditions as possible in order to be comparable with the information presented earlier. The main reason for this investigation, in addition to providing the characteristics of each compound in a given system, has been to evaluate more closely the connection between the chemical structure of the compounds and their different colour reactions, and the relationship between the structure and R_F values in different solvent systems. In general the values are in agreement with previous findings, with few exceptions so far, and the recorded data fit the picture fairly well, as regards the 1,2-, 1,3- and 1,4-dihydric phenol derivatives, which give characteristic colour patterns. The chromatographic behaviour of monohydroxylated pyridines, quinolines and more complex ring systems such as phenothiazines and acridines show a typical maximum of R_F values in solvent B, when compared with solvent A; this seems to hold as a general rule. The alkaloids investigated in the previous paper behave similarly in this respect. The R_F values and the colour code provide a method for distinguishing between these heterocyclic types of compounds.

* For previous papers in this series, see refs. 1 and 2.

MATERIALS AND METHODS

For one-dimensional descending chromatography rectangular glass jars of size 20 × 30 × 60 cm were used. Whatman No. 1 filter paper of dimensions 24 × 48 cm was used throughout the experiments and the composition of the six solvent systems is given in the section "Abbreviations used in the Tables".

Spray reagents

The following twelve standard spray reagents were used to detect the compounds:

Diazotized sulphanilic acid (obtained from Th. Schuchart Co., Munich); diazotized 4-benzoylamino-2,5-dimethoxyaniline (Light Chemicals Co., England); diazotized *o*-dianisidine (Light Co.); *p*-nitrobenzenediazonium fluoborate (Eastman Kodak, U.S.A.); 2,6-dibromoquinone-4-chloroimide (British Drug Houses Ltd., England); 2,4-dinitrophenylhydrazine; ferric chloride; phosphomolybdic acid; potassium permanganate; bromophenol blue; *p*-dimethylaminobenzaldehyde; and *p*-dimethylaminocinnamaldehyde (Heidenheimer Chemisches Laboratorium, Heidenheim-Brenz, Germany). For the composition of these reagents, see the section "Abbreviations used in the Tables".

Additional reagents

For detection of indoles the following three reagents (Frinton Laboratories Inc., Vineland, N.J.) were tried out: *p*-N,N-bis(2-chloroethyl)-aminobenzaldehyde (abbreviated as E3), 2-chloro-4-N,N-bis(2-chloroethyl)-aminobenzaldehyde (E4) and 4-N,N-bis(2-chloroethyl)-amino-2-tolualdehyde (E5). The best results with respect to colour development were obtained by dissolving these compounds in conc. HCl. Reagents E3 and E5 behaved similarly giving red-violet or blue-violet colours, although there were a few marked differences with certain compounds. Reagent E4 gives predominantly blue to green shades. A comparison of these reagents with the reagents EH and DAC used earlier is given in Table XVI. However, some caution should be exercised in interpreting the colour differences as these reagents react very rapidly in this medium and in most cases ran through many shades in a few seconds. They should therefore be tested together with a number of known compounds, when applying them to unknown substances. Consequently, the values in Table XVI serve only as guiding values among several other possibilities, it being almost impossible to record them all. Reagent E4 should be prepared immediately before use, in order to avoid background colorization. When these three reagents were dissolved in 1 *N* HCl colour development was too slow.

For detecting aldehydes, a number of aromatic amines were tested. The overall picture is presented in Table XVII. Primary aromatic amines are known to condense in acidic solution with aldehydes forming coloured compounds, *e.g.* Schiff bases[3,4]. The formation of coloured Schiff bases was instantaneous in most cases, and any colour changes with time were recorded as indicated in the footnote to Table XVII. No heating was required to develop the colours. The freshly recrystallized amines were made up as a 1 % solution in 2 *N* HCl prior to spraying.* In the cases of benzidine, *o*-tolidine and *o*-dianisidine, saturated solutions in 2 *N* HCl were used. The following

* Caution in handling of aromatic amines, see ref. 11.

amines were used for the detection and recording of the colour sequences given in Table XVII: *o*-phenylenediamine, *m*-phenylenediamine, *p*-phenylenediamine, 2,4-diaminophenol, *p*-anisidine, *o*-toluidine, *p*-toluidine, α-naphthylamine, β-naphthylamine, benzidine, *o*-tolidine, dimethyl-*p*-phenylenediamine, *p*-aminoacetophenone, 4-amino-antipyrine, 1,2-diaminonaphthalene and 2,7-diaminofluorene. Yellow, orange, red and brown colours were generally obtained. *o*-Phthalaldehyde gave predominantly grey and green colours, except with *o*-toluidine where a transient red-violet colour was observed. Another red-violet coloration was obtained when 1,2-diaminonaphthalene reacted with cinnamaldehyde.

The following compounds were also tested under similar conditions, but are not recorded in the tables. However, some useful information is given below, when marked differences were noted.

Phenylhydrazine. This reagent gave green colours with terephthalaldehyde, cinnamaldehyde, salicylaldehyde, 3-hydroxybenzaldehyde, and 2,4-dihydroxybenzaldehyde and grey colours with piperonal and 4-methoxybenzaldehyde.

N-(1-Naphthyl)-ethylenediamine. This reagent gave a red-violet colour with *o*-phthalaldehyde and was rose pink with cinnamaldehyde and *o*-veratraldehyde.

Azobenzene-phenylhydrazinesulphonic acid. The reaction with 3-indolealdehyde and 2,4-dihydroxybenzaldehyde was green.

1,5-Diaminonaphthalene. There was a rose-pink colour with coniferylaldehyde.

2,3-Diaminonaphthalene. A brown coloration was given with 4-acetoxy-5-methoxy-isophthalaldehyde and 2,5-dihydroxybenzaldehyde.

2,7-Diaminonaphthalene. This reagent was green with *o*-phthalaldehyde, brown with terephthalaldehyde, and carmine with 4-acetoxy-5-methoxy-isophthalaldehyde.

3,3'-Diaminobenzidine. Orange colours were given with 3-indolealdehyde and 2,5-dihydroxybenzaldehyde; with coniferylaldehyde the colour was rose-pink and brown with cinnamaldehyde and 4-acetoxy-5-methoxy-isophthaldehyde.

2,6-Dibromoquinone-4-chloroimide (DB) reagent was found to be useful for the detection of phenylthiohydantoin (PTH) derivatives of amino acids (Table XV), producing red and brown colours, and in some cases it enabled one to distinguish between different PTH-derivatives. The sensitivity of the reagent seemed to be acceptable; furthermore it was easy to prepare, and the spots appeared almost instantaneously.

For detecting hippuric acid derivatives a 2 % solution of recrystallized *p*-diethylaminobenzaldehyde (DAB) in acetic anhydride was used[2,5] and the results are presented in Table XIII. In a few cases weak colorations were observed with compounds other than the hippuric acid derivatives described in this paper. Pale yellow colours were produced by: 3,4,5-trimethoxy-aniline, β-3,4-dimethoxyphenyl-ethylamine, 2,3-diaminopyridine, 2-quinoline-4-carboxylic acid, 3-indoleacetic acid methyl ester, 5-methoxygramine and N-methylanthranilic acid. Compounds such as 5-hydroxy-3-indoleacetamide, N-methyltryptamine, N,N-diethyltryptamine, 5-fluorotryptamine, tryptophol, tryptophan methyl ester and ergotamine, after treatment with DAB reagent, showed yellow colours only under U.V. light.

The general paper-chromatographic procedure followed essentially the same principles as described earlier[1]. The colour reactions were recorded as numbers, following the "Derwent" coloured pencils index of Cumberland Pencil Co., Keswick, England. The descriptions of these colours is found under the heading "Colour index for the Tables".

The compounds listed in the tables, except the PTH- and DNP-amino acid derivatives, were mainly obtained from commercial sources.

The hippuric acid derivatives listed in Table XIII were synthesized by the author using the methods of SHEEHAN AND HESS[7] and ITO AND NEILANDS[8], whereby glycine ester is condensed with an aromatic acid, in the presence of dicyclohexyl-carbodiimide, and the ester is then hydrolysed to yield the corresponding aroylglycine.

GUIDE TO TABLES I–XV

Tables I–XIII present the information compiled for approximately 170 organic compounds, investigated by the procedure outlined above. Tables XIV and XV give only the R_F values for 35 amino acid derivatives. The R_F values are recorded in six different solvent systems designated by F, E, A, B, C and D. For the composition of these solvents, see the list of abbreviations given below. Under the heading "Detection" (columns 2–13) the colour reactions are recorded for 12 different reagents used for the identification of each compound; in addition, the colour produced in ultraviolet light is indicated in the first column under this heading. The amount of substance used in these experiments was 25–50 γ per spot, which produced different colours when the reagents were applied. These colours are referred to by numbers, the explanation of which is found in the colour index. For abbreviations and the composition of the reagents, the reader is referred to the following section.

Table I	Monohydric phenols and their derivatives.
Table II	1,2-Dihydric phenols and their derivatives.
Table III	1,3-Dihydric phenols and their derivatives.
Table IV	1,4-Dihydric phenols and their derivatives.
Table V	Trihydric phenols.
Table VI	Naphthalene and quinoline derivatives.
Table VII	Benzoic and phthalic acid derivatives.
Table VIII	Aromatic non-phenolic and heterocyclic monocarboxylic acids with the COOH group in the side chain, and their derivatives.
Table IX	Aliphatic and heterocyclic amino acid derivatives.
Table X	Biologically active nitrogen compounds, *e.g.* biogenic amines etc.
Table XI	Indole derivatives.
Table XII	Miscellaneous compounds.
Table XIII	Hippuric acid derivatives.
Table XIV	2,4-Dinitrophenyl derivatives of amino acids.
Table XV	Phenylthiohydantoins of amino acids.

Owing to the lack of space in the tables, the R_F values have been multiplied by 100 and recorded as 12, 56, 88, but should be read: 0.12, 0.56, 0.88 etc. The colours produced by the reagent are recorded as numbers and the corresponding shades can be found in the colour index. The –-sign indicates an uncertain reaction, which was too weak to deserve colour estimation. Reactions with the reagents Mn and Ind are only indicated by the signs: —, + or ++. The ++ sign means that a positive reaction was obtained immediately. In the few cases where the colours are recorded by a number placed on top of another number, *e.g.* $\left.\begin{matrix}24\\57\end{matrix}\right\}$, this indicates that immediately upon spraying a violet spot (24 in the colour index) appears which, within a few seconds, turns to light brown (57 in the colour index). Usually most colours are unstable and after some time take on a brownish tone; this is to some extent caused by the chemical influence of other reagents used in the vicinity. This change in colour is not recorded in the tables, nor is there any column for those compounds that, at this low concentration, are visible on the chromatograms because of their own colour.

The various compounds listed in the tables have been arranged as far as possible according to the following principles:

(1) Free phenols together with their methoxy- and alkyl-derivatives; (2) the corresponding amino- and nitro-derivatives; and (3) further derivatives in the order: hydroxymethyl, keto, aldehyde and finally monocarboxylic acid derivatives, derivatives with a carboxylic acid group in the side chain and dicarboxylic acids.

ABBREVIATIONS USED IN TABLES I–XVII

Chromatographic solvent systems

A = Methyl isobutyl ketone–formic acid–water (10 parts ketone saturated with 1 part 4 % formic acid)
B = Chloroform–methanol–formic acid–water (10 parts of chloroform saturated with a mixture of 1 part methanol and 1 part 4 % formic acid)
C = Benzene–ethyl methyl ketone–formic acid–water (a mixture of 9 parts benzene and 1 part ketone saturated with 1 part 2 % formic acid)
D = Benzene–formic acid–water (10 parts benzene saturated with 1 part 2 % formic acid)
E = Ethyl methyl ketone–diethylamine–water (921:2:77 parts by volume)
F = Ethyl methyl ketone–acetone–formic acid–water (40:2:1:6 parts by volume)

Reagents used for detection

U.V. = Ultraviolet light
D1 = Diazotized sulphanilic acid (0.3 % solution in dioxane–water 1:2)
D2 = Diazotized 4-benzoylamino-2,5-dimethoxyaniline (0.6 % solution in dioxane–water 1:2)
D3 = Diazotized *o*-dianisidine (0.6 % solution in dioxane–water 1:2)
D4 = *p*-Nitrobenzenediazonium fluoborate (0.4 % solution in dioxane–water 1:2)
DB = 2,6-Dibromoquinone-4-chloroimide (0.5 % solution in dioxane–acetone 4:1)
DN = 2,4-Dinitrophenylhydrazine (*ca.* 0.1 % solution in 1 *N* HCl)
Fe = Ferric chloride (2 % aqueous solution)
Mo = Phosphomolybdic acid (2 % aqueous solution)
Mn = Potassium permanganate (1 % aqueous solution)
Ind = Bromophenol blue (*ca.* 0.05 % solution in ethanol)
EH = Ehrlich reagent (1 % *p*-dimethylaminobenzaldehyde in 1 *N* HCl)
DAC = *p*-Dimethylaminocinnamaldehyde (0.1 % solution in 1 *N* HCl)
DAB = *p*-Dimethylaminobenzaldehyde (2 % solution in acetic anhydride)

Special reagents used for detection

E3 = *p*-N,N-Bis-(2-chloroethyl)-aminobenzaldehyde (2 % in conc. HCl)
E4 = 2-Chloro-4-N,N-bis-(2-chloroethyl)-aminobenzaldehyde (2 % in conc. HCl)
E5 = 4-N,N-Bis-(2-chloroethyl)-amino-2-tolualdehyde (2 % in conc. HCl)

COLOUR INDEX FOR THE TABLES

The colours produced by the action of different reagents on the investigated compounds, presented in Tables I–XVII, have been recorded as numbers, according to the following code:

01	Zinc Yellow	25	Dark Violet	49	Sap Green
02	Lemon Cadmium	26	Light Violet	50	Cedar Green
03	Gold	27	Blue Violet Lake	51	Olive Green
04	Primrose Yellow	28	Delft Blue	52	Bronze
05	Straw Yellow	29	Ultramarine	53	Sepia
06	Deep Cadmium	30	Smalt Blue	54	Burnt Umber
07	Naples Yellow	31	Cobalt Blue	55	Vandyke Brown
08	Middle Chrome	32	Spectrum Blue	56	Raw Umber
09	Deep Chrome	33	Light Blue	57	Brown Ochre
10	Orange Chrome	34	Sky Blue	58	Raw Sienna
11	Spectrum Orange	35	Prussian Blue	59	Golden Brown
12	Scarlet Lake	36	Indigo	60	Burnt Yellow Ochre
13	Pale Vermilion	37	Oriental Blue	61	Copper Beech
14	Deep Vermilion	38	Kingfisher Blue	62	Burnt Sienna
15	Geranium Lake	39	Turquoise Blue	63	Venetian Red
16	Flesh Pink	40	Turquoise Green	64	Terra Cotta
17	Pink Madder Lake	41	Jade Green	65	Burnt Carmine
18	Rose Pink	42	Juniper Green	66	Chocolate
19	Madder Carmine	43	Bottle Green	67	Ivory Black
20	Crimson Lake	44	Water Green	68	Blue Grey
21	Rose Madder Lake	45	Mineral Green	69	Gunmetal
22	Magenta	46	Emerald Green	70	French Grey
23	Imperial Purple	47	Grass Green	71	Silver Grey
24	Red Violet Lake	48	May Green	72	White=colourless

TABLE I

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME MONOHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection											
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*	*EH*	*DAC*
95	93	95	95	95	94	*o-tert.*-Butylphenol	—	07	08	63 56	08 24	43 35	—	—	43 68	++	—	—	—
95	93	94	95	95	94	2,6-Di-*tert.*-butylphenol	—	17	17	17	09 28	28 25	—	—	43	+	—	—	—
89	85	84	75	64	30	*o*-Acetaminophenol	—	06 10	09 15	19 65	07 23	41 37	—	26 70	69	43 66	—	—	—
87	84	79	20	17	02	*m*-Acetaminophenol	—	06 09	15	19 15	09 15	38 35	—	70	71	++	—	03	+
83	81	72	09	07	00	*p*-Acetaminophenol	—	14	12 64	63 65	62 65	—	—	27	30	++	—	—	60
34	85	13	16	00	00	*p*-Methylaminophenol	56	—	—	+	63	53	—	+	68	++	—	52	21
40	82	10	12	03	00	4-Amino-3-methylphenol	55	—	—	64	07	—	—	—	69	++	—	07	21
92	65	92	56	57	08	2-Amino-4-nitrophenol	55	+	58	55	58	55	—	60	43 68	++	—	06	23
94	89	92	56	57	08	2-Amino-5-nitrophenol	59	63	63	55 65	55	21 63	—	55	55 69	++	—	15 12	25 28
91	82	87	86	78	70	4-Amino-2-nitrophenol	55	56	62	63	64	—	—	—	63 68	++	—	09	19
49	86	27	79	24	17	*p*-Anisidine	70	08	59	65 63	59	—	—	24	50 53	++	—	09	19
72	88	48	93	53	41	*o*-Dianisidine	56	63	—	—	56	50	—	43 68	53	++	—	15 20	23 65
92	90	94	90	92	86	4-Nitro-*o*-anisidine	65	—	—	—	—	—	—	—	—	+	—	12	65 25
92	91	95	91	92	86	5-Nitro-*o*-anisidine	65	—	—	—	—	—	—	—	—	+	—	07	19
93	91	92	92	89	86	2-Nitro-*p*-anisidine	64	—	—	—	—	57	—	—	—	+	—	11	24 25
92	91	87	43	59	22	5-Hydroxyindole	—	64	55 65	24 25	56 63	28	16	55	43 68	++	—	23	43 28
96	53	94	93	89	88	2-Nitro-3-hydroxyaceto-phenone	57	—	—	—	+	—	+	—	—	—	—	—	—
95	91	93	89	88	68	Salicylaldoxime	—	—	—	63	—	03 41	—	54	68	++	—	—	—
92	79	86	76	69	44	Salicylamide	33	07	08	65	10	41	—	23	—	++	—	06	21
95	65	93	88	87	66	5-Bromosalicylic acid	34	—	—	—	—	—	—	24	—	+	++	—	—
93	67	87	76	77	24	5-Nitrosalicylic acid	26	—	—	—	—	—	—	64	—	—	++	—	—
89	92	91	84	87	71	3-Hydroxybenzoic acid methyl ester	—	08	62	63 65	12	43	—	+	—	++	—	—	—
92	06	92	83	73	26	3-Hydroxy-4-nitrobenzoic acid	59	+	08	56	63	48	—	+	08 34	+	+	—	57
94	93	93	74	76	15	3,5-Dichloro-4-hydroxy-benzoic acid	—	—	—	—	08 63	40	—	57	—	++	+	—	—
81	00	48	00	00	00	5-Hydroxyanthranilic acid	34	07	—	24 65	03 64	—	—	—	34	+	+	—	06
93	14	89	88	87	84	*o*-Methoxyphenylacetic acid	—	—	—	—	—	—	—	—	—	—	+	—	—
91	05	87	19	22	01	*m*-Hydroxyphenylacetic acid	—	06 09	14	15 65	08 65	37 25	—	—	71	++	+	—	—
93	10	88	37	41	03	*m*-Hydroxyphenylpropionic acid	—	06 10	12	65	09 65	38 25	—	—	30 71	++	+	—	—
95	16	94	91	90	76	*o*-Methoxycinnamic acid	33	—	—	—	—	—	—	—	—	++	+	—	—
95	14	94	90	84	69	*m*-Methoxycinnamic acid	33	—	—	—	—	—	—	—	—	++	+	—	—
85	04	57	02	02	00	*dl-p*-Hydroxymandelic acid	—	08	12 64	65	06 65	38 25	—	03	71	++	+	—	—
87	62	78	76	39	08	*o*-Methoxymandelic acid	—	—	—	—	—	—	—	58	—	+	++	—	—

(continued on p. 481)

TABLE I (*continued*)

R_F values × 100						Compounds	Detection											
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*	*EH*	*DAC*
90	18	78	68	46	05	*m*-Methoxymandelic acid	—	—	—	—	—	—	—	+	—	+	++	—	—
92	09	76	54	35	04	*p*-Methoxymandelic acid	—	—	—	—	—	—	—	—	—	+	+	—	—
93	91	89	75	63	25	5-Hydroxy-3-indolylacetic acid methyl ester	—	17	+	+	+	—	—	—	—	—	—	34	30 27
91	10	91	71	53	11	5-Methoxy-3-indolylacetic acid	—	03	—	+	07	—	60	26 23	44 71	++	+	26 27	27 30
68	57	20	01	00	00	5-Hydroxy-3-indolylacetamide	+	64	23	23 65	64	68	—	70	71	++	—	51	25 28
88	00	83	00	00	00	5-Hydroxyisophthalic acid	—	09	—	63	60	38 25	—	+	—	+	+	—	—
91	76	88	88	88	87	2-Chloro-6-hydroxypyridine	—	—	—	—	—	—	—	57	—	—	—	—	—
72	64	58	68	24	15	2-Mercaptopyridine	63	—	—	62	—	08 63	—	—	51 69	++	—	—	—
45	15	12	14	00	00	4-Mercaptopyridine	03	05	09	11	06	59	—	—	45 68	++	—	—	—

TABLE II

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection											
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*	*EH*	*DAC*
94	94	92	86	86	66	4-Isopropylpyrocatechol	—	59 09	24	56 24	64 65	28	+	+	43 68	++	—	17	17
93	69	93	88	83	75	3-Nitropyrocatechol	56	07 19	63	65	63 68	68	—	45	69 09	+	—	03	58
90	04	82	59	52	12	3-Methoxy-4-hydroxyphenylacetic acid = homovanillic acid	—	59 17	—	—	63	33	—	—	69	+	+	—	—
91	04	79	59	28	05	3-Hydroxy-4-methoxycinnamic acid	30	09 56	15 08	63 65	11 55	38 70	—	57	35 69	++	+	—	—
87	01	76	13	05	00	3,4-Dihydroxyphenylpropionic acid = hydrocaffeic acid	—	06 64	23 19	23 19	09 65	25	—	53	43 68	++	+	—	—
60	00	25	00	00	00	*dl*-3,4-Dihydroxymandelic acid	—	58 17	17	17	08 65	28	—	43	68	++	+	—	—
77	03	47	04	02	00	3-Hydroxy-4-methoxymandelic acid	—	07 10	08 15	11 25	13 65	38 35	—	58	71	+	+	—	—
79	03	48	05	04	00	4-Hydroxy-3-methoxymandelic acid	—	63	60	+	24 03	38 28	—	—	69	+	+	—	—

TABLE III

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,3-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D_1	D_2	D_3	D_4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
87	32	67	16	11	00	4-Nitrosoresorcinol	56	08, 56	23, 62	65	10, 65	+	—	—	69	++	—	—	—
64	05	32	17	04	00	2,4-Dinitrosoresorcinol	55	58	58	59	+	52	+	43	43	++	—	—	—
89	80	90	88	87	68	2,4-Dihydroxypropiophenone	34	08, 56	56, 64	23, 65	09, 65	06, 28	06	23, 65	34, 71	++	—	—	—
94	20	92	36	49	03	5-Bromo-2,4-dihydroxybenzoic acid	—	06, 59	19, 63	24, 65	12	—	—	24, 27	—	++	+	—	—
94	35	94	92	90	71	4-Ethoxy-2-hydroxybenzoic acid	—	56, 08	62	64	09	—	—	23	+	+	+	—	—
92	24	89	90	85	74	2,4-Dimethoxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+	—	—
58	06	26	81	04	04	2,6-Dihydroxypyridine	40, 56	06, 10	65, 63	66	60, 35	43, 28	—	55	52, 68	++	—	18, 21	05, 52
62	00	10	00	00	00	2,6-Dihydroxyisonicotinic acid	40	06, 08	14	14, 62	06	25	—	—	+	+	+	—	—

TABLE IV

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,4-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D_1	D_2	D_3	D_4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
94	85	92	46	61	12	2-Bromohydroquinone	—	—	56	60	59	—	—	—	41, 69	+	—	—	—
87	84	81	93	83	78	2,6-Dimethoxy-1,4-benzoquinone	57	—	—	—	—	—	—	—	—	—	—	—	—
92	88	89	88	85	83	2,5-Dimethoxybenzoic acid	+	—	—	—	—	—	—	—	—	—	++	—	—
73	05	42	08	04	02	3,6-Dihydroxyphthalimide	40	07	11	11	60	56	—	51	69, 03	++	—	—	—

TABLE V

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME TRIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D_1	D_2	D_3	D_4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
78	08	62	00	02	00	1,2,4-Trihydroxybenzene	56	+	+	56	56	56	55	54	68, 55	++	—	+	27, 44
89	39	81	56	26	06	3,4-Dihydroxy-5-methoxybenzaldehyde	—	57	59	60	59	43, 70	09	70	43, 68	++	—	—	—
85	41	64	03	01	00	2,4,6-Trihydroxybenzoic acid	—	10	60, 63	23, 25	12, 65	26, 70	—	25, 26	39, 70	++	++	18, 23	30, 25
88	03	77	75	28	06	3,5-Dimethoxy-4-hydroxycinnamic acid = sinapic acid	38	15, 23	63	25, 65	14, 62	25	—	21	52, 28	++	+	—	—
75	83	52	94	51	35	3,4,5-Trimethoxyaniline	+	58	58	65, 64	64	71	—	—	52, 70	++	—	07	19
08	00	00	00	00	00	Tetrahydroxy-*p*-benzoquinone	+	—	58	—	—	—	—	—	70	—	—	—	—

TABLE VI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NAPHTHALENE AND QUINOLINE DERIVATIVES

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
95	94	83	77	06	04	1-Amino-2-naphthol	57	60	56	56	56	—	23	56	53	++	—	06	64
88	91	80	55	36	09	2-Amino-5-naphthol	33	12, 23	23, 65	65	65, 64	63, 28	—	56	68	++	—	08	30, 24
93	09	90	53	37	01	2-Hydroxy-6-naphthoic acid	33	13	24, 65	23, 65	12, 15	43, 63	—	60	30	++	+	—	—
94	36	92	33	36	01	3,5-Dihydroxy-2-naphthoic acid	06	15, 23	24	25, 28	64, 68	63, 29	—	28, 69	43, 68	++	+	05	—
57	85	29	56	07	01	5-Hydroxyisoquinoline	34	12, 15	20	23, 65	64, 55	35, 28	—	71	68	++	—	—	—
84	68	68	25	06	00	1,5-Dihydroxyisoquinoline	34	17, 15	63, 25	25, 28	15, 65	39	—	26	68	++	—	—	—
79	00	37	08	05	00	2-Hydroxyquinoline-4-carboxylic acid	34	—	—	—	—	—	—	—	—	—	+	—	—

TABLE VII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BENZOIC ACID DERIVATIVES

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
95	90	85	84	88	83	*m*-Nitrobenzyl alcohol	27	—	—	—	—	—	—	—	—	+	—	+	—
94	93	93	93	93	93	*o*-Aminoacetophenone	39	—	—	—	—	—	+	—	34	+	—	08	24
90	89	81	91	74	66	*m*-Aminoacetophenone	57	08	—	64	62	—	07	—	+	+	—	06	19
93	92	85	92	78	70	*m*-Aminobenzaldehyde	—	—	—	—	—	—	06	—	—	+	—	06	22
96	94	93	95	93	92	*o*-Nitrobenzaldehyde	+	—	—	—	—	—	06	—	—	+	—	04	23
94	58	92	88	87	86	Thiobenzoic acid	—	+	60	+	+	64	—	65, 72	69	++	+	—	—
91	39	82	84	84	65	2,6-Dichlorobenzoic acid	—	—	—	—	—	—	—	—	—	—	++	—	—
89	06	80	49	24	05	*p*-Hydrazinobenzoic acid	39	—	59	63, 56	—	—	—	—	—	+	—	05	17
94	31	86	93	86	84	N-Methylanthranilic acid	33	06	—	12, 62	08	38	—	26, 51	45, 50	++	+	07, 03	21, 15
94	14	92	87	78	50	2-Amino-4-methylbenzoic acid	33	08	—	64	12	45	—	—	+	+	—	07	24
94	18	91	84	78	52	2-Amino-5-methylbenzoic acid	33	06	—	62	08	60	—	58	30	++	+	09	19, 65
90	04	80	66	33	07	3-Amino-4-methylbenzoic acid	34	08	—	64	12	43	—	—	—	+	—	05	22
91	06	88	68	46	15	4-Amino-3-methylbenzoic acid	—	08	—	64	10	—	—	—	—	+	—	06	23
93	15	89	92	85	58	3,5-Dimethylanthranilic acid	39	—	—	64	06	—	—	—	+	+	—	—	+
88	86	33	06	03	00	3,4-Diaminobenzoic acid	38	+	58	23, 56	59	56	—	63, 56	69	++	—	09	23, 25
92	48	89	67	79	24	2,5-Dinitrobenzoic acid	26	—	—	—	—	—	—	—	—	—	++	58	—
85	02	83	45	07	00	3-Nitrophthalic acid	26	—	—	—	—	—	—	—	—	—	+	—	—

TABLE VIII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC NON-PHENOLIC AND HETEROCYCLIC MONOCARBOXYLIC ACIDS WITH THE COOH GROUP IN THE SIDE CHAIN, AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection											
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*	*EH*	*DAC*
94	19	93	90	88	82	4-Chlorocinnamic acid	—	—	—	—	—	—	—	—	—	+	+	—	—
93	42	91	75	67	12	4-Bromomandelic acid	—	—	—	—	—	—	—	—	—	+	+	—	—
88	06	90	79	61	18	3-Methyl-3-phenylglutaric acid	—	—	—	—	—	—	—	—	—	—	+	—	—
34	01	07	34	00	00	3-Pyridylacetic acid	—	—	—	—	—	—	—	—	—	—	+	—	—
00	03	00	00	00	00	4,5-Imidazole-dicarboxylic acid	—	—	—	—	+	—	—	—	—	—	+	—	—
05	00	00	00	00	00	Imidazole-4-acetic acid	34	15 60	62	64	08 65	—	—	—	—	—	+	—	—
06	00	00	00	00	00	1-Methylimidazole-4-acetic acid	—	—	+	56	+	—	—	—	—	—	+	—	—
08	00	00	00	00	00	Dihydrourocanic acid	—	15	60	65	07	—	—	—	—	—	+	—	—
87	73	82	44	34	09	Pyrrole-2-carboxylic acid	—	07 11	64	23	12 64	—	—	62	—	+	+	22 23	39 69
93	94	84	88	87	84	3-Indolylacetic acid methyl ester	+	—	60	60	08	—	—	—	—	++	—	18 69	24 25
88	08	86	18	24	05	3-Indolylglyoxylic acid	—	—	—	56	—	+	03	64	03 72	—	++	—	—
91	07	00	23	28	04	3-Indolylpyruvic acid	03	58	62	63	+	63	+	24	69	+	+	17	23 65

TABLE IX

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME ALIPHATIC AND HETEROCYCLIC AMINO ACID DERIVATIVES

R_F values × 100						Compounds	Detection											
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*	*EH*	*DAC*
08	00	00	00	00	00	DL-γ-Aminobutyric acid	—	—	—	—	—	—	—	—	—	—	30	—	—
02	00	00	00	00	00	DL-Methionine sulfoximine	—	—	—	—	—	—	—	—	—	—	33	05	—
20	01	00	00	00	00	DL-Ethionine	—	—	—	—	—	—	—	—	—	++	—	02	—
00	00	00	00	00	00	Cysteic acid	—	—	—	—	—	—	—	—	—	—	+	04	—
87	55	67	60	10	01	N^{α}-Acetylkynurenine	39	—	—	—	—	—	—	—	70	+	+	08	23
38	00	03	01	00	00	Hydantoin-5-acetic acid	—	—	—	—	—	—	—	—	—	—	30	—	—
05	00	00	00	00	00	N-Acetyl-L-histidine	—	15 08	62	68 56	07 24	—	—	—	—	—	—	—	—
59	00	07	04	00	00	N-Acetyl-L-glutamic acid	—	—	—	—	—	—	—	—	—	—	+	—	—

TABLE X

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BIOLOGICALLY ACTIVE NITROGEN COMPOUNDS

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
85	82	73	52	21	05	N-Acetyltyramine	—	07	60	64	23 08	—	—	—	68	+	—	—	—
06	15	00	00	00	00	N-Acetylhistamine	—	15 08	62	64 56	07 24	—	—	—	—	—	—	—	—
00	02	00	00	00	00	1-Methylhistamine	34	—	57	56	+	—	—	—	—	—	—	—	—
52	82	04	25	00	00	*dl*-Desoxyephedrine*	—	72	—	—	—	—	—	—	—	—	—	—	—
87	94	63	94	47	30	Cinnamylephedrine*	—	—	—	—	—	—	—	—	—	+	—	—	—
42	85	07	11	05	02	β-(*p*-Methoxyphenyl)-ethylamine	34	58	60	07 64	08 63	—	—	58	58 71	+	33	20	60 14
12	69	00	00	00	00	β-(3-Methoxy-4-hydroxyphenyl)-ethylamine	—	17	59	59	58 25	—	—	—	68	++	—	—	—
23	05	02	03	00	00	β-(3,4-Dimethoxyphenyl)-ethylamine	—	06	—	58 60	06 60	23 56	—	57	47	+	—	05	57 08
21	07	00	00	00	00	Noradrenalone	—	57 18	57 18	60	57	52	+	43	68	++	—	05	62
27	04	00	00	00	00	*dl*-Metanephrine	—	59 09	12	60 64	06 23	35	—	71	30	++	—	—	—
15	71	00	00	00	00	*dl*-Normetanephrine	—	59	10	56 23	58 23	35	—	—	30	+	—	—	—
36	14	00	00	00	00	2,3-Diaminopyridine	33	08 59	56	56	19 63	23	—	60	69	+	—	06	23
11	02	00	00	00	00	3-Hydroxy-N-methyl-piperidine	—	—	—	—	—	—	—	—	—	+	—	—	17
49	33	13	02	00	00	Thiourea	—	—	—	60	58	24	—	—	71	++	—	05	—
35	25	08	03	00	00	Biuret = carbamylurea	—	—	—	—	—	—	—	—	—	—	—	05	17

* Detected by $KI \cdot BiI_3$ reagent, see ref. 2.

TABLE XI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME INDOLE DERIVATIVES

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
37	35	00	00	00	00	Indican	—	—	—	55	08	25	—	—	—	+	—	63	26 23
92	91	87	43	59	22	5-Hydroxyindole	—	64	55 05	24 25	56 63	28	16	55	43 68	++	—	23	43 28
59	08	07	07	00	00	Gramine-N-oxide	—	—	—	64	07	+	17	58	59 71	+	33	58	22 24
48	86	02	01	00	00	5-Methoxygramine	—	—	—	—	08	23	—	—	—	+	33	04	26 71
90	88	87	85	67	45	N-Acetyltryptamine	33	57	—	—	10	—	06	—	30	++	—	08 24	24 25
51	80	03	00	00	00	N-Methyltryptamine	—	—	—	13	08	+	—	—	+	+	33	17 71	23 25
48	92	05	05	02	01	N,N-Dimethyltryptamine	—	—	—	58	58	—	—	58	52	++	—	58 23	23

(continued on p. 486)

TABLE XI (*continued*)

R_F values × 100						Compounds	Detection											
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*	*EH*	*DAC*
68	93	04	06	00	00	N,N-Diethyltryptamine	—	—	—	+	08	+	—	—	70	+	33	17 23	23
67	87	04	00	00	00	5-Fluorotryptamine	—	—	—	—	—	—	—	58	—	—	—	17	24 27
08	00	00	00	00	00	Psylocybin	—	—	—	—	—	—	—	—	70	—	—	16 26	27
52	83	02	00	00	00	5-Methoxytryptamine	—	06	—	60	08	—	—	—	70	++	—	26	27 25
83	82	60	18	01	00	N-Acetyl-5-hydroxy-tryptamine	—	17 60	24	25	18	+	—	—	30	+	—	69	30 43
90	88	78	84	48	28	N-Acetyl-5-methoxy-tryptamine = melantonin	—	06	—	—	08	—	—	—	70	++	—	26	25 28
94	93	93	93	92	92	Indole-3-carbinol	57	—	—	64	09	—	58	17	57 70	++	—	58 17	24
90	88	90	81	72	59	Tryptophol	34	58	—	—	08	24	70	—	71	++	—	07 63	23 25
51	84	01	01	00	00	L-Tryptophan methyl ester	—	—	—	—	08	23	—	—	—	++	33	03	23 25
93	19	92	72	68	15	5-Chloroindole-2-carboxylic acid	34	06	24	25	09 63	—	—	57	—	—	+	—	26
91	09	90	63	51	15	Indole-5-carboxylic acid	30	06	59	64	10	—	—	57	+	++	+	23	30
93	94	84	88	87	84	3-Indolylacetic acid methyl ester	+	—	60	60	08	—	—	—	—	++	—	18 69	24 25
85	77	65	50	19	07	3-Indolylacetamide	—	06	—	58	09	—	—	—	44 71	++	—	26 69	23 25
88	08	86	18	24	05	3-Indolylglyoxylic acid	—	—	—	56	—	+	03	64	03 72	—	++	—	—
94	93	92	93	92	88	3-Indolylglyoxylamide	—	—	—	—	—	—	07	—	—	+	—	—	17
95	93	93	94	92	92	3-Indolylglyoxyldimethyl-amide	—	—	—	—	—	—	07	—	—	+	33	—	+
91	07	00	23	28	04	3-Indolylpyruvic acid	03	58	62	63	+	63	—	24	69	+	+	17	23 65
94	08	91	66	50	14	3-Indolylacrylic acid	34	63 23	—	24 64	64	23	58	63	70	++	+	40	23
93	91	89	75	63	25	5-Hydroxy-3-indolylacetic acid methyl ester	—	17	+	+	+	—	—	—	—	—	—	34	30 27
68	57	20	01	00	00	5-Hydroxy-3-indolyl-acetamide	+	64	23	23 65	64	68	—	70	71	++	—	51	25 28
91	10	91	71	53	11	5-Methoxy-3-indolylacetic acid	—	03	—	+	07	—	60	26 23	44 71	++	+	26 27	27 30
86	74	63	54	15	05	5-Methoxy-3-indolyl-acetamide	—	—	—	—	07	—	—	—	71	+	—	26 27	27 24
42	45	00	00	00	00	Harmalol	39	17 64	64	71 67	64	43 71	—	—	71	—	—	—	—
52	88	02	12	00	00	Harmine	32	—	—	—	—	—	—	57	03 71	—	—	—	+
62	88	05	15	00	00	Harmane	33	—	—	—	—	—	—	—	—	—	—	—	—
80	91	16	47	00	00	Ergotamine	34	—	—	—	—	+	—	—	—	+	—	26 70	+

TABLE XII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF MISCELLANEOUS COMPOUNDS

R_F values × 100						Compounds	Detection											
F	E	A	B	C	D		U.V.	D_1	D_2	D_3	D_4	DB	DN	Fe	Mo	Mn	Ind	EH	DAC
17	65	00	01	00	00	4-Aminopyridine	—	—	—	60	59	—	—	59	07, 72	—	33	06	17, 23
28	77	04	23	00	00	2-Amino-4-picoline	34	—	—	62	—	—	—	57	08, 72	—	33	—	22
38	80	04	22	00	00	2-Amino-6-picoline	34	06	58	62	09	+	—	58	09, 72	—	33	+	17
95	03	00	94	00	00	Bilirubin	58	64	41, 25	44, 68	—	48	—	—	71	+	—	+	07
95	00	00	95	00	00	Biliverdin	34	—	—	—	—	71	—	—	71	—	—	—	—
10	00	00	00	00	00	Folic acid	44	—	—	60	—	—	—	—	—	+	+	—	17
92	90	87	74	63	11	7-Hydroxycoumarin	39	11	18, 23	23, 63	59	—	—	—	44, 71	++	—	—	—
64	63	60	23	14	00	Isoniazid = isonicotinoyl-hydrazine	05	—	59	65	03, 64	55	—	60	45, 68	++	33	58	64
70	91	17	29	46	00	Dromoran	—	09	—	—	—	—	—	—	71	+	—	—	—
81	64	42	88	22	15	Chlorpromazine	39	—	—	—	60	52	—	17	18, 49	++	—	—	—
88	80	73	92	53	33	Phenothiazine-5-oxide	—	—	—	—	—	—	42	41	41, 30	+	—	59, 39	57, 56
76	90	70	95	79	73	Acridine	07	58	—	—	+	—	—	03	03, 71	—	—	58	—
79	42	03	00	00	00	2-Ethoxy-6,9-diamino-acridine = rivanol	48	09	64	63	62	—	—	—	—	++	—	12	65
47	92	01	07	00	00	Atebrin = quinacrine	03	—	—	—	64	—	—	—	—	+	—	—	60

TABLE XIII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME HIPPURIC ACID DERIVATIVES

R_F values × 100						Compounds	Detection												
F	E	A	B	C	D		U.V.	D_1	D_2	D_3	D_4	DB	DAB*	Fe	Mo	Mn	Ind	EH	DAC
86	06	76	83	34	05	o-Methoxyphenaceturic acid	—	—	—	—	—	—	05	—	—	—	+	—	—
85	05	76	10	05	00	2,3-Dihydroxyhippuric acid	30	08	62	62	10	28	13	28, 70	68	++	+	—	—
84	04	62	02	01	00	2,5-Dihydroxyhippuric acid	33	—	—	—	—	—	12, 13	70	30	+	+	—	—
76	02	43	09	01	00	Vanilloylglycine	—	08	—	65	08, 65	28	10	71	70	++	+	—	—
69	02	24	00	00	00	Caffeoylglycine	33	—	63	58	08, 63	+	17	03, 51	69	++	+	—	—
76	02	45	13	01	00	Feruoylglycine	33	06, 24	23, 13	23, 43	13	25	17, 15	—	69	++	+	—	—
85	05	65	34	08	01	Anthranylglycine	33	—	—	56	08	—	13, 17	—	—	++	+	07	22
92	89	87	92	77	67	o-Aminohippuric acid methyl ester	33	—	—	—	06	—	06	—	—	+	—	06	22
91	08	84	18	01	00	3-Hydroxyanthranylglycine	38	—	—	17	58	43	06	56	43, 69	++	+	05	22
85	05	73	26	07	00	Indole-3-acetylglycine	—	—	—	—	07	—	—	—	—	++	+	28	27
77	03	28	01	00	00	5-Hydroxyindole-3-acetyl-glycine	—	63	24	25	63, 65	71, 27	—	59	70	++	+	71, 28	30, 33
91	06	74	14	03	00	Indole-3-acrylglycine	34	06	63	23	58	—	17	—	70	++	+	13	23
90	07	87	82	58	10	Quinaldylglycine	34	—	—	—	—	—	22	—	—	+	+	—	—

* DAB = hippuric acid reagent. DN reagent gave no reaction with the compounds listed in this table.

TABLE XIV

PAPER-CHROMATOGRAPHIC SEPARATION OF SOME 2,4-DINITROPHENYLAMINO ACIDS

R_F values × 100						Compounds	Detection
F	E	A	B	C	D		U.V.
94	40	92	73	83	35	DNP-alanine	+
93	18	90	85	82	33	DNP-β-alanine	+
92	29	93	92	91	46	DNP-β-aminobutyric acid	+
56	02	00	00	00	00	DNP-arginine	+
88	09	72	03	02	00	DNP-asparagine	+
94	01	89	10	24	00	DNP-aspartic acid	+
87	14	53	02	00	00	DNP-citrulline	+
90	12	68	05	04	00	DNP-glutamine	+
91	26	91	48	73	08	DNP-glycine	+
87	58	00	00	00	00	DNP-histidine	+
91	65	92	92	91	55	DNP-isoleucine	+
92	56	92	92	90	43	DNP-methionine	+
92	68	92	92	91	56	DNP-norleucine	+
92	57	92	92	91	51	DNP-norvaline	+
94	78	93	84	72	05	DNP-ornithine	+
94	68	93	92	91	49	DNP-phenylalanine	+
92	24	88	06	24	01	DNP-serine	+
93	85	93	92	90	33	DNP-tyrosine	+

TABLE XV

PAPER-CHROMATOGRAPHIC SEPARATION OF PHENYLTHIOHYDANTOIN DERIVATIVES OF SOME AMINO ACIDS

R_F values × 100						Compounds	Detection
F	E	A	B	C	D		DB
91	91	90	90	88	83	PTH-alanine	18
92	17	92	81	50	10	PTH-β-aminobutyric acid	18
65	14	03	00	00	00	PTH-arginine	18
85	84	66	44	11	00	PTH-asparagine	18
91	10	88	63	38	02	PTH-aspartic acid	18
83	32	56	37	05	00	PTH-citrulline	18
84	80	68	54	11	01	PTH-glutamine	18
92	92	91	59	35	10	PTH-glycine	62, 18
92	92	91	85	82	64	PTH-hydroxyproline	64
93	92	92	93	93	89	PTH-isoleucine	58
93	92	92	92	90	90	PTH-leucine	58
92	91	92	92	93	88	PTH-methionine	58
92	55	91	85	33	02	PTH-ornithine	18
94	93	92	92	80	16	PTH-phenylalanine	62, 18
92	90	90	81	59	10	PTH-proline	58, 18
90	90	88	57	36	07	PTH-serine	64
91	93	92	84	80	29	PTH-tyrosine	62

TABLE XVI

COMPARISON OF COLOUR REACTIONS FOR SOME INDOLE DERIVATIVES WITH DIFFERENT MODIFIED EHRLICH REAGENTS

Compounds	Detection					Exposure in air
	EH	DAC	E3	E4	E5	
	in 1 N HCl		in conc. HCl			
Indole*	24	28, 33	19, 23	65	19	62
2-Methylindole*	22	35, 25	15, 22	19	19	62
5-Methylindole*	22	23, 28	+	+	59	+
Indoxyl acetate*	57	23	23	69	23, 56	23
Indican = indoxyl-3-sulphate ester	63	26, 23	60	51	52, 23	34
5-Hydroxyindole	23	43, 28	23	23	23	56
5-Methoxyindole*	24, 20	38, 28	23	23	23	62
5-Benzyloxyindole*	18, 22	40, 25	19	23	23	63
Isatin*	—	62	—	+	—	—
Indole-3-carbinol	58, 17	24	62	56	+	60
Tryptophol = indole-3-ethanol	07, 63	23, 25	65, 51	65	65, 35	58
3-Indolealdehyde*	26	22, 63	+	46	57	+
5-Methoxy-3-indolealdehyde*	26	26	57	46	57	+
α-(3-Indolyl)-acetic acid*	60, 70	22	63	69	63	58
3-Indolylacetic acid methyl ester	18, 69	24, 25	65, 51	65, 68	24, 65	58
3-Indolylacetamide	26, 69	23, 25	23	42	23	—
5-Hydroxy-3-indolylacetic acid*	68	24	69	43	70	63
5-Methoxy-3-indolylacetic acid	26, 27	27, 30	25, 28	28	25, 28	57
5-Hydroxy-3-indolylacetamide	51	25, 28	69, 43	42	69	58
5-Methoxy-3-indolylacetamide	26, 27	27, 24	25, 28	23, 38	25, 28	+
5-Chloro-2-carboxy-indole	—	26	22, 24	41	17, 26	—
5-Methoxy-2-carboxy-indole*	26, 25	70	25, 69	28, 70	23, 27	+
Indole-5-carboxylic acid	23	30	22	23	22, 23	+
3-Indolylglyoxylic acid	—	—	—	46	60	+
β-(3-Indolyl)-propionic acid*	23, 71	23	23, 46	28	23, 71	+
γ-(3-Indolyl)-butyric acid*	23, 70	23	24	41	24, 71	—
DL-2-(3-Indolyl)-lactic acid*	26	25	23, 39	23, 41	23, 27	—
3-Indolylpyruvic acid	17	23, 65	—	46	60	+
3-Indolylacrylic acid	40	23	62	46	62	18

(continued on p. 490)

TABLE XVI (*continued*)

Compounds	Detection					Exposure in air
	EH	DAC	E_3	E_4	E_5	
	in 1 N HCl		in conc. HCl			
L-Tryptophan*	57	23	23 40	28	23	—
DL-Acetyltryptophan*	60 26	25 22	23 38	25 28	23 37	—
L-Tryptophan methyl ester	03	23 25	23 41	25 47	23 44	—
DL-4-Methyltryptophan*	—	23	23	28	23	—
DL-4-Hydroxytryptophan*	71	26 52	68	46	68	+
DL-5-Methyltryptophan*	—	23 25	—	44	—	—
DL-5-Hydroxytryptophan*	—	26	28 41	38	25 28	+
Gramine*	57	22	62	46	62	—
Gramine-N-oxide	58	22 24	60	+	60	+
5-Methoxygramine	04	26 71	—	46	—	—
Tryptamine*	70 51	23	23 51	28	23	+
N-Acetyltryptamine	08 24	24 25	23 51	28	23	+
5-Fluorotryptamine	17	24 27	23	+	62	—
5-Hydroxytryptamine = serotonine*	71	26	25 51	41	25	+
5-Methoxytryptamine	26	27 25	28 41	28	23 25	04
N-Acetyl-5-hydroxytryptamine	69	30 43	23	42	23	—
N-Acetyl-5-methoxytryptamine = melantonin	26	25 28	28 38	28	23 25	60
N-Methyltryptamine	17 71	23 25	23 38	25	23 30	—
N,N-Dimethyltryptamine	58 23	23	23 38	25	23 30	58
N,N-Diethyltryptamine	17 23	23	23 38	25	23 30	+
Bufotenine = 5-hydroxy-N,N-dimethyl-tryptamine*	27 71	30 25	26	46	26	—
Psylocybin	16 26	27	23 40	43 38	18	—
Indole-3-acetylglycine	28	27	23	42	23	—
Indole-3-acrylglycine	13	23	60	46	60	—
5-Hydroxyindole-3-acetylglycine	71 28	30 33	69	46	70	58

For compounds marked with an asterisk (*) other chromatographic data can be found in ref. 2, while for the others further data are given in Tables XI and XIII of this paper.

TABLE XVII

SOME COLOUR REACTIONS FOR ALDEHYDES DETECTED WITH DIFFERENT AMINES

Compounds	*o*-Phenylenediamine	*m*-Phenylenediamine	*p*-Phenylenediamine	2,4-Diaminophenol	*p*-Anisidine	*o*-Dianisidine	*o*-Toluidine	*p*-Toluidine	α-Naphthylamine	β-Naphthylamine	Benzidine	*o*-Tolidine	Dimethyl-*p*-phenylenediamine	*p*-Aminoacetophenone	4-Aminoantipyrine	1,2-Diaminonaphthalene	2,7-Diaminofluorene
Benzaldehyde*	—	—	—	—	+ / —	—	—	—	—	05 / —	—	—	—	—	60	18 / —	04 / —
p-Tolualdehyde*	—	—	—	—	+ / —	—	—	—	—	05 / —	05 / —	—	—	—	+ / —	18 / —	04 / —
Cumaldehyde*	—	—	—	—	05 / —	—	—	—	—	05 / —	05 / —	—	—	—	+ / —	—	04 / —
o-Chlorobenzaldehyde	+ / 18 / —	—	—	05 / —	05	05 / —	—	— / 05	—	— / 05 / —	— / 05 / —	05 / —	—	—	06 / —	—	—
m-Chlorobenzaldehyde*	—	—	—	—	+	—	—	—	—	—	05 / —	—	—	—	+ / —	18 / —	—
p-Chlorobenzaldehyde**	—	—	—	—	05 / —	—	—	—	—	05 / —	05 / —	—	—	—	+ / —	18 / —	04 / —
m-Nitrobenzaldehyde*	—	—	+	—	05 / —	—	—	—	—	05 / —	—	— / 05	— / 64	—	+ / —	—	04 / —
o-Phthalaldehyde*	—	70	52 / 53	27 / 69	51 / 53	70	23 / 51	51 / 53	63 / 53	51 / 52	52 / 70	18 / 70	52 / 51	59 / —	50 / 52	— / 70	51 / 70
Terephthalaldehyde**	—	—	— / 58 / 63	— / 05	06 / 08	— / 05 / 62	— / 05	— / 05	— / 05 / —	07 / 08	— / 08 / 62	— / 07	— / 23	—	09	06	58 / 65
o-Carboxybenzaldehyde**	—	—	—	—	— / 05 / —	—	—	—	—	—	— / 05 / —	—	—	—	—	—	+
Cinnamaldehyde*	09 / 60	06 / 60	10 / 62	08 / —	07	11 / 12	05	06	08	07	09	08	13 / 63	06 / —	08	23	09 / 12
3-Indolealdehyde*	05 / —	06	07 / —	05 / —	05	05 / 07	05 / —	05	— / 05	05 / 03	06	05	05 / 09	06 / —	06 / 60	08	05 / 08
Salicylaldehyde*	06 / —	06 / —	07 / —	06 / —	06	09 / 56	05	05 / —	07 / —	05 / —	06 / —	07 / —	+ / —	05 / 15	06 / —	08 / 64	06

(continued on p. 492)

TABLE XVII (*continued*)

Compounds	*o*-Phenylenediamine	*m*-Phenylenediamine	*p*-Phenylenediamine	2,4-Diaminophenol	*p*-Anisidine	*o*-Dianisidine	*o*-Toluidine	*p*-Toluidine	α-Naphthylamine	β-Naphthylamine	Benzidine	*o*-Tolidine	Dimethyl-*p*-phenylenediamine	*p*-Aminoacetophenone	4-Aminoantipyrine	1,2-Diaminonaphthalene	2,7-Diaminofluorene
3-Methylsalicylaldehyde*	—	—	+ —	05 —	05 —	06 —	05 —	—	—	05 —	05 —	05 —	—	05 12	58	—	06
5-Formylsalicylic acid**	—	—	—	—	— 05	+ —	—	—	—	05 —	05 07	+ 05	— 17	05 —	58 —	—	06
3-Hydroxybenzaldehyde*	—	—	— 62	—	04	05 —	05 —	—	—	05 —	06	—	+ 14	04 62	06 —	18 —	06 08
4-Hydroxybenzaldehyde*	06 —	06 —	07 63	07	05	08	06 —	06	05 —	06	06	05	58 14	05 62	06 —	—	— 07
4-Methoxybenzaldehyde*	04 —	—	08 64	07 —	06	08	05 —	05	05 —	06	07 —	05	— 14	05 62	06 —	62 —	08
5-Methoxy-3-indolealdehyde**	05 —	06	08 62	07	— 05	05 08	05	05	—	05	06	05	06 62	06 —	06 —	— 08	06 08
Protocatechualdehyde*	05 ·	06	08 62	07	06	08	05	05	05 —	06	07 08	05	06 13	05 62	06 —	—	07 08
Piperonal*	04 —	04 —	07 62	06 —	06	05 —	05 —	06	04 —		07 08	05	05 14	05 62	06	—	08
Veratraldehyde*	04 —	04 —	09 62	06 —	06	05 07	05 —	05	05 —	04 —	08	05	05 14	05 60	06	—	08 09
o-Veratraldehyde*	05 —	05 —	09 64	06 —	06	05 07	04 —	05	05 ·	04 —	08 09	06	06 14	05 60	06	—	08 09
2-Acetoxy-3-methoxybenzaldehyde*	04 —	—	+ 62	—	04	05 07	04 —	04	04 —	—	05 07	05 07	— 13	— 13	—	—	06 08
Coniferyl aldehyde*	58 —	58 —	08 62	08 —	58 —	13	06	07	08 —	08 —	09	09	07 17	08 —	58 —	62 —	09
o-Vanillin*	06 —	06 —	08 64	08 62	06	09 62	06 07	06	06 —	06 —	08	09 12	07 14	07 19	06	—	09
Isovanillin*	06 —	06 —	08 64	07 62	06	08	06	06	06 —	06 —	07	07	06 14	06 64	06	—	07 08
Vanillin*	06	06	09 62	08 62	06	05 07	05	06	06 —	07	08	05	05 13	04 60	07	—	08 09
3-Ethoxy-4-hydroxybenzaldehyde**	06	06	09 62	08 62	06	05 07	05	06	06 —	08	08	05	05 13	05 60	08	—	08

5-Propenylvanillin	06	06	09 62	08 62	06	06	05	06	06 —	08	08	05	05 13	05 60	06	—	08 09
5,6-Dimethoxy-2-formylbenzoic acid**	—	—	—	—	— 05	—	—	—	—	—	—	—	—	—	+	—	+
4-Acetoxy-5-methoxyisophthalaldehyde*	08 17	58 09	64 65	09 64	07 09	09 19	07	06 09	07 09	08 11	11 14	09 11	06 63	08 62	06 08	62	10 19
5-Methoxy-6-hydroxyisophthalaldehydic acid*	+	—	62 —	58 —	05 07	08 63	05	05	—	07	09	06	— 62	58 —	58 —	—	58 13
4-Methoxy-5-hydroxyisophthalaldehydic acid*	—	—	—	—	05	05 07	— 05	— 05	—	— 05	06 —	06	—	05 —	58 —	—	05 60
2,4-Dihydroxybenzaldehyde*	06	03 08	08 62	07 09	06	08 09	05	05	06	07	07	08	58 12	06 14	03	— 08	07 09
4-Methyl-2,6-dihydroxybenzaldehyde*	—	—	+ —	+ —	—	05	—	—	04 —	04 —	05 —	05	— 17	04 13	58 —	—	05 —
Orsellinic aldehyde**	05 06	05 06	08 09	06	05	06 09	05	05	05 03	05	06	06	58 09	05 15	06	— 07	06 08
2,4-Dimethoxybenzaldehyde*	05	05	09 —	07 —	06	10	05	05	05 03	05	08	09 10	58 13	05 08	07	— 07	09
2,5-Dihydroxybenzaldehyde**	07	59	10 63	08 64	07	11	08	07	07 62	08 62	09 14	09 12	60 64	09 19	07	—	09 10
2,5-Dimethoxybenzaldehyde**	06 —	06	10 63	08 62	07	12	07	07	07 —	08 62	09 13	09 12	07 64	08	07	63 —	12
Phloroglucinaldehyde*	05	05	07 —	06 —	05	07	05	05	05 03	05	06	06	58 08	06 62	07 03	08	06
2,4,6-Trimethoxybenzaldehyde*	—	—	05 —	06 —	05	07	05	05	04 —	04 —	06	06	— 17	04	58 07	—	05 06
3,4-Dihydroxy-5-methoxybenzaldehyde	06 —	03	14 64	07	06	09	02	06	— 03	07	09	06	06 62	06	06	—	08 09
Trimethoxygallaldehyde*	—	—	08 62	—	04	05 —	—	—	—	04 —	08	—	— 62	— 05	—	—	06 08
p-Dimethylaminobenzaldehyde	09	09	15	12	09	11 14 20	06	08	08 60	10	15	11	08 62	09	08	08 09	12 14
p-Dimethylaminocinnamaldehyde	19 64	19	65 25	19	14 19	23 65	14 19	14 19	22 19	19	19 65	22 23	15 23	65	12	23	19 65
2-Chloro-4-dimethylaminobenzaldehyde	09	09	12 15	10	09	09 14	06	08	08 60	10	12	11	08 63	09 64	08	09	12

The first number indicates instantaneous colour development, or during one minute. The second number shows the colour change after one hour. The third number, when present, gives the change during 24 hours. If only one or two numbers are present, no change has been noticed. The sign — indicates no detection or that the colour becomes identical with that of the background. Further identification data for the compounds marked by * and ** can be found in ref. 1 and ref. 2 respectively, while for the other compounds such data is given in this paper.

RESULTS AND DISCUSSION

As mentioned earlier, the number of standard reagents has been increased to twelve, including two Ehrlich reagents. This provides information as to the possible interference from compounds which are not usually expected to react with Ehrlich reagent and yet give some unusual colour reactions. In Table II we find two of the pyrocatechols, which show a light but definite reaction. Further exceptions are: 2,6-dihydroxypyridine (Table III), 1,2,4-trihydroxybenzene and 2,4,6-trihydroxybenzoic acid (Table V), and isoniazid and phenothiazine-N-oxide (Table XII). 2,4,6-Trihydroxybenzoic acid gives shades very similar to indole compounds, *e.g.* red-violet and blue; the others, however, have different red and brown colours. The colour formation of Ehrlich reagent with phloroglucinol has been observed earlier by STEELNIK[9]; some resorcinol derivatives with free 4 or 6 position give purple Ehrlich reaction[10].

The colour patterns with diazonium reagents for 1,2-, 1,3- and 1,4-dihydric phenol derivatives (Tables II–IV) do not appear as clearly as they did earlier. But since only a few of these compounds have been listed this time, results obtained previously should be taken into consideration when evaluating these patterns. The general picture still holds, even for these compounds, indicating three distinct colour patterns. Hydroxylated naphthalenes and also 5-hydroxyisoquinoline (Table VI), show typical strong coloured patterns with diazonium reagents, as observed before. From Table XIII it seems that glycine-conjugates with nitrogen-containing heterocyclic carboxylic acids have a tendency to give strong red to magenta colours with hippuric acid reagent. The example quoted here is quinaldic acid glycine-conjugate; earlier it was nicotinuric acid.

In Table XV the DB reagent has been used to detect amino acid phenylthiohydantoins. The colours produced varied between pink and brown. It has been noticed that certain thio-derivatives, such as thiosalicylic acid, thiobenzoic acid and mercaptopyridines, give similar types of colours including yellow, orange and brown shades, thus a characteristic difference between hydroxylated aromatic and heterocyclic compounds is established. The latter compounds have variations only in red-violet, violet, blue and green shades.

Data obtained by spraying indole derivatives with five modifications of Ehrlich reagent are collected in Table XVI. It is obvious that these values can only serve as guide, because a variety of colours were observed in several cases. The main feature seems to be that purple and red-violet colours are produced with indole derivatives substituted in the 2- or 3-position (the 5-position being free). Blue shades appear to be more dominant with 5-hydroxylation or methoxylation. However, there is some overlapping in the case of N-alkylated tryptamines which also give blue colours. Indican, indole-3-carbinol, gramine, gramine-N-oxide and indole-3-acrylglycine tend to show brown spots. A column was also included in Table XVI in order to demonstrate that untreated spots became visible after one week and were coloured owing to the oxidation of the compounds in the air.

In Table XVII the results of colour reactions with aromatic amines were recorded for 47 aromatic aldehydes. The colour development at room temperature was in most cases instantaneous. However, on standing, a slow increase in the intensity of the colours was observed. Yellow to orange colours dominated the overall picture, but in a few compounds red and violet colours occurred. The best reagents were: *p*-phenylenediamine, *p*-anisidine, *o*-dianisidine, *β*-naphthylamine, benzidine, dimethyl-*p*-phe-

nylenediamine, *p*-aminoacetophenone and 2,7-diaminofluorene. Among the aldehydes tested *o*-phthalaldehyde showed green and grey colours; other compounds which were relatively more reactive were cinnamaldehyde, 2,5-dihydroxybenzaldehyde and 4-acetoxy-5-methoxyisophthalaldehyde. The last three aldehydes in Table XVII are constituents of Ehrlich reagents, consequently the code is also useful for detecting aromatic amines by spraying with Ehrlich reagents EH, DAC and 2-Chloro-EH (as abbreviated in this paper).

As regards the distribution of the R_F values in the solvent systems, it is worth noting the interesting behaviour of the pyridine derivatives. Following the R_F values in the order as presented in the tables we find two maximum values for these derivatives, one for solvent E (or F) and another for solvent B according to the distribution criterion R_F in E $>$ R_F in A $<$ R_F in B. This double peak is more clearly demonstrated if the values are visualised in a diagram. This regularity has been observed earlier[2] and has occurred when monohydroxy derivatives of nitrogen-containing heterocyclic compounds were involved. Pyridines, quinolines, isoquinolines, but not indoles and imidazoles, seem to belong to this group; of the more complex ring structures, alkaloids behaved in the same way. In this paper 2- and 4-mercaptopyridines (Table I), 2,6-dihydroxypyridine (Table III), 5-hydroxyisoquinoline (Table VI), 3-pyridylacetic acid (Table VIII), 2-amino-4-methylpyridine and 2-amino-6-methylpyridine (Table XII), follow this rule, together with other heterocyclic compounds like chlorpromazine, phenothiazine-5-oxide, acridine and atebrin (Table XII) and harmine, harmane and ergotamine (Table XI). Of the approximately 60 simple indole derivatives investigated by this procedure, only N-acetyl-5-methoxytryptamine shows that a similar distribution of the R_F values in the solvents is due to the R_F-elevating effect of methylation in solvent B of the parent compound N-acetylhydroxytryptamine. The similar effect is also noted in the case of *p*-anisidine and *o*-dianisidine which are the exceptions, since the non-methoxylated corresponding amino phenols behave normally, gradually lowering the R_F values towards the solvents A, B, C and D. 3-Indolepyruvic acid, which shows another maximum R_F value in C is not considered to interfere in this reasoning, it even satisfies the distribution of R_F values according to E $>$ A $<$ B. It is also interesting to note an addition to the group of compounds which show a jump of R_F values between solvent B and solvent C (R_F in B $<$ R_F in C), *viz.* 5-hydroxyindole, which was found to behave like some of the resorcinol and hydroquinone derivatives.

In general the R_F values were in accordance with previous observations giving low values for free phenolic carboxylic acids in solvent E and high values in F and A slowly decreasing the values in solvents B, C and D. Amino derivatives of phenols showed relatively high values in solvents F, E and A; thereafter much lower values in B, C and D. Aromatic amines have, as a rule, low to medium values in solvent F, followed by a maximum value in solvent E, continuing rapidly to low values in A, B, C and D. N-Acetylation has an elevating effect on all values, except those from solvent E. Imidazole derivatives are practically immobile in these solvents.

In Tables XIV and XV the R_F values are recorded for some 2,4-dinitrophenyl and phenylthiohydantoin derivatives of amino acids. Although the solvents are not very suitable for the separation of these compounds, causing the spots to diffuse, they might be used in some cases. The R_F values should be regarded as approximate and merely as indicators of their relative rates of movement.

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SUMMARY

Paper-chromatographic data are presented for an additional approx. 170 compounds in six solvent systems together with their colour reactions with twelve standard reagents. The main types of compounds presented in 15 tables are: mono-, di- and trihydric phenol derivatives, naphthalene derivatives, biogenic amines, indole derivatives and hippuric acids. Thirteen hippuric acid derivatives were synthetized for this purpose. The relationship between the colour reactions and the chemical structure of the compounds on the one hand, and the connection between the R_F values and chemical structure on the other hand, is discussed. Special attention was devoted to the detection and characterisation of aromatic aldehydes and indole derivatives, whereby an additional number of reagents was used. For indoles the following reagents were found to be useful in distinguishing between different compounds with the same R_F value: *p*-N,N-bis(2-chloroethyl)-aminobenzaldehyde, 2-chloro-4-N,N-bis(2-chloroethyl)-aminobenzaldehyde and 4-N,N-bis(2-chloroethyl)-amino-2-tolualdehyde. For detection of PTH-amino acid derivatives DB reagent (2,6-dibromoquinone-4-chloroimide) was found suitable, producing red and brown colours.

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SUR LE MARQUAGE DU DIPHÉNYL-MERCURE PAR ^{203}Hg: ÉTUDE PAR CHROMATOGRAPHIE SUR PAPIER DU SYSTÈME $Hg(C_6H_5)_2$–$^{203}HgCl_2$

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INTRODUCTION

La synthèse chimique de molécules marquées par des atomes radioactifs peut parfois être évitée grâce à des réactions d'échange isotopique. C'est ainsi, par exemple, que des hydrocarbures aromatiques ont été marqués par le tritium par échange isotopique avec de l'eau tritiée. Cette technique ne semble pas encore avoir été utilisée pour le marquage de composés organométalliques par des atomes radioactifs du métal. Pourtant, dans certaines conditions, il est possible d'obtenir par cette méthode des produits marqués avec un excellent rendement, c'est le cas en particulier des composés organomercuriques.

Les réactions d'échange isotopique du mercure entre des composés organomercuriques, leurs sels et des composés minéraux du mercure ont fait l'objet de plusieurs travaux. GROSS ET PINAJIAN[1], les premiers, ont étudié l'échange du mercure entre l'acétate de phényl-mercure et l'acétate mercurique marqué par ^{203}Hg. Ces auteurs auraient observé un échange très rapide dans l'acide acétique glacial, l'eau, l'éthanol à 50% et le benzène; l'échange du mercure entre le chlorure de phényl-mercure et le chlorure mercurique serait plus lent. Ces résultats ont été réfutés par BELMONDI ET ANSALONI[2], puis par ANSALONI, BELMONDI ET CROATTO[3]. Selon ces deux groupes d'auteurs, c'est la technique de séparation par l'eau des composés organiques et minéraux du mercure, utilisée par GROSS ET PINAJIAN[1], qui induit un échange extremement rapide du mercure.

REUTOV et ses collaborateurs ont étudié systématiquement les réactions d'échange isotopique du mercure en chimie organométallique[4–8] en employant généralement des techniques de séparation chimiques courantes, bien que les avantages de la chromatographie sur papier eussent été soulignés par REUTOV ET SOKOLOV[9]: simplicité, gain de temps appréciable, possibilité de travailler avec de petites quantités de produits marqués. La chromatographie sur papier a été utilisée pour l'étude cinétique de l'échange du mercure entre des dérivés du bromure de benzyl-mercure et le bromure mercurique[9].

Nous nous sommes proposés d'étudier par chromatographie sur papier les conditions du marquage du diphényl-mercure $Hg(C_6H_5)_2$ (en abrégé $HgPh_2$) par le mercure radioactif ^{203}Hg (T = 46 jours; β 0.21 MeV, γ 0.279 MeV) en présence de $^{203}HgCl_2$.

La séparation de composés organomercuriques par chromatographie sur papier a été réalisée la première fois par KANAZAWA *et al.*[10] qui ont utilisé comme solvant du butanol saturé avec une solution d'ammoniaque N. Avec cet éluant le R_F du chlorure de phényl-mercure C_6H_5HgCl (ou PhHgCl) est égal à 0.40. Dans le travail déjà cité[9] le bromure mercurique ($R_F = 0$) est séparé des organomercuriques ($R_F = 1$) par un mélange octane–benzène (3:2) et un papier imprégné d'une solution à 10 % d'éthylène glycol dans l'acétone.

BRODERSEN ET SCHLENKER[11] ont utilisé un mélange de tétrahydrofurane, butanol et ammoniaque N (15:35:20) pour séparer les bromures de phényl-mercure et de *p*-méthoxyphényl-mercure, ainsi que des halogénures d'alcoyl-mercure dont les premiers termes ont également été séparés par chromatographie en phase gazeuse. Selon BARTLETT ET CURTIS[12], le meilleur solvant est un mélange de butanol–éthanol–ammoniaque (8:1:3) avec lequel le R_F du chlorure de phényl-mercure est 0.39 tandis que les sels minéraux du mercure restent fixés au point dépôt. Nous avons employé ce dernier solvant qui avait déjà été utilisé avec succès pour l'analyse des produits de décomposition radiolytique du diphényl-mercure[13].

ANALYSE CHROMATOGRAPHIQUE DU SYSTÈME $HgPh_2$–$^{203}HgCl_2$

En milieu homogène non aqueux, le diphényl-mercure est rapidement marqué par ^{203}Hg si le système contient du chlorure mercurique radioactif $^{203}HgCl_2$. C'est ainsi qu'à partir d'une solution dans le mélange alcool–benzène (1:1) de $HgPh_2$ $2 \cdot 10^{-2}$ M et $HgCl_2$ $2.5 \cdot 10^{-4}$ M il est possible de recueillir au bout de quelques minutes, à la température de 40°, un composé dont le point de fusion de 122° est caractéristique de $HgPh_2$ est qui entraîne 96 % de l'activité totale, chiffre proche de la valeur théorique à l'équilibre d'un échange isotopique apparent selon la réaction:

$$HgPh_2 + {}^{203}HgCl_2 \rightleftharpoons {}^{203}HgPh_2 + HgCl_2 \qquad (1)$$

Un résultat identique est obtenu à partir d'une solution de $HgPh_2$ et $Hg(NO_3)_2$ marqué.

Cependant, pour un mélange équimoléculaire de $HgPh_2$ et $HgCl_2$ on observe la formation d'un nouveau composé également marqué, le chlorure de phényl-mercure (point de fusion 251°):

$$HgPh_2 + {}^{203}HgCl_2 \longrightarrow 2\ Ph^{203}HgCl \qquad (2)$$

Une réaction d'échange isotopique du type (1) avec substitution directe des atomes de mercure nécessitant des ruptures de liaison entre le mercure et les radicaux phényles est peu probable, et la réaction (2) doit être une étape décisive dans le marquage de $HgPh_2$. Cette hypothèse a été verifiée par l'analyse chromatographique du mélange $HgPh_2$–$^{203}HgCl_2$ pour plusieurs concentrations initiales en $HgCl_2$ lorsque la réaction de marquage est achevée, c'est-à-dire après 45 min de contact à 40°.

A cet effet, on dépose une goutte de la solution de $HgPh_2 + HgCl_2$ dans le mélange alcool–benzène, sur une bande de papier Whatman No. 1 (20 cm × 2.7 cm) et effectue une chromatographie ascendante, à la température de 25°, avec le solvant butanol–éthanol–ammoniaque (8:1:3). Le front de l'éluant se déplace de 15 cm en 6 h

environ. Après séchage, la bande de papier est découpée cm par cm et l'activité des divers échantillons est déterminée à l'aide d'un cristal-puits NaI (Tl). Les valeurs moyennes des R_F sont: $HgCl_2$ 0.04; $HgPh_2$ 1.0; PhHgCl 0.39.

Les radiochromatogrammes de la Fig. 1 montrent nettement la formation de chlorure de phényl-mercure marqué quel que soit le rapport des concentrations de

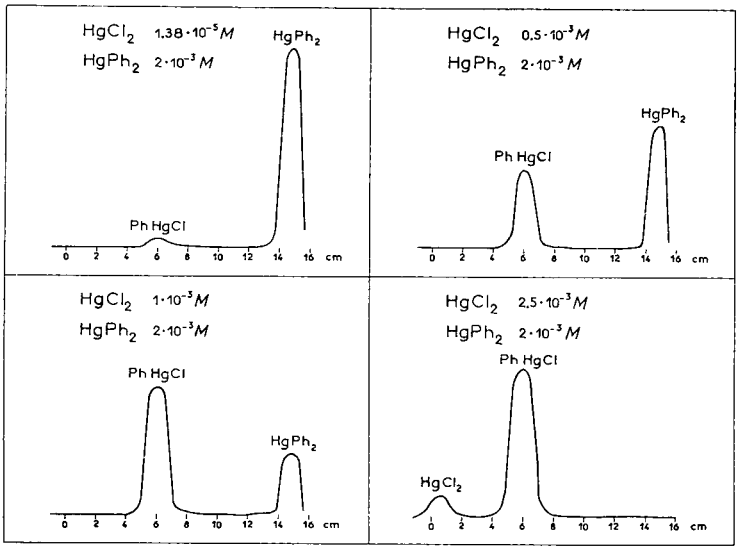

Fig. 1. Radiochromatogrammes de solutions de $HgPh_2$–$^{203}HgCl_2$.

$HgPh_2$ et $HgCl_2$. Pour toutes les expériences la concentration initiale de $HgPh_2$ est $2 \cdot 10^{-3}$ M, celle du chlorure mercurique varie entre $1.4 \cdot 10^{-5}$ M et $2.5 \cdot 10^{-3}$ M.

La réaction (2) constitue la première étape du marquage de $HgPh_2$; elle est suivie de la réaction d'échange isotopique:

$$Ph^{203}HgCl + HgPh_2 \rightleftharpoons PhHgCl + {}^{203}HgPh_2 \qquad (3)$$

Le partage de ^{203}Hg entre $HgPh_2$ et PhHgCl déduit des courbes de la Fig. 1 est en bon accord avec les valeurs théoriques que l'on calcule pour l'équilibre de la réaction d'échange (3) succédant à la réaction (2) (Tableau I).

Lorsque le chlorure mercurique est en excès par rapport au diphényl-mercure ($HgCl_2$ $2.5 \cdot 10^{-3}$ M) on observe sur le chromatogramme un pic de $R_F = 0.04$ attribué à $^{203}HgCl_2$. Cet excès de réactif a pour conséquence une nouvelle réaction d'échange isotopique:

$$Ph^{203}HgCl + HgCl_2 \rightleftharpoons PhHgCl + {}^{203}HgCl_2 \qquad (4)$$

dont le mécanisme a été étudié par ANSALONI *et al.*[3]: dans le benzène saturé d'eau la durée de demi-échange, à 40° et pour des concentrations $0.3 \cdot 10^{-2}$ M est de 45 min; l'énergie d'activation de la réaction est de 3.5 ± 0.3 kcal/mole. Le calcul du partage

TABLEAU I

PARTAGE DE ^{203}Hg ENTRE LES COMPOSÉS ORGANOMERCURIQUES À L'ÉQUILIBRE

Concentration initiale en $HgPh_2$ $2 \cdot 10^{-3}$ M. Les chiffres entre parenthèses représentent les valeurs expérimentales. Durée de la réaction 45 min; température 40°.

Concentration initiale en $HgCl_2$	*% de ^{203}Hg*	
	$HgPh_2$	*$PhHgCl$*
$1.4 \cdot 10^{-5}$ M	98.7 (93.5)	1.3 (6.5)
$0.5 \cdot 10^{-3}$ M	60 (57)	40 (40.9)
$1 \cdot 10^{-3}$ M	33.3 (33.7)	66.7 (65)
$2.5 \cdot 10^{-3}$ M	— (0)	— (85.6)

TABLEAU I BIS

Concentration initiale en $HgPh_2$ $2 \cdot 10^{-3}$ M. Les chiffres entre parenthèses représentent les valeurs expérimentales. Durée de la réaction 45 min; température 40°.

Concentration initiale en $Hg(NO_3)_2$	*% de ^{203}Hg*	
	$HgPh_2$	*$PhHgNO_3$*
$0.5 \cdot 10^{-3}$ M	60 (53)	40 (43.1)
$1 \cdot 10^{-3}$ M	33.4 (33.4)	66.6 (54.6)
$2.5 \cdot 10^{-3}$ M	— (0)	— (74.5)

de ^{203}Hg pour la concentration intitiale $HgCl_2$ $2.5 \cdot 10^{-3}$ M donne les chiffres suivants: PhHgCl 88.9%; $HgCl_2$ 11.1% en très bon accord avec les valeurs expérimentales (85.6% et 11.1% respectivement) ce qui confirme la réalité de la réaction (4). L'étude similaire du système $HgPh_2$–$^{203}Hg(NO_3)_2$ dans le mélange acétone–benzène (1:1) a donné les résultats du Tableau I bis.

Une étude du système $^{203}HgCl_2$–PhHgCl effectuée dans les conditions expérimentales déjà décrites a permis d'établir des chromatogrammes analogues à ceux représentés sur la Fig. 2. Le partage à l'équilibre de ^{203}Hg a été déterminé pour différen-

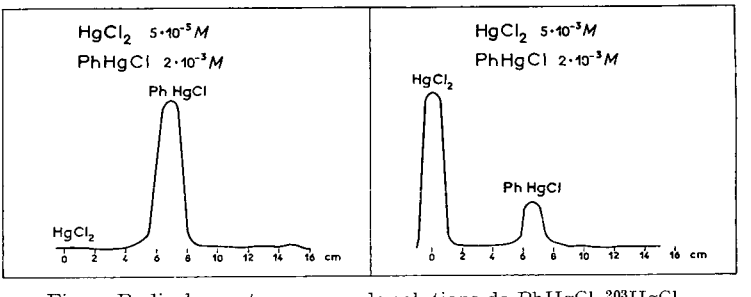

Fig. 2. Radiochromatogrammes de solutions de PhHgCl–$^{203}HgCl_2$.

tes concentrations de $HgCl_2$ et PhHgCl dans le mélange alcool-benzène. Ces résultats sont reproduits dans le Tableau II tandis que le Tableau II bis correspond au système $PhHgNO_3$–$^{203}Hg(NO_3)_2$ dans le mélange acétone–benzène.

TABLEAU II

PARTAGE DE ^{203}Hg ENTRE LES FORMES ORGANIQUE ET MINERALE DU MERCURE, À L'ÉQUILIBRE
Concentration en PhHgCl $2 \cdot 10^{-3}$ *M*. Les chiffres entre parenthèses représentent les valeurs expérimentales. Durée de la réaction 45 min; température 40°.

Concentration en $HgCl_2$	% *de* ^{203}Hg	
	$HgCl_2$	*PhHgCl*
$1.40 \cdot 10^{-5}$ *M*	0.7 (1.2)	99.3 (95.4)
$1 \cdot 10^{-3}$ *M*	33.3 (25.5)	66.7 (71.1)
$2.5 \cdot 10^{-3}$ *M*	55.6 (51)	44 (46.6)
$0.5 \cdot 10^{-2}$ *M*	71.4 (67.6)	28.6 (30.6)

TABLEAU II BIS

Concentration en $Hg(NO_3)_2$ $5 \cdot 10^{-3}$ *M*. Les chiffres entre parenthèses représentent les valeurs expérimentales. Durée de la réaction 45 min; température 40°.

Concentration en $PhHgNO_3$	% *de* ^{203}Hg	
	$Hg(NO_3)_2$	$PhHgNO_3$
$3 \cdot 10^{-3}$ *M*	55.1 (47.7)	44.9 (47.7)
$3.5 \cdot 10^{-3}$ *M*	49 (44.2)	51 (51.7)
$4 \cdot 10^{-3}$ *M*	43.5 (43)	56.5 (50.1)
$4.5 \cdot 10^{-3}$ *M*	39 (37)	61 (59.1)

Ces expériences confirment le mécanisme proposé par d'autres auteurs et montrent l'apport de la chromatographie sur papier à l'élucidation du mécanisme de réactions d'échanges isotopiques.

REMERCIEMENT

Nour remercions Madame G. PAULUS pour l'aide technique qu'elle nous a apportée au cours de ce travail.

RÉSUMÉ

L'analyse par chromatographie sur papier de solutions de $^{203}HgCl_2$ et $Hg(C_6H_5)_2$ dans le mélange alcool–benzène a permis de mettre en évidence la formation intermédiaire de C_6H_5HgCl radioactif dans le mécanisme du marquage du diphényl-mercure par ^{203}Hg.

SUMMARY

The labelling of diphenylmercury by exchange with $^{203}HgCl_2$ has been studied by paper chromatography using the solvent system alcohol–benzene. Labelled phenylmercuric chloride is an intermediate species in the reaction.

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ION EXCHANGE PROCEDURES

I. CATION EXCHANGE IN CONCENTRATED HCl AND $HClO_4$ SOLUTIONS*

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Approximately three years ago systematic studies oriented toward development of a general ion exchange analysis scheme were initiated at this laboratory. While the ultimate objective has so far not been achieved, considerable new information has been accumulated and a variety of procedures developed, many of which are expected to be part of such a scheme. The purpose of the present series of papers is to present these procedures and other pertinent results as they are developed.

The studies were initiated because there seems to be an ever growing need for development of new comprehensive separations schemes by which any mixture of elements can rapidly and cleanly be separated into groups and individual components. Schemes for separation of elements based on precipitation methods (*e.g.*, the NOYES AND BRAY scheme) are, for most purposes, too slow or cumbersome and are usually not applied to specific problems without drastic modification. Further, separations based largely on precipitation methods normally require addition of carriers in tracer and other radiochemical work.

Schemes handling a variety of tracer mixtures have become of increasing importance in recent years in such fields as activation analysis, fission product analysis and in the isolation of specific radio-isotopes from targets. A number of methods have been developed, some with broad coverage of elements. However, it appears that a still more general scheme, adaptable to both tracer and macro concentrations and embracing essentially all elements, remains an important goal. One would hope that such a scheme might combine reasonable speed, theoretical and experimental simplicity, high product purity, good recovery of the elements, and adaptability to simultaneous multiple analysis without excessive labor.

Ion exchange techniques seem to meet most of these requirements. They can be operated with (carrier-free) tracer and macro quantities. They are applicable to essentially all the elements through proper choice of resins, media, oxidation–reduction and complexing reactions. The techniques are simple and intrinsically rapid, at least when short columns can be used at moderately rapid flow rates. Several columns can be operated simultaneously. Purity and yield of separated elements are usually high.

Ion exchange as a separations tool has been intensively studied for the last 20

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years. A large body of basic information is available regarding the anion and cation exchange behavior of essentially all the elements. One might thus have expected that a new analysis scheme could be developed simply by properly arranging available information. However, from an examination of these data we concluded that insufficient information was available to permit development of a generalized scheme, applicable to (initial) samples containing any number of elements. In particular, it did not appear possible to specify, on the basis of existing information, a set of conditions under which elements could be separated into groups without overlapping.

As mentioned in a previous review[1] a gap existed in our knowledge of cation exchange of the elements at high ionic strength. Judging from the success of high ionic strength anion exchange, which during the last 10 years has become very popular, a thorough examination of high ionic strength cation exchange now seemed required.

There was reason to expect on the basis of a number of earlier publications[2–8] that such studies would be rewarding. DJURFELDT AND SAMUELSON[2] called attention to "anomalous" cation exchange behavior of iron in moderately concentrated HCl solutions; adsorbability did not decrease with increasing ionic strength as expected from simple mass action considerations—rather, there was significant adsorption of iron by cation exchangers from moderately concentrated HCl solutions. STREET, SEABORG AND DIAMOND[3–5] pointed out "anomalous" behavior in adsorbability studies of some rare earths and alkaline earths in HCl solutions. A few years ago, it was found in this laboratory[6] that strong adsorption on cation exchangers may occur from concentrated HCl solutions with some elements which exist substantially only as anionic complexes (of the type MCl_4^-) in the aqueous phase. Simultaneously with the work to be reported here and independently of it, CHOPPIN AND DINIUS[8] found very high adsorbabilities for a number of elements in perchloric acid solutions.

A rather broad survey of adsorbabilities of the elements in perchloric acid solutions has now been completed and the results are given in the present paper. These data are compared with adsorbabilities from HCl solutions which were obtained with the same ion exchange resin. The large differences in adsorbabilities of the elements in these two media at high ionic strength are striking and lead to many interesting separations. Through use of mixtures of these and other acids, many new techniques for separations become possible. A few of these will be mentioned here; the broad survey of high ionic strength separations in mixed acid systems, which were carried out at this laboratory, will be discussed in subsequent papers.

EXPERIMENTAL

1. Exchanger

A sulfonic acid–polystyrene–divinylbenzene resin of moderate cross linkage (Dowex 50-X4) was used. Originally, it was hoped that this resin might have reasonably rapid ion exchange rates even in concentrated electrolyte solutions. However, presumably because of the reduced water content of the resin in concentrated HCl and $HClO_4$ solutions, exchange rates in these media are unfavorable, although they are satisfactory at lower ionic strength. Because of these slow equilibration rates, very fine particles (270–325 mesh water-wet) were selected for column operation. On the basis of a number of preliminary experiments, still finer resins would give substantially improved performance in concentrated acids. For batch equilibrations, a somewhat coarser fraction (200–270 mesh) of the same resin was preferred.

The resin was screened, washed, and air dried for convenience in weighing. Moisture content and capacity were determined by standard methods (see *e.g.*, KRAUS AND RARIDON[9]). Capacity was 5.12 equivalents per kg dry hydrogen form resin.

2. Procedure

Adsorbabilities were determined by column elution, preloaded column, and batch equilibration techniques[10]. They will be expressed as weight distribution coefficients, D (amount per kg dry resin/amount per liter of solution) or volume distribution coefficients, D_v (amount per liter of bed/amount per liter of solution); D is related to D_v by the relationship $D = D_v/\rho$, where ρ is the bed density in the medium of interest.

Bed densities were determined in HCl and $HClO_4$ solutions. Approximately 1 g samples of resin were placed in a graduated tube of 0.25 cm^2 cross-sectional area; the beds were treated with water or electrolyte solutions and the bed volumes measured. The results are summarized in Fig. 1 as plots of $1/\rho$ (l of bed/kg dry resin) *versus*

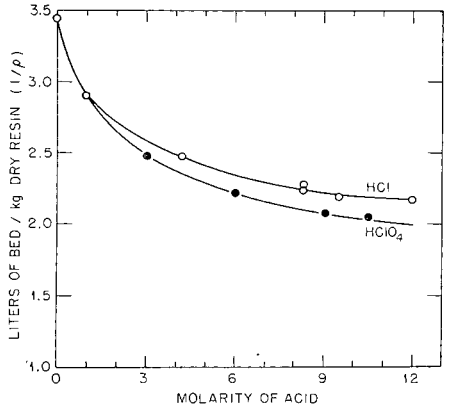


Fig. 1. Bed volume of Dowex 50-X4 in HCl and $HClO_4$ solutions.

molarity M (moles/l of solution) of acid. The resin shrinks greatly with increasing acid concentration from $1/\rho = 3.45$ l/kg in water to 2.18 and 2.00 l/kg in 12 M HCl and 12 M $HClO_4$ respectively.

For measurement of distribution coefficients by the column elution method, small aliquots of the solution containing the ions of interest were added to small columns (*ca.* 4 cm × 0.25 cm^2) of the resin pretreated with the appropriate HCl or $HClO_4$ solutions; the columns were eluted with the same acid solutions. Values of D_v were computed from the number of (geometric) column volumes (height × cross-sectional area) of effluent at which the metal appeared in maximum concentration; appropriate corrections were made for interstitial volume. As discussed earlier[10], this method is particularly suited when D_v is less than *ca.* 10.

For measurement of larger distribution coefficients, batch equilibration techniques were used. Weighed amounts of resin were agitated with known volumes of solution until there was no significant change in metal concentration with time. From analysis of the solution phase before and after equilibration, the weight distribution coefficients, D, were computed. Usually, 10 h equilibration was sufficient; however,

for the most concentrated acid solutions, agitation times of 2–3 days were sometimes required.

For measurement of very high distrubution coefficients, the preloaded column technique was used (see also KRAUS, PHILLIPS AND NELSON[11]). A tracer of the element was first uniformly adsorbed on a weighted amount of resin; a column was prepared from the loaded resin and eluting solution passed through it. Distribution coefficients were computed from the tracer concentration in the effluent and its known concentration in the preloaded bed.

Distribution coefficients were usually determined at metal concentrations sufficiently low so that the capacity of the resin utilized for adsorption (loading) was less than 1 %. In some experiments with concentrated $HClO_4$ solutions and metals which form very strong chloride complexes, *e.g.*, Ag(I), Hg(II), Bi(III), Tl(III), adsorption of tracers tended to be erratic presumably because trace amounts of chloride (*ca.* 10^{-4} *M*) initially present in the perchloric acid used strongly affect distribution coefficients. After adding the elements in sufficient excess (*ca.* 10^{-3} *M*) to override such effect, the results were reproducible. This caused substantially higher loading when adsorbability was high and distribution coefficients reported for some conditions thus refer to loading greater than 1 %.

Except when otherwise noted, the experiments were carried out at 25°. Temperature of the columns was controlled by jacketing them and pumping thermostated water through the jacket[12]. With the fine mesh resins used, gravity flow rates were usually too slow. Hence, the columns were operated under modest (< 2 p.s.i.) pressure.

3. *Analytical methods*

Most analyses were carried out radiometrically. Aliquots of solutions containing γ-emitters or energetic β-emitters were counted in a well-type scintillation counter, usually by integral counting techniques. When more than one tracer was present or in analyses of parent–daughter pairs, gamma spectrometry was used for identification and quantitation. Gamma energies were resolved with a 200 channel RIDL (Radiation Instrument Development Laboratory) Gamma Spectrometer, Model 34-8.

Alpha counting was used for analysis of radium, plutonium, curium and most americium samples. Aliquots of HCl and $HClO_4$ solutions were evaporated to dryness in small glass beakers, the residue taken up with hot 8 *M* HNO_3, transferred to stainless steel plates, evaporated and flamed before counting in an α-proportional counter. When necessary, pulse analysis techniques were used.

Aluminum analyses were carried out by spot testing with alizarin when the band elution method was used. Such semi-quantitative methods are satisfactory for determining D_v by establishing peak positions of the elution bands. In equilibration experiments, Al(III) concentrations were determined by EDTA titration with Eriochromeblack T as indicator. The samples were evaporated, the residue dissolved in 1 *M* HCl, excess EDTA added, and the mixture adjusted to pH 10 with ammonia. Back titration was carried out with standardized Zn(II) solution.

Several other elements were analyzed only semi-quantitatively when the band elution method was used: lithium and boron (borate) were analyzed by flame testing the effluent fractions; HNO_3, by noting appearance of brown fumes on warming the effluent samples with concentrated HCl; HF, by precipitation of LaF_3 on addition of $La(NO_3)_3$ solution; chloride, by precipitation of AgCl with $AgNO_3$; H_2SO_4, by pre-

cipitation of $BaSO_4$ on addition of $Ba(NO_3)_2$ solution. Adsorbability (D_v) of Cr(VI) and V(V), which in HCl and $HClO_4$ solutions tend to reduce, was determined by visually estimating the rate at which their bands (colored) moved down the column. The stock solutions were prepared immediately before use by dissolving CrO_3 or NH_4VO_3 in the appropriate acids.

In a number of column separations, millimolar rather than tracer concentrations of the elements were used. Pertinent details of solution preparation and analytical methods are given in the discussion.

4. Radioactive tracers

A list of the tracers used is shown in Table I. Most of the tracers were obtained from the Isotopes Division of Oak Ridge National Laboratory. The tracers, ^{7}Be, ^{22}Na, ^{28}Mg,

TABLE I

RADIOISOTOPES USED IN STUDY

Atomic No.	*Isotope*	*Half-life**	*Atomic No.*	*Isotope*	*Half-life**	*Atomic No.*	*Isotope*	*Half-life**
4	^{7}Be	53 d	37	^{86}Rb	18.7 d	71	^{177}Lu	6.8 d
11	^{22}Na	2.6 y	38	^{85}Sr	64 d	72	^{181}Hf	43 d
	^{24}Na	15 h	39	^{91}Y	59 d	73	^{182}Ta	115 d
12	^{28}Mg	21.3 h	40	^{95}Zr	65 d	74	^{187}W	24 h
14	^{31}Si	2.62 h	41	^{95}Nb	35 d	75	^{186}Re	90 h
15	^{32}P	14.3 d	42	^{99}Mo	66 h	76	^{191}Os	15 d
19	^{42}K	12.4 h	43	^{99m}Tc	6 h	77	^{192}Ir	74 d
20	^{47}Ca	4.5 d	44	^{103}Ru	40 d	78	^{191}Pt	3.0 d
21	^{44}Sc	4.0 h		^{106}Ru	1.0 y		^{197}Pt	20 h
	^{46}Sc	84 d	45	^{102}Rh	206 d	79	^{198}Au	64.8 h
	^{47}Sc	3.4 d	46	^{109}Pd	13.6 h	80	^{203}Hg	47 d
22	^{44}Ti	10^3 y	47	^{110m}Ag	249 d	81	^{204}Tl	3.9 y
23	^{48}V	16.1 d		^{111}Ag	7.6 d	82	^{210}Pb	21 y
24	^{51}Cr	27.8 d	48	^{115m}Cd	43 d	83	^{207}Bi	30 y
25	^{54}Mn	314 d	49	^{114m}In	50 d		^{210}Bi	5 d
	^{56}Mn	2.58 h	50	^{113}Sn	118 d	84	^{210}Po	138.4 d
26	^{59}Fe	45 d	51	^{124}Sb	60 d	88	^{226}Ra	1620 y
27	^{60}Co	5.27 y		^{125}Sb	2.7 y	90	^{234}Th	24.1 d
28	^{65}Ni	2.56 h	52	^{123m}Te	104 d	91	^{233}Pa	27.4 d
29	^{64}Cu	12.9 h	53	^{131}I	8.05 d	92	^{237}U	6.75 d
30	^{65}Zn	245 d	55	^{134}Cs	2.1 y	93	^{238}Np	2.1 d
31	^{72}Ga	14.1 h		^{137}Cs	30 y		^{239}Np	2.35 d
32	^{77}Ge	11 h	56	^{133}Ba	7.5 y	94	^{239}Pu	$2.44 \cdot 10^4$ y
33	^{76}As	25.5 h	57	^{140}La	40.2 h	95	^{241}Am	458 y
	^{77}As	39 h	58	^{141}Ce	32.5 d	96	^{244}Cm	17.6 y
34	^{75}Se	120 d	63	^{155}Eu	1.7 y			
35	^{82}Br	35.7 h	70	^{169}Yb	32 d			

* From *Chart of the Nuclides*, Knolls Atomic Power Laboratory, December, 1961

^{44}Ti–^{44}Sc, ^{48}V, ^{54}Mn, ^{102}Rh and ^{191}Pt, which are prepared by cyclotron irradiation, were purchased* as radiochemically pure stock solutions or prepared by the Electronuclear Research Division with the ORNL 86-Inch Cyclotron.

* ^{7}Be, ^{22}Na, ^{44}Ti–^{44}Sc, ^{54}Mn: Nuclear Science and Engineering Corporation, Pittsburgh, Pa.; ^{28}Mg: Brookhaven National Laboratory.

Short-lived tracers and others not routinely available from the Isotopes Division were prepared by neutron irradiation of appropriate target materials in the Low Intensity Training Reactor (LITR) or the Oak Ridge Research Reactor (ORR) at fluxes between 10^{12} and 10^{14} neutrons/cm^2/sec.

Most tracers were initially in HCl or HNO_3 solutions; $HClO_4$ "stock" solutions were prepared from these by fuming with concentrated $HClO_4$.

5. Purification of tracers

Most tracers were of sufficient radiochemical purity to be used directly. When this was not the case, they were usually purified by "standard" ion exchange techniques. Some (non-standard) purification procedures are given below.

The ^{48}V tracer, prepared by proton irradiation of TiO_2, was separated and purified by cation exchange in HCl–HF as described earlier[13].

^{191}Pt, obtained by proton irradiation of Ir metal was separated and purified by anion exchange after converting the target to soluble Ir(IV) and Pt(IV) chlorides by fusion in NaCl in an atmosphere of chlorine gas. The NaCl melt was dissolved in 1 *M* HCl and shaken with Ag metal to reduce Ir(IV) to Ir(III). The solution was passed into a small column of Dowex 1-X10 anion exchange resin. The column was washed with several column volumes of 6 *M* HCl containing Fe(II). Under these conditions, Ir(III) is eluted, while the ^{191}Pt remains adsorbed[14], presumably as the Pt(II) complex. Removal of ^{191}Pt was effected with *ca.* 8 column volumes of 11 *M* HCl at 50°. The effluent was evaporated with hydrogen peroxide to oxidize Fe(II) to Fe(III), adjusted to *ca.* 0.5 *M* HCl by water addition and passed into a small column of Dowex 50-X10 to remove Fe(III). On washing the column with 0.5 *M* HCl, ^{191}Pt appeared in the effluent while Fe(III) remained adsorbed.

The ^{226}Ra tracer* contained its decay products, primilarily Rn, Po, Pb and Bi. The impurities were removed immediately before use by passing a 2 *M* HCl solution of the tracer through small columns of Dowex-1 anion exchange resin[15].

^{234}Th was isolated from natural uranium by a method similar to that described by DYRSSEN[16]. Approximately 400 g of uranium nitrate hexahydrate were dissolved in 0.1 *M* HNO_3. The solution was passed through a 1.8 cm^2 × 5 cm (10 c.c.) bed of Dowex 50-X8 which strongly adsorbs Th(IV) under these conditions and concentrates it together with some uranium. Most of the uranium was selectively eluted with 4 *M* HCl after which Th(IV) was removed with 6 *M* HCl–1 *M* HF. The Th(IV) fraction was evaporated to near dryness, taken up in 12 *M* HCl and passed through a small bed of Dowex-1 anion exchanger to remove the last traces of U(VI).

Uranium analyses were carried out with ^{237}U rather than the more commonly available α-emitting isotopes in order to substitute more convenient γ-counting for tedious α-counting. The ^{237}U tracer was prepared by neutron irradiation of enriched ^{236}U. Since the preparation contained some ^{235}U and ^{238}U, separation of ^{237}U from fission products and ^{239}Np was required. The separation method, which will be described in detail in a separate publication[17] makes use of the strong adsorption of U(VI) by cation exchangers from $HClO_4$ media (see also Section 2 of RESULTS AND DISCUSSION). Final purification of ^{237}U was carried out by anion exchange in HCl using a modification of the procedure of CROUCH AND COOK[18].

* We are indebted to Dr. P. S. RUDOLPH of the ORNL Chemistry Division for this tracer.

RESULTS AND DISCUSSION

1. *Adsorbabilities in 9 M HCl and 9 M $HClO_4$*

To evaluate rapidly some of the significant aspects of cation exchange at high ionic strength, μ, one may examine adsorbabilities in 9 *M* $HClO_4$ and 9 *M* HCl solutions. The corresponding distribution coefficients are given in Table II; values of D_v are tabulated because of their direct relationship to elution band maxima, $c.v._{max}$, expressed as (geometric) column volumes ($D_v + i = c.v._{max}$, where $i \approx 0.4$ is the fractional interstitial volume).

Table II gives information on most of the elements. A few relatively volatile or, highly insoluble elements were not studied. Some elements which hydrolyze and precipitate in perchloric acid or which are normally handled as chloride complexes, were studied only in HCl solutions. Inclusion of these elements (as well as some of the elements listed) in a separations scheme based on the use of $HClO_4$ requires presence of appropriate complexing agents to assure reproducibility of properties, *i.e.*, they should be handled in acid mixtures.

From a cursory examination of Table II, a number of striking features concerning cation exchange at high ionic strength becomes apparent:

(i) A large number of elements show at least moderate adsorbabilities ($D_v > 1$) in either 9 *M* HCl or 9 *M* $HClO_4$. Thus a large number of high ionic strength cation exchange separations are feasible.

(ii) Adsorbabilities are usually substantially larger in 9 *M* $HClO_4$ than in 9 *M* HCl. The principal exceptions are Fe(III), Ga(III), and presumably also Au(III). These are the elements which form complexes of the type MCl_4^-, whose special adsorption characteristics on cation exchangers were discussed earlier[6]. In addition, Sb(V) also shows very high adsorption from 9 *M* HCl; its adsorbability seems to be very much higher than reported earlier[6]. D_v of Tl(III) in HCl is also somewhat greater than in $HClO_4$ as is D_v of Ra(II).

(iii) In 9 *M* HCl only 6 elements were found to have $D_v > 10$ (Au(III), Fe(III), Ga(III), Sb(V), Sc(III), Th(IV)). Most of these have $D_v > 100$; Sc(III) and Th(IV) have $10 < D_v < 100$.

(iv) In 9 *M* $HClO_4$ a large number of elements have $D_v > 10$. This large group of elements includes the rare earths, Sc(III), Y(III) and, interestingly, all the actinides. It also includes some alkaline earths: Ca(II), Sr(II); other divalent ions: Mn(II), Cd(II), Pb(II); trivalent ions: Fe(III), Ga(III), Bi(III); tetravalent ions: Ti(IV), Zr(IV), Hf(IV), V(IV); and ions of oxidation number five: V(V), Nb(V), Ta(V), Pa(V); and six: Cr(VI), Mo(VI), W(VI), U(VI). For most of these elements, D_v is larger than 100.

(v) The large differences in adsorbabilities of many elements in these media make high ionic strength solutions attractive for separations and isolations. A few such applications will be described below. However, the number of such examples has been intentionally kept small since it appears that the full benefits of high ionic strength cation exchange can best be developed through use of mixtures of these and other acids; properties of these mixtures will be discussed in subsequent publications.

2. *Some characteristics of cation exchange at high ionic strength*

(*a*) *Distribution coefficients.* The differences in intrinsic adsorbabilities of the various

TABLE II

DISTRIBUTION COEFFICIENTS IN 9 M HCl AND 9 M $HClO_4$

Element	D_v		Element	D_v	
	9 M HCl	9 M $HClO_4$		9 M HCl	9 M $HClO_4$
Ac(III)	$\sim 10^*$		Os(IV)	< 1	
Ag(I)	< 1	2.4			
Al(III)	0.6	3.8	P(H_3PO_4)	< 1	1
Am(III)	1.2	$2.0 \cdot 10^3$	Pa(V)	< 1	$> 1 \cdot 10^3$
As(III)	< 1	0.4	Pb(II)	< 0.5	$< 1.9 \cdot 10^1$
As(V)	< 1	1.4	Pd(II)	< 1	
Au(III)	$1.2 \cdot 10^2$		Po(IV)	< 1	< 1
			Pt(IV)	< 1	
B(H_3BO_3)	< 1	1	Pu(VI)	< 1	$> 1 \cdot 10^2$
Ba(II)	4.5	4.9			
Be(II)	0.3	1.3	R.E.(III)		
Bi(III)	< 1	$8.6 \cdot 10^2$	La(III)	4.5	$3.3 \cdot 10^3$
Br(Br^-)	< 1	< 1	Eu(III)		$2.0 \cdot 10^3$
			Yb(III)	1.0	$1.3 \cdot 10^2$
Ca(II)	3.2	$1.4 \cdot 10^2$	Lu(III)	0.8	$8.7 \cdot 10^1$
Cd(II)	< 1	$1.7 \cdot 10^1$	Ra(II)	2.5	2.0
Cl(Cl^-)	< 1	< 1	Rb(I)	< 1	< 0.5
Cm(III)	1	$> 1 \cdot 10^3$	Re(VII)	< 0.8	< 1
Co(II)	0.3	1.5	Rh(III)	< 1	
Cr(III)	< 1	3.5	Ru(IV)	< 1	
Cr(VI)	< 1	$> 1.0 \cdot 10^2$			
Cs(I)	< 1	0.6	S(HSO_4^-)	< 1	1
Cu(II)	0.4	1.2	Sb(III)	< 1	1
			Sb(V)	$2 \cdot 10^3$	
F(F^-)	< 1	< 1	Sc(III)	$1.7 \cdot 10^1$	$7.2 \cdot 10^5$
Fe(III)	$3 \cdot 10^2$	$1.8 \cdot 10^1$	Se(IV)	< 1	0.7
Fr(I)	$< 1^*$	$< 1^*$	Sn(IV)	< 1	
			Sr(II)	3.5	$2.9 \cdot 10^1$
Ga(III)	$3.3 \cdot 10^2$	$1.3 \cdot 10^1$			
			Ta(V)	1	$> 1 \cdot 10^3$
Hf(IV)	< 1	$9.6 \cdot 10^2$	Tc(VII)	< 0.5	< 1
Hg(II)	< 1	7	Te(IV)	< 1	2.8
			Th(IV)	$2.8 \cdot 10^1$	$> 1 \cdot 10^7$
I(I^-)	< 1	< 1	Ti(IV)	1	$1.9 \cdot 10^3$
In(III)	< 1	9.5	Tl(III)	5.3	1
Ir(III)	< 1				
Ir(IV)	< 1		U(VI)	0.5	$3.8 \cdot 10^3$
K(I)	< 1	0.3	V(IV)	< 1	$2.1 \cdot 10^1$
			V(V)	2	$> 1 \cdot 10^2$
Li(I)	< 1	0.5			
			W(VI)	< 1	$> 5 \cdot 10^2$
Mg(II)	0.3	1.9			
Mn(II)	0.4	$1.9 \cdot 10^1$	Y(III)	1.6	$5.8 \cdot 10^2$
Mo(VI)	< 1	$8.7 \cdot 10^2$			
			Zn(II)	< 1	1.8
N(NO_3^-)	< 1	1.5	Zr(IV)	1	$2.1 \cdot 10^3$
Na(I)	< 1	< 0.5			
Nb(V)	1.6	$> 1 \cdot 10^3$			
Ni(II)	< 1	1.0			
Np(VI)	< 1	$> 1.0 \cdot 10^2$			

* Estimated.

elements, as characterized by the distribution coefficients at low ionic strength, μ, under non-complexing conditions, while significant and of principal interest in earlier ion exchange studies do not concern us here since the differences at high μ are often very much larger. For example, adsorbabilities of the alkaline earths at low μ vary substantially less than a factor of 10 and in a regular manner in the series Be(II) to Ra(II), with Ra(II) most strongly adsorbed[5,19]; in 9 M $HClO_4$ adsorbabilities differ by more than a factor of 100 and Ca(II) is most strongly adsorbed.

In addition to inversions of elution order, other striking departures from "ideal" ion exchange behavior may occur at high μ, as illustrated in Fig. 2 with Sc(III).

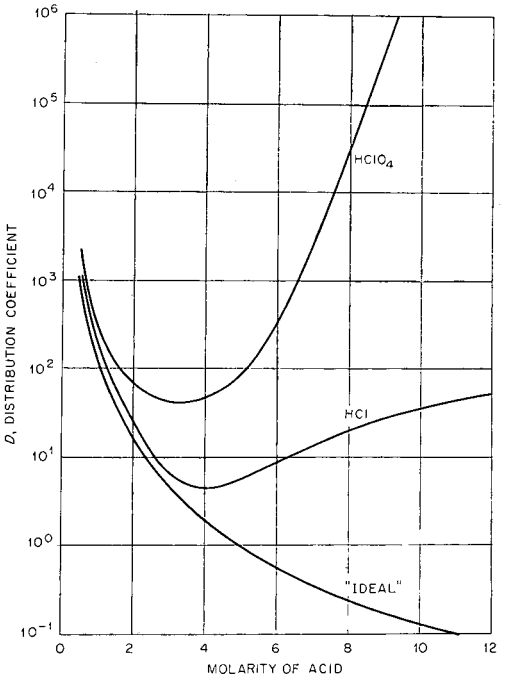


Fig. 2. Non-ideality at high ionic strength (Sc(III), Dowex 50-X4, 25°).

Its adsorption functions in HCl and $HClO_4$ are compared with an "ideal" function computed from the equilibrium expression for the exchange of the ions $M^{+\nu}$ and B^{+} with the assumptions (1) that $M^{+\nu}$ is a trace ion, (2) that there is no invasion of the resin, and (3) that the appropriate activity coefficient ratio is constant.

The equilibrium expression for the exchange reaction, using the same standard states in both phases, is:

$$\frac{m_{M(r)}m_B^{\nu}}{m_M m_{B^{\nu}(r)}} \frac{\gamma_{M(r)}\gamma_B^{\nu}}{\gamma_M \gamma_{B^{\nu}(r)}} = 1 \qquad (1)$$

where m is the concentration, γ the activity coefficient and subscript (r) designates the resin phase. (For discussion of the more general case of exchange with an ion B^{+b} see KRAUS AND NELSON[20]). After setting:

$$D_M = \frac{m_{M(r)}}{m_M} \text{ and } \Gamma_{M/B} = \frac{\gamma_{M(r)}\gamma_B^{\nu}}{\gamma_M\gamma_{B(r)}^{\nu}} = \frac{\gamma_{\pm MX_\nu(r)}^{\nu+1}\gamma_{\pm BX}^{2\nu}}{\gamma_{\pm MX_\nu}^{\nu+1}\gamma_{\pm BX(r)}^{2\nu}}$$

eqn. (1) becomes:

$$\log D_M = -\nu \log m_B + \nu \log m_{B(r)} - \log \Gamma_{M/B} \tag{2}$$

and

$$\frac{d \log D_M}{d \log m_B} = -\nu + \nu \frac{d \log m_{B(r)}}{d \log m_B} - \frac{d \log \Gamma_{M/B}}{d \log m_B} \tag{3}$$

The limiting "ideal" conditions (Γ = constant, $m_{B(r)} \approx$ constant, $m_{M(r)} \ll m_{B(r)}$) are equivalent to the simplifications:

$$\log D_M = -\nu \log m_B - \text{const.} \tag{2a}$$

and

$$\frac{d \log D_M}{d \log m_B} = -\nu \tag{3a}$$

The "ideal" curve of Fig. 2 was calculated on the basis of these assumptions with $\nu = 3$ after matching distribution coefficients at low μ; it, of course, does not show the minima and rapid rises of D with m which Sc(III) and many other elements show at high μ. Such positive slopes can only arise if d log Γ/d log m_B (eqn. (3)) is sufficiently negative since the resin invasion term d log $m_{B(r)}$/d log m_B is unlikely to become larger than unity.

Consideration of these non-ideality effects and estimation of their magnitude at high μ is simplified if one considers distribution of the components MX_ν and BX individually[21]. Using the same standard states in both phases, the conditions of equilibrium are:

$$a_{MX_\nu} = a_{MX_\nu(r)} \tag{4}$$

$$a_{BX} = a_{BX(r)} \tag{4a}$$

where a is the activity.
From eqn. (4):

$$D_M = \frac{m_{M(r)}}{m_M} = \frac{m_X^{\nu}\gamma_{\pm MX_\nu}^{\nu+1}}{m_{X(r)}^{\nu}\gamma_{\pm MX_\nu(r)}^{\nu+1}} \tag{5}$$

or:

$$\frac{d \log D_M}{d \log m_X} = \nu\left(1 - \frac{d \log m_{X(r)}}{d \log m_X}\right) - (\nu + 1)\frac{d \log \Gamma_{MX_\nu}}{d \log m_X} \tag{5a}$$

where $\Gamma_{MX_\nu} = \gamma_{\pm MX_\nu(r)}/\gamma_{\pm MX_\nu}$. The terms $m_X^{\nu}/m_{X(r)}^{\nu}$ or d log $m_{X(r)}$/d log m_X can readily be estimated if $m_M \ll m_X$ and $m_{M(r)} \ll m_{B(r)}$ since then $m_X \approx m_{BX}$, $m_{B(r)} \approx C + m_{X(r)}$, where C is the capacity. These simplifications together with eqn. (4a) lead to:

$$\frac{d \log m_{X(r)}}{d \log m_X} = \frac{d \log m_{BX(r)}}{d \log m_{BX}} = 2 - \frac{d \log m_{B(r)}}{d \log m_{BX}} - 2\frac{d \log \Gamma_{BX}}{d \log m_{BX}} \tag{5b}$$

When Γ_{MX_ν} and $\Gamma_{BX} = \gamma_{\pm BX(r)}/\gamma_{\pm BX}$ are substantially constant, and when $m_{B(r)} \approx C$, eqns. (5) yield d log D_M/d log $m_{BX} \approx -\nu$, as did eqn. (1) under similar assumptions.

At high electrolyte concentrations these simplifications do not hold; instead $m_{BX(r)} \approx m_{BX}$ and $d \log m_{BX(r)}/d \log m_{BX} \approx 1$, provided concentrations are expressed in moles/1000 g water in both phases. This, at least, was the case with a number of simple electrolytes in anion exchangers[21]. It presumably is also a good approximation for describing the invasion of cation exchangers by simple electrolytes as indicated by recent measurements of CHOPPIN AND DINIUS[22] on invasion of Dowex 50-X4 by perchloric acid.

Eqn. (4) then becomes:

$$D_M \approx \frac{\gamma_{\pm MX_\nu}^{\nu+1}}{\gamma_{\pm MX_{\nu(r)}}^{\nu+1}} = \frac{1}{\Gamma_{MX_\nu}^{\nu+1}} \tag{6}$$

and

$$\frac{d \log D_M}{dm_{BX}} \approx (\nu + 1)\left[\frac{d \log \gamma_{\pm MX_\nu}}{dm_{BX}} - \frac{d \log \gamma_{\pm MX_{\nu(r)}}}{dm_{BX}}\right] = -(\nu + 1)\frac{d \log \Gamma_{MX_\nu}}{dm_{BX}} \tag{6a}$$

Thus, distribution coefficients at high μ are determined largely by the activity coefficient ratio Γ_{MX_ν}. If this ratio were near unity, as it appears to be for the supporting electrolyte, no unusual adsorption effects should occur at high μ even if the individual activity coefficients are much larger than unity. As seen from Table II, this condition seems to apply for a surprisingly large number of elements. However, for Sc(III) and the many other elements which show considerable adsorption, particularly in concentrated perchloric acid solutions, Γ_{MX_ν} must be considerably smaller than unity; of course, the distribution coefficient is given by the $(\nu + 1)$ power of this ratio. Examination of the distribution functions of these adsorbable elements shows an approximately linear increase of log D with m_{HClO_4} after D has gone through the minimum. This is not surprising since it is reasonable to expect that (eqn. (6a)) both log $\gamma_{\pm MX_\nu}$ and log $\gamma_{\pm MX_{\nu(r)}}$ vary linearly with m_{HClO_4}.

More detailed quantitative comparisons and evaluation of $\gamma_{\pm MX_{\nu(r)}}$, which is required for a deeper understanding of the phenomena, seem impossible at present. Not only is accurate information lacking on electrolyte invasion of the resin used here and its water content (note that concentrations in the resin should be expressed as moles/kg water) but the pertinent activity coefficients for the aqueous phase are also not available. Needed are the values of $\gamma_\pm$ of the trace components (MX_ν) in the supporting electrolyte ($HClO_4$ or HCl). Some qualitative information, however, can be obtained by comparison of the distribution coefficients in 9 M $HClO_4$ (14.5 molal) of a series of divalent ions for which the activity coefficients of the perchlorates in their two component (water–salt) systems are known[23] at the same ionic strength.

Fig. 3 gives such a comparison for Ba, Pb, Sr, Ca, Mg, Zn, and UO_2^{2+}; for their perchlorate salts ($\mu = 14.5$) $\gamma_\pm$ is estimated to be 2.10, 3.75, 9.1, 23.5, 103, 117 and 610, respectively. Most of the points fall reasonably well on a straight line; it can be described by the relationship $\log D_M \approx 0.40 + 1.155 \log \gamma_{\pm MX_2(m = 4.8)}$.

Probably the most striking feature of Fig. 3 is the fact that Zn(II) and Mg(II) which have very high values of $\gamma_\pm$, have distribution coefficients $D = ca.\ 4$; from the line defined by the other salts $D \approx 500$ would have been predicted. We have no explanation for these large apparent differences in the interactions of these salts with the ion exchangers which lead to such large differences in selectivity at high ionic

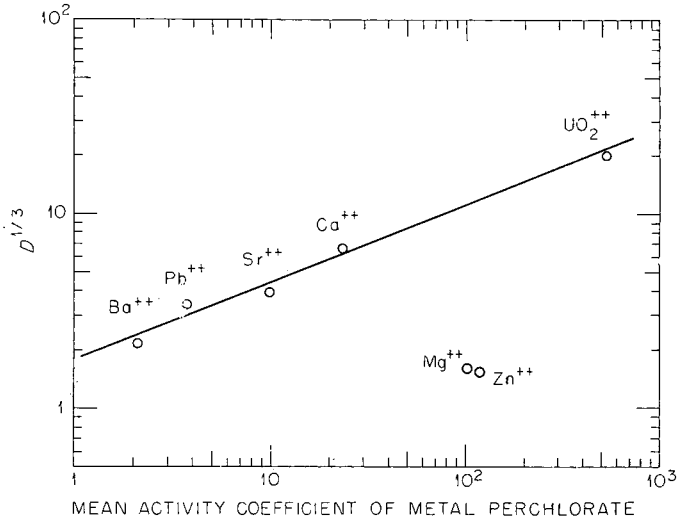


Fig. 3. Adsorption of trace metals from $HClO_4$ solution (9 M $HClO_4$, Dowex 50-X4, 25°).

strength. In a previous paper[6] in which the unusually high adsorption of some complex anions by cation exchangers was discussed, we proposed that these probably interact with the resin network. For the adsorbable cations in perchlorate solutions one might propose interaction (complex formation?) with the sulfonate groups of the exchangers. But even such a hypothesis leaves unresolved the question as to why this interaction should be so specific nor can it be readily reconciled with our observations (see next section) that distribution coefficients vary in an essentially ideal manner over a wide range of loading conditions.

(*b*) *Loading effects.* To allow direct comparison of observed distribution coefficients, most adsorption data were obtained under conditions of low loading, L, defined as:

$$L = \frac{\nu m_{M(r)}}{C_{(r)}} \tag{7}$$

where $C_{(r)}$ is the resin capacity expressed in the same concentration units as $m_{M(r)}$. With the tracer techniques used, loading of the high capacity exchanger (5.12 equivalents/kg dry resin) was generally less than $L = 0.01$. To establish that the unusually high adsorbabilities exhibited by some elements in concentrated electrolyte solutions were not anomalous "tracer" effects, a variety of "loading" experiments was carried out.

Two typical break-through experiments are illustrated in Fig. 4. For the first, a 9.2 M $HClO_4$ solution containing 0.1 M Ce(III) (with ^{144}Ce) was passed into a 0.25 cm^2 × 4 cm column of Dowex 50-X4 at 50° at a flow rate of 0.2 cm/min. No significant Ce(III) break-through occurred during the first 6 column volumes (c.v.); the 50% break-through point (where the concentration of Ce(III) in the effluent is half its concentration in the feed) occurred at 7.7 c.v. This corresponds (after correcting for interstitial volume) to a total uptake of 0.73 moles of Ce(III)/l of bed. Since the ex-

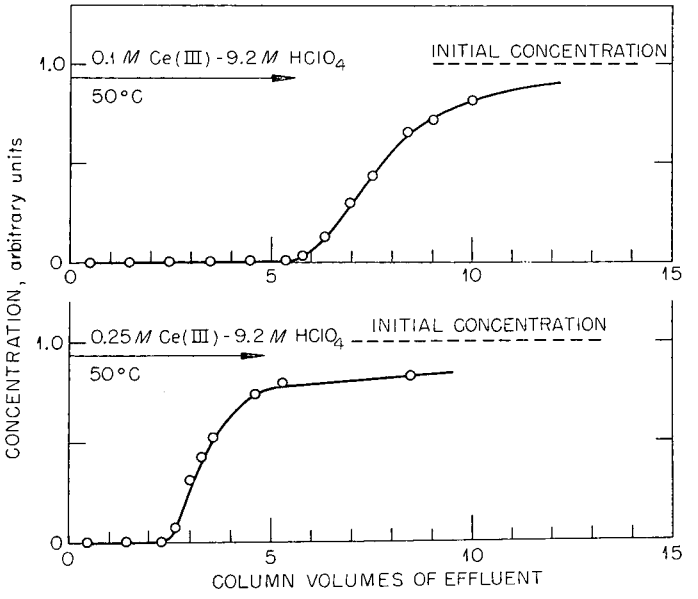
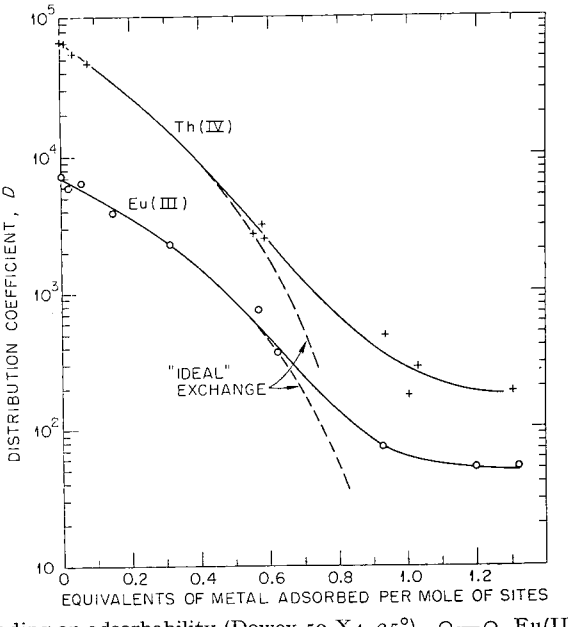


Fig. 4. Adsorption of Ce(III) from $HClO_4$ solution (Dowex 50-X4, —400 mesh, 25°).



5. Effect of loading on adsorbability (Dowex 50-X4, 25°). O—O Eu(III) in 9.2 *M* $HClO_4$; +—+ Th(IV) in 6.0 *M* $HClO_4$.

change capacity was 2.47 equivalents/l of bed, this corresponds to 89 % loading of the column ($L = 0.89$).

In the second example, a 0.25 M Ce(III) solution (with ^{144}Ce) in 9.2 M $HClO_4$ was passed through a small column under similar conditions; 50 % break-through occurred at 3.6 c.v., corresponding to an uptake of 0.8 moles/l of bed, or $L = ca.\ 0.97$. In neither experiment were the break-through curves symmetrical; beyond 50 % break-through the effluent concentration slowly approached the feed concentration. The exchange rates became increasingly unfavorable as the columns became heavily loaded. Nevertheless, high utilization of the columns can be achieved at moderate flow rates.

In more detailed studies of loading effects, measurements were carried out with Eu(III) in 9.1 M $HClO_4$ and Th(IV) in 6.0 M $HClO_4$*. The batch equilibration method was used; different loadings were obtained through control of the initial metal concentration and the ratio of solution volume to resin weight. Loading, L, was computed according to eqn. (7) with $\nu = 3$ for Eu(III) and $\nu = 4$ for Th(IV).

A plot of log D *versus* L is shown in Fig. 5. At low loading ($L < 0.01$). D was essentially independent of L, as expected. Between 5 and 75 % loading, D falls off rapidly; at still higher loading, D decreases only slowly. Values of L significantly larger than unity can be reached with $D \gg 1$.

The shape of the loading curve is given by the derivative either of eqn. (1) or eqn. (5):

$$\frac{1}{\nu}\frac{d \ln D'_M}{dL} = \frac{d \ln m'_{B(r)}}{dL} - \frac{d \ln m_B}{dL} + \frac{\nu - 1}{\nu}\frac{d \ln f}{dL} - \frac{1}{\nu}\frac{d \ln \Gamma_{M/B}}{dL} \qquad (8)$$

$$\frac{1}{\nu}\frac{d \ln D'_M}{dL} = -\frac{d \ln m'_{X(r)}}{dL} + \frac{d \ln m_X}{dL} - \frac{\nu + 1}{\nu}\frac{d \ln f\Gamma_{MX_\nu}}{dL} \qquad (9)$$

For eqns. (8) and (9) and the rest of this section we shall distinguish between resin concentrations expressed as moles/kg dry resin (primed quantities) and moles/kg water in the resin (unprimed quantities); the relation between these two concentration units is $m = m'f$ where $1/f$ is the water content (kg water/kg dry resin).

We may define an ideal loading curve as one for which the water content, the activity coefficient ratios and the concentration of the supporting electrolyte are constant and where $m'_{B(r)} = C' - \nu m'_{M(r)} = C'(1 - L)$. This leads to:

$$\frac{d \log D'_M}{dL} = -\frac{\nu}{2.303\,(1 - L)} \qquad (8a)$$

The curves of Fig. 5 marked "ideal" are drawn on the basis of these simplifications. Agreement between observed and calculated curves is surprisingly good for both Eu(III) and Th(IV) up to $L = ca.\ 0.6$ in spite of the fact that because of electrolyte invasion $m'_{B(r)} > (C' - \nu m'_{M(r)})$. Further, under the conditions of these experiments, d ln $\Gamma_{M/B}$ and d ln f almost certainly are not zero.

Indeed, adherance of even the initial loading curve (near $L = 0$) to the highly

* The Th(IV) loading experiments were carried out by Dr. E. L. LIND, Central Washington State College, Ellensburg, Washington, ORINS Research Participant at ORNL, 1962–1963. We are indebted to Dr. LIND for permission to quote these results.

restrictive "ideal" conditions is very difficult to understand at high μ. Thus, in addition to the equilibrium equation for the distribution of the components BX and MX_ν, the electro-neutrality restriction:

$$\nu m'_{M(r)} + m'_{B(r)} = C' + m'_{X(r)} \tag{10}$$

or its equivalent:

$$(1 - L) = \frac{1}{C'}\left(m'_{B(r)} - m'_{X(r)}\right) \tag{11}$$

must apply. If we evaluate $d \ln m'_{B(r)}/dL$ from eqn. (10), eqn. (8) becomes:

$$\frac{1}{\nu}\frac{d \ln D'_M}{dL} = \frac{C'}{m'_{B(r)}}\left[\frac{dm'_{X(r)}}{\nu dm'_{M(r)}} - 1\right] - \frac{d \ln m_B}{dL} + \frac{\nu - 1}{\nu}\frac{d \ln f}{dL} - \frac{1}{\nu}\frac{d \ln \Gamma_{M/B}}{dL} \tag{12}$$

If we further make the assumptions, justifiable at high values of D'_M, that m_B, m_X, $\gamma_{\pm BX}$ and $\gamma_{\pm MX_\nu}$ in the aqueous phase are independent of L, eqn. (12) becomes:

$$\frac{1}{\nu}\frac{d \ln D'_M}{dL} = \frac{C'}{m'_{B(r)}}\left[\frac{dm'_{X(r)}}{\nu dm'_{M(r)}} - 1\right] + \frac{\nu - 1}{\nu}\frac{d \ln f}{dL} - \frac{\nu + 1}{\nu}\frac{d \ln \gamma_{\pm MX_\nu(r)}}{dL} + \frac{d \ln \gamma_{\pm BX(r)}}{2\,dL} \tag{12a}$$

This clearly reduces to eqn. (8a) if there is no invasion ($dm'_{X(r)} = 0$) and if $d \ln f = d \ln \gamma_{\pm MX_\nu(r)} = d \ln \gamma_{\pm BX(r)} = 0$. However at finite invasion the simultaneous assumptions that $d\, m'_{X(r)}/\nu\, d\, m'_{M(r)} = 0$, $d \ln f/d\, L = 0$ and that the activity coefficient derivatives are zero lead to a logical inconsistency. Thus, with $d\, m'_{X(r)}/\nu\, d\, m'_{M(r)} = 0$ electro-neutrality requires $d\, m'_{B(r)}/\nu\, d\, m'_{M(r)} = -1$. Since we assumed operation at constant activity of BX, invariance of $m'_{X(r)}$ can be reconciled with a decrease in $m'_{B(r)}$ only if $\gamma_{\pm BX(r)}$ changes appropriately. Thus the apparent ideality of the loading curves at low values of L implies an interesting compensation of the various derivatives of eqns. (12). Their experimental evaluation should be of considerable interest.

3. *The adsorption functions in HCl and $HClO_4$ solutions*

The adsorption functions of the elements are described below together with a few illustrations of their use for separations. Measurements and discussions emphasize, as mentioned, results for the more concentrated solutions. In those cases in which dilute solutions were studied, agreement with published data was satisfactory (see *e.g.*, refs. 24 and 25).

(*a*) *Alkali metals.* The alkali metals Li through Cs (and presumably Fr) show negligible adsorption in concentrated HCl and $HClO_4$ solutions. In the current context, the minor variations and inversions in adsorbabilities ($D_{Cs} < D_{Li}$) are not of interest. The non-adsorbability of alkali metals at high μ is in agreement with earlier observations on some of these elements[4-6,8].

(*b*) *Alkaline earths* (*Figs. 6 and 7*). These elements show striking differences in adsorbabilities, particularly in perchlorate solutions.

Beryllium(II) and Mg(II) show little adsorption at high ionic strength.

Calcium is strongly adsorbed from concentrated $HClO_4$ and less, though still significantly, from concentrated HCl. In HCl, the adsorption minimum, $D = ca.\ 2$, is located near 6 M; in $HClO_4$ the minimum with $D = ca.\ 7$ is near 4.5 M. In HCl, D reaches *ca.* 30 near 12 M; in $HClO_4$, D is *ca.* 10^3 near 11 M, but appears to decrease slightly at higher concentrations.

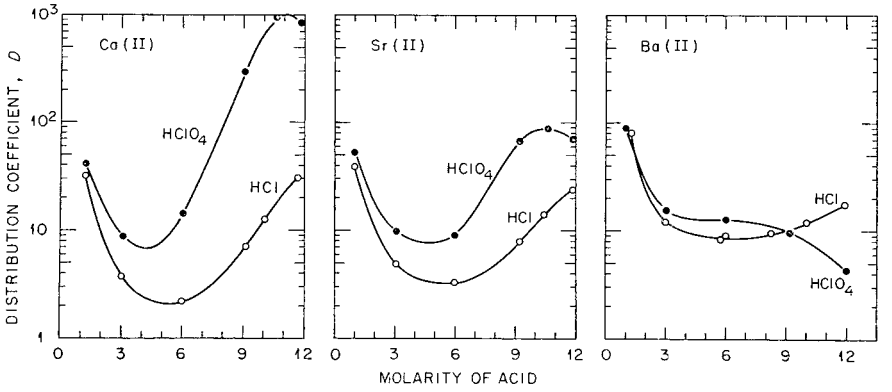


Fig. 6. Adsorption of Ca(II), Sr(II) and Ba(II) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

The behavior of Sr(II) is similar to that of Ca(II), but adsorbabilities at high μ are significantly lower. In HCl the adsorption minimum occurs at 6 M with $D = ca.\ 3$; in $HClO_4$ the minimum occurs near 5 M with $D = ca.\ 8$. In 12 M HCl, D becomes *ca.* 25; in 11 M $HClO_4$, $D = 90$. As with Ca(II), there is evidence for a slight decrease in adsorbability above 11 M $HClO_4$.

Adsorbability of Ba(II) at high μ is substantially less than that of Sr(II). In HCl a shallow minimum with $D = 9$ occurs near 6 M; D then increases to *ca.* 18 in 12 M HCl. In $HClO_4$, D decreases with M $HClO_4$ from *ca.* 90 in 1 M to *ca.* 4 in 12 M; there is no adsorption minimum.

Radium is slightly adsorbed from 9 M HCl and 9 M $HClO_4$ with $D = 5.3$ and 4.2 respectively. There are no adsorption minima.

Our measurements at high μ agree reasonably well with data reported by others. Thus, our results for Mg(II) are in agreement with those of MANN[26] for HCl and $HClO_4$ solutions. The results for HCl with Be(II), Ca(II), Sr(II), Ba(II), and Ra(II) are in reasonable agreement with those of DIAMOND[5] who determined adsorbabilities with a 10–12 % DVB resin. However, the adsorption functions obtained by CHOPPIN AND

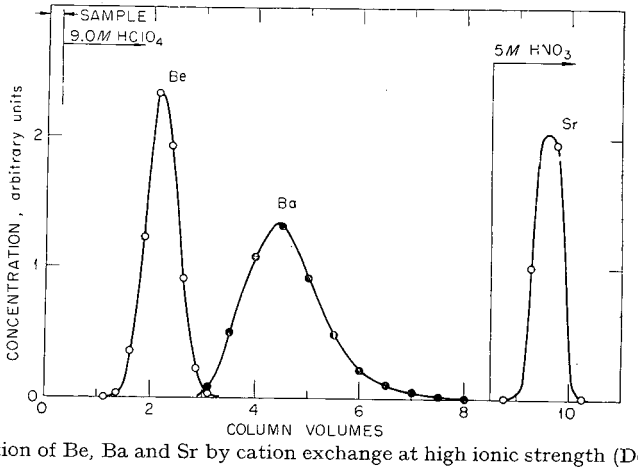


Fig. 7. Separation of Be, Ba and Sr by cation exchange at high ionic strength (Dowex 50-X4, 50°).

DINIUS[8] for Ca(II) and Ba(II) in $HClO_4$ differ substantially from ours for M $HClO_4$ > 2. The reason for the disagreement is not known.

The differences in adsorbabilities of the alkaline earths in concentrated perchloric acid are particularly noteworthy and lead to striking separations. Separation of Mg(II) from Ca(II) was recently reported[27]; that of Be(II), Ba(II), and Sr(II) using $HClO_4$ for adsorption and HNO_3 for elution is shown in Fig. 7. While the Be–Ba separation is marginal with the short column used (4 cm), that of Ba(II) from Sr(II) is excellent.

(*c*) *Scandium(III) and Yttrium(III)* (*Figs. 8 and 11*). Adsorbability of Sc(III) from concentrated $HClO_4$ is larger than that of any other trivalent ion. Adsorbability rises from a minimum $D = 57$ in 3 M to $D > 10^6$ in 9 M $HClO_4$. In HCl adsorbability rises much less dramatically from a minimum $D = 6$ at 5 M to $D = 70$ at 12 M.

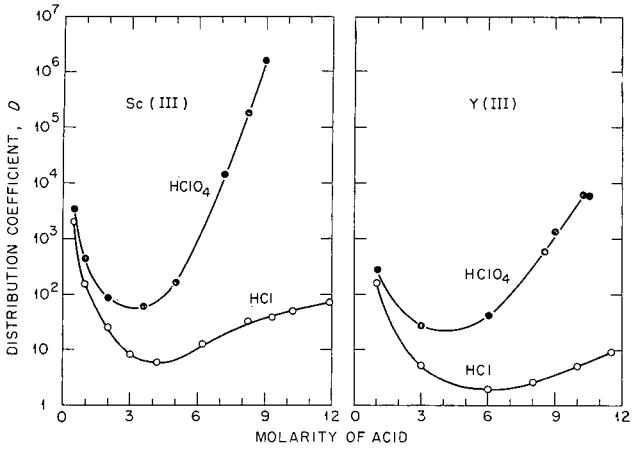


Fig. 8. Adsorption of Sc(III) and Y(III) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

A separation which makes use of the strong adsorption of Sc(III) from 9 M $HClO_4$ (and 9 M HCl) is shown in Fig. 11 and discussed in Section 3e.

The adsorption function of Y(III) is similar to that of Sc(III), but at high ionic strength adsorbability is much less. For Y(III) in 9 M $HClO_4$, D is 10^3; in 9 M HCl, D is only 4. Adsorption minima are at 4 M $HClO_4$ and 6 M HCl with $D = 23$ and 1.9, respectively.

(*d*) *Rare earths* (*Fig. 9*). The adsorption functions of the lightest and heaviest rare earths, La(III) and Lu(III), apparently define the region of adsorbability of the rare earths. At high μ, as with Sc(III) and Y(III), D is much greater in $HClO_4$ than in HCl. In $HClO_4$ the adsorption functions show minima near 4–5 M and rise steeply thereafter. In HCl the minima are very shallow and occur near 6 M.

For the four rare earths studied, D decreases fairly regularly, for a given medium, with increasing atomic number. Study of other rare earths did not seem warranted since other authors have established that adsorbability of the rare earths in HCl*,[4] and $HClO_4$[8] decreases monotonically with atomic number. Where comparisons can be made, our results are in good agreement with those published.

* We are indebted to Dr. A. CHETHAM-STRODE for permission to quote these unpublished results.

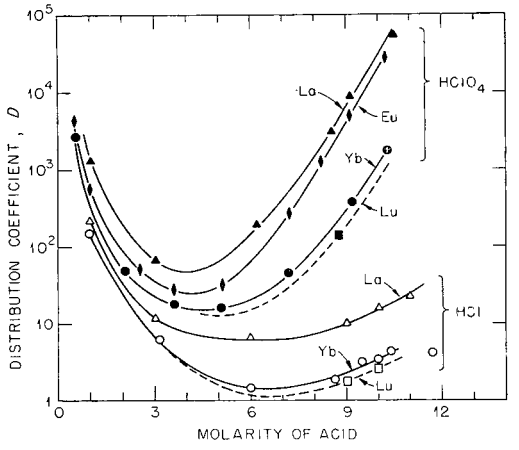


Fig. 9. Adsorption of rare earths from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

(*e*) *Titanium(IV)* (*Figs. 10 and 11*). Adsorbability of Ti(IV) in HCl first decreases with increasing M to a shallow minimum, $D < 1$, near 6 M and then increases slowly to $D = 10$ in 12 M HCl.

In $HClO_4$ adsorbability of Ti(IV) first decreases to a minimum $D = 6$ near 3 M and then increases markedly with increasing M $HClO_4$ to $D > 10^4$ near 10 M.

A separation involving Ti(IV) in $HClO_4$ is illustrated in Fig. 11. A solution containing the tracers ^{42}K, ^{44}Ti–^{44}Sc in $2.5 \cdot 10^{-3}$ M Ti(IV)–12 M HCl was evaporated with 0.5 ml of conc. $HClO_4$ to remove HCl. The solution was adjusted to a final volume of 1 ml with 9 M $HClO_4$ and a small aliquot was added to a 3 cm × 0.2 cm^2 column of Dowex 50-X4 which had been pretreated with 9 M $HClO_4$. On elution with 9 M $HClO_4$, K^+ appeared in a sharp band near one column volume (c.v.) while Ti(IV) and Sc(III) (see Section 3c) were retained by the resin. Ti(IV) was removed in a reasonably sharp band with 9 M HCl and Sc(III) with 4 M HCl–0.1 M HF. Although

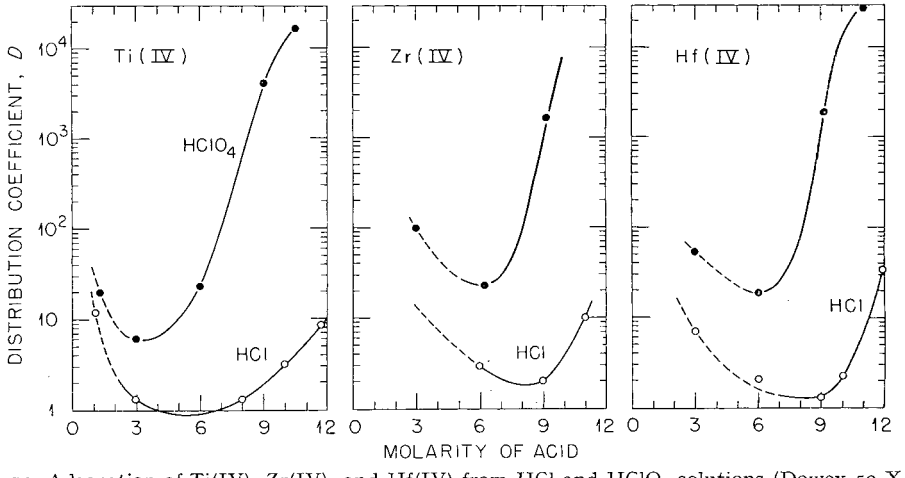


Fig. 10. Adsorption of Ti(IV), Zr(IV), and Hf(IV) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

Sc(III) could also have been eluted with 4 *M* HCl, an eluent containing HF was selected since it permits more rapid removal of Sc(III) through formation of weakly adsorbed fluoride complexes.

Attempts to carry out similar separations with *ca.* 0.1 *M* Ti(IV) solutions were unsuccessful; precipitation of Ti(IV) occurred as HCl was removed during the fuming step with concentrated $HClO_4$.

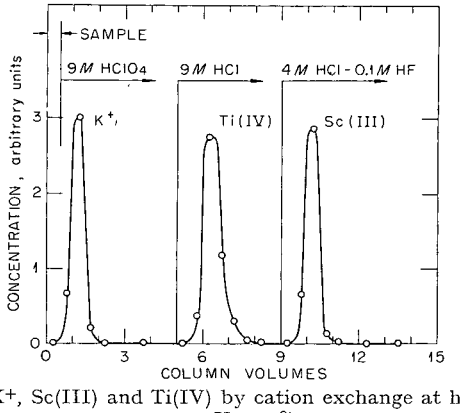


Fig. 11. Separation of K^+, Sc(III) and Ti(IV) by cation exchange at high ionic strength (Dowex 50-X4, 25°).

(f) Zirconium(IV) and hafnium(IV) (*Figs. 10 and 12*). The cation exchange behavior of Zr(IV) and Hf(IV) in dilute or moderately concentrated HCl or $HClO_4$ solutions has been extensively studied (see *e.g.*, ref. 28). Both elements are relatively strongly adsorbed from the dilute acids; while adsorbability falls off rapidly with increasing acidity it is also dependent on metal concentration at a given acidity.

Adsorbability of Zr(IV) and Hf(IV) in HCl reaches a minimum with $D = ca.\ 2$ at 6 *M*; *D* then rises to *ca.* 40 in 12 *M* HCl. Our results are in good agreement with those

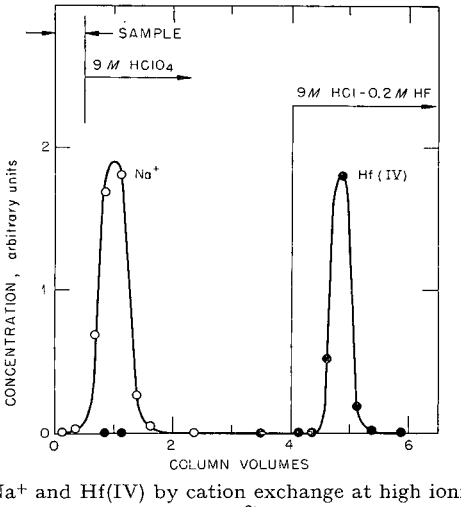


Fig. 12. Separation of Na^+ and Hf(IV) by cation exchange at high ionic strength (Dowex 50-X4, 50°).

of BENEDICT, SCHUMB AND CORYELL[29]. The adsorption function of Hf(IV) is slightly below that of Zr(IV); the differences are sufficient for chromatographic separation of the elements as first demonstrated by STREET AND SEABORG[30].

In $HClO_4$ the adsorbability minimum of Zr(IV) and Hf(IV) is near 6 M with D = *ca.* 20; D then increases markedly with M $HClO_4$ to *ca.* $3 \cdot 10^4$ in 11 M. Again, Hf(IV) is slightly less adsorbed than Zr(IV).

A separation involving Hf(IV) in concentrated $HClO_4$ is shown in Fig. 12. It serves to illustrate that Hf(IV) (and presumably also Zr(IV)) in millimolar concentrations may be quantitatively adsorbed from concentrated $HClO_4$ solutions; the usual tendency of these elements to undergo precipitation and other complicating hydrolytic reactions in the absence of complexing agents seems not to interfere at these high acidities.

For the separation of Fig. 12, a 9 M $HClO_4$ solution (0.5 ml) containing $6 \cdot 10^{-3}$ M Hf(IV) with ^{181}Hf tracer and a typical weakly adsorbed element, ^{22}Na, was added to a 4 cm $\times$ 0.25 cm^2 column of Dowex 50-X4 which had been pretreated with 9 M $HClO_4$. To improve exchange rates, the column was operated at 50°. On elution with 9 M $HClO_4$, sodium appeared in a sharp band near 1 c.v. while Hf(IV) remained adsorbed. Elution of Hf(IV) was carried out with 9 M HCl–0.2 M HF; this complexing medium conveniently removes Hf(IV) (and Zr(IV)).

(*g*) *Thorium(IV)* (*Fig. 13*). Traditionally, Th(IV) is the element most difficult to remove from cation exchangers without use of complexing agents. In HCl, D decreases from *ca.* 10^5 at 0.5 M to *ca.* 70 at 6 M where its extremely shallow adsorption minimum is located. These results agree reasonably well with data reported by others[4].

The adsorption function in $HClO_4$ is similar to that in HCl at low concentrations. However, because of an extemely rapid increase of D with M $HClO_4$ at high concentration, the distribution coefficient at the adsorption minimum (located at M = *ca.* 3) is very large, D = *ca.* 10^3.

The principal cation exchange problem with Th(IV) thus is development of convenient elution methods. Elution in few column volumes requires complexing agents

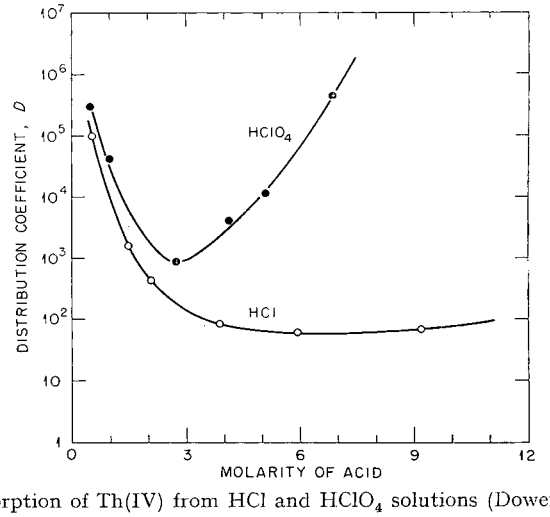


Fig. 13. Adsorption of Th(IV) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

such as organic acids, H_2SO_4 and $HClO_4$–HF or HCl–HF mixtures. We shall discuss some of these in later reports.

(h) Vanadium(IV) and (V) (Figs. 14 and 15). V(*IV*) is much more strongly adsorbed from concentrated $HClO_4$ than from HCl. Adsorbability in HCl solutions de-

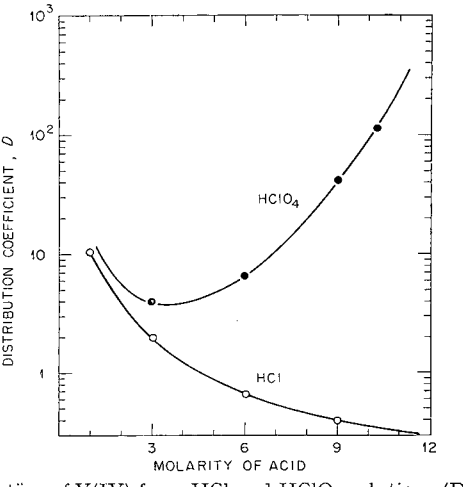


Fig. 14. Adsorption of V(IV) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

creases with increasing *M* and becomes negligible at high *M* HCl. In $HClO_4$, after an initial decrease adsorbability increases rapidly from a minimum $D = 4$ near 3 *M* to $D = ca.$ 100 near 10 *M* $HClO_4$.

The strong adsorption of V(IV) from concentrated $HClO_4$ is utilized in the separation shown in Fig. 15. A mixture containing V(IV) and two relatively weakly adsorbed elements, Cr(III) and Ni(II) (see Sections 3*j* and 3*o*) in 9 *M* $HClO_4$ was added to a small column of Dowex 50-X4. The weakly adsorbed elements were removed in

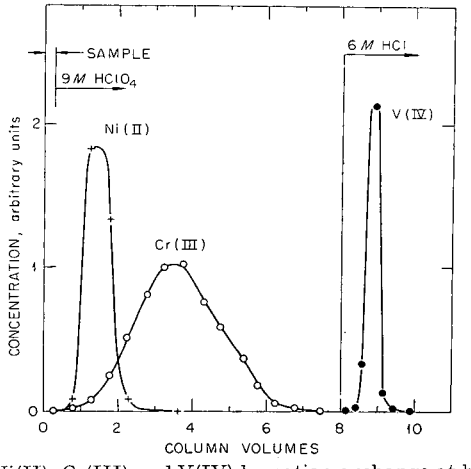


Fig. 15. Separation of Ni(II), Cr(III) and V(IV) by cation exchange at high ionic strength (Dowex 50-X4, 25°).

overlapping bands with 9 c.v. of 9 *M* $HClO_4$ while V(IV) remained adsorbed; V(IV) was removed in a sharp band with 6 *M* HCl.

Vanadium(V), like V(IV), is strongly adsorbed from 9 *M* $HClO_4$ ($D_v > 10^2$) and weakly adsorbed from 9 *M* HCl, ($D_v = ca.\ 2$). In 9 *M* $HClO_4$, V(V) adsorbs as an intensely colored (brown) band which slowly reduces to a blue V(IV) species. In HCl, V(V) adsorbs as a reddish-brown band, presumably a chloride complex, which rapidly reduces to V(IV).

(*i*) *Niobium(V), tantalum(V) and protactinium(V)*. Hydrolysis of these elements is particularly troublesome in $HClO_4$ solutions, including concentrated $HClO_4$. Even at tracer concentrations, these elements tend to adsorb irreversibly and irreproducibly on the walls of containers. To estimate *D* in 9 *M* $HClO_4$, adsorption measurements were carried out in the presence of small and varying amounts of HF. At 9 *M* $HClO_4$ and low *M* HF, the elements are strongly adsorbed and adsorbabilities increase rapidly with decreasing *M* HF at constant *M* $HClO_4$. The distribution coefficients at the lowest HF concentration ($3 \cdot 10^{-4}$ *M*) were all larger than 10^3; these are presumably lower limits for the adsorbabilities in 9 *M* $HClO_4$.

In 9 *M* HCl, Nb(V), Ta(V) and Pa(V) are weakly adsorbed. The stock tracer solutions used in the study were prepared by heating appropriate aliquots of ^{95}Nb, ^{181}Ta, and ^{233}Pa in HCl–HF media with excess H_3BO_3 to volatilize fluoride as BF_3. The residual solutions were taken up in excess 9 *M* HCl and added to small columns of resin which had been pretreated with 9 *M* HCl–0.1 *M* H_3BO_3. In some experiments, particularly with Ta(V), the elution bands tailed suggesting that slowly reversible and adsorbable species tend to form in 9 *M* HCl media. With 12 *M* HCl, D_v values of the order of 1–2 were observed for Nb(V), Ta(V) and Pa(V).

(*j*) *Chromium(III) and (VI)*. Cr(III) is weakly adsorbed ($D_v < 1$) above *ca.* 6 *M* HCl. In dilute HCl, Cr(III) is strongly adsorbed. However, on heating Cr(III) solutions in HCl, weakly adsorbed species may be formed. Thus in a typical experiment, Cr(III) tracer in 0.5 *M* HCl was heated several minutes near 95°, cooled to room temperature and added to a small column of Dowex 50-X4 resin. On elution with 0.5 *M* HCl a small amount of the Cr(III) immediately passed through the column while most of the Cr(III) was strongly adsorbed.

In perchloric acid solutions, the adsorption function of Cr (III)shows a shallow minimum, $D_v = 3.3$ near 6 *M* and increases slightly with further increase of *M* $HClO_4$.

Adsorption of Cr(VI) from dilute HCl is negligible ($D_v < 1$). For *M* HCl > *ca.* 4 adsorbability increases slightly to $D_v = ca.\ 1.6$ near 8 *M*.

In $HClO_4$ adsorption of Cr(VI) is also negligible for *M* < *ca.* 4, however, at higher acid concentration, *D* increases markedly; in 9 *M* $HClO_4$, D_v is probably considerably larger than 10^2, a surprisingly high value for a negatively charged ion. Since Cr(VI) is reduced by HCl and, presumably by the resin in $HClO_4$, these media cannot be utilized for separations. Estimates of the distribution coefficients were made by adding *ca.* 0.05 *M* Cr(VI) solutions to the columns and noting, in band elution experiments, the behavior of the unreduced portions.

(*k*) *Molybdenum(VI)* (*Fig. 16*). Adsorbability of Mo(VI) is negligible from 2 *M* HCl ($D_v < 1$) and increases only slightly with increasing *M* HCl to $D = 1.6$ at 12 *M*. Measurements were not attempted with more dilute HCl solutions because of the tendency of this element to form slowly reversible hydrolytic species[31].

In perchloric acid, adsorbability of Mo(VI) is low, $D_v < 1$, in the region 2–4 *M*

$HClO_4$. Measurements were not carried out below 2 M $HClO_4$ because of expected difficulties from hydrolytic polymerization. Adsorbability of Mo(VI) becomes significant in concentrated $HClO_4$ solutions, increasing from $D = 9$ near 6 M to $D > 10^3$ in 10 M.

(*l*) *Tungsten(VI)* (*Fig. 16*). Adsorbability of W(VI) is low in concentrated HCl; it increases slightly from $D = 1.1$ in 6 M to $D = 2$ in 12 M. Measurements were not carried out below 6 M HCl because difficulties from hydrolytic reactions were expected[31].

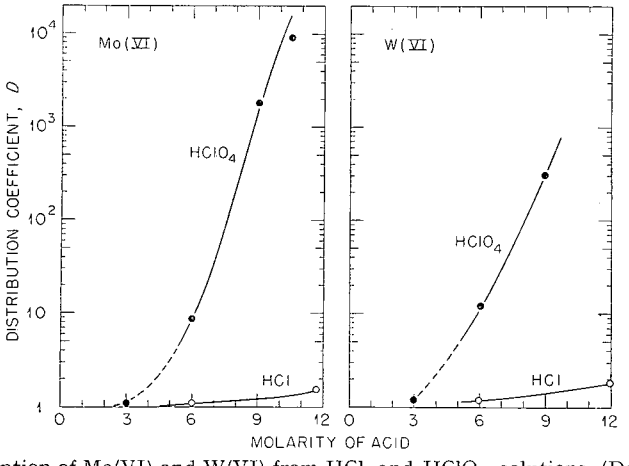


Fig. 16. Adsorption of Mo(VI) and W(VI) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

In perchloric acid adsorbability of W(VI) is low near 3 M and increases rapidly with increasing M to $D > 10^2$ near 9 M.

Adsorption data for perchloric acid concentrations below 6 M $HClO_4$ tended to be erratic (hydrolysis) even at the tracer W(VI) concentrations studied (*ca.* $4 \cdot 10^{-8}$ M). Further, although satisfactory column adsorption of W(VI) could be obtained with *ca.* 10^{-7} M W(VI) in 9 M $HClO_4$, attempts to carry out separations with *ca.* 10^{-4} M W(VI) in 9 M $HClO_4$ were unsuccessful because of precipitation of tungstic acid.

(*m*) *Uranium(VI), neptunium(VI) and plutonium(VI)* (*Fig. 17*). U(VI) is strongly

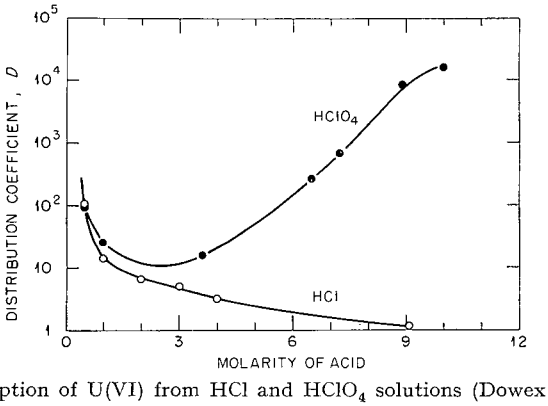


Fig. 17. Adsorption of U(VI) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

adsorbed from dilute HCl and adsorbability decreases with increasing M HCl; above 6 M HCl adsorbability is negligible, $D_v < 1$. Our results for U(VI) are in good agreement with similar data of DIAMOND, STREET AND SEABORG[4]. As might be expected, Np(VI) and Pu(VI) adsorb very similarly[4]. In the present study, low adsorbability ($D_v < 1$) was confirmed for Np(VI) and Pu(VI) in 9 M HCl solutions.

In $HClO_4$, adsorbability of U(VI) decreases from $D = 100$ near 0.5 M to a minimum $D = 10$ near 2.5 M, then increases to $D > 10^4$ in 10 M $HClO_4$. In 9 M $HClO_4$, (containing small amounts of Cl_2 gas) Np(VI) and Pu(VI) were found to be strongly adsorbed, $D > 100$. However, complete adsorption functions for Np(VI) and Pu(VI) in $HClO_4$ were not determined since they are probably very similar to that of U(VI).

In other oxidation states U, Np and Pu are only slightly adsorbed from HCl at high μ according to published data[4] for U(IV), Np(IV), Np(V), Pu(III) and Pu(IV). However, in conc. $HClO_4$ strong adsorption will probably occur for all oxidation states, including, presumably, the + 5 state.

(*n*) *Transplutonium elements*. The adsorption behavior of the trivalent transplutonium elements in HCl has been extensively studied by others[4,7]. At low M HCl their adsorption is similar to that of the rare earths, but at high M HCl the trivalent transplutonium elements are less strongly adsorbed than the rare earths and a group separation becomes possible. We have included Am(III) and Cm(III) in our studies and found, in agreement with the earlier work with similar Dowex 50 resins that $D_v < 1$ for both elements at M HCl > *ca.* 6.

In $HClO_4$, the adsorption function of Am(III) parallels that in HCl at low acidity; however, near 4 M $HClO_4$ adsorbability increases rapidly from a minimum D = *ca.* 10 to $D > 10^4$ near 10 M $HClO_4$. Our results for Am(III) are in excellent agreement with similar data of CHOPPIN AND DINIUS[8]. Strong adsorption was also found for Cm(III) in 9 M $HClO_4$; presumably all of the trivalent trans-plutonium elements are strongly adsorbed by Dowex 50 from concentrated $HClO_4$.

Concentrated $HClO_4$ solutions thus appear to be unique media from which all actinides (and indeed most α-emitters) from Ac(III) through the transplutonium elements may be adsorbed.

(*o*) *Manganese(II), cobalt(II), copper(II) and nickel(II)*. (*Figs. 18 and 19*). The divalent transition elements, Mn(II), Co(II), Ni(II) and Cu(II) have very similar adsorption functions in HCl. They are adsorbed from dilute HCl; but above 6 M HCl, D becomes less than unity. In $HClO_4$ adsorbability of Co(II), Cu(II) and Ni(II) is only slightly greater than from HCl solutions of the same concentration. However, Mn(II) is markedly more strongly adsorbed from concentrated $HClO_4$ solutions than from the corresponding HCl solutions. Adsorbability of Mn(II) increases from a minimum, $D = 5.5$ near 5 M $HClO_4$ to D = *ca.* 700 in 12 M $HClO_4$.

The strong adsorption of Mn(II) from concentrated $HClO_4$ leads to interesting separations. A typical experiment is shown in Fig. 19 which illustrates the separation of Mn(II) from a relatively weakly adsorbed element, Co(II). A small aliquot of solution containing ^{54}Mn and ^{60}Co tracers in 9 M $HClO_4$ was added to a 0.25 $cm^2 \times$ 3 cm column of Dowex 50-X4 pretreated with 9 M $HClO_4$. On elution with the same eluent, Co(II) appeared in the effluent in peak concentration near 1.7 c.v. while Mn(II) remained adsorbed. It was eluted in a sharp band with 9 M HCl.

(*p*) *Iron(III)* (*Fig. 20*). Adsorbability of Fe(III) from HCl solutions first decreases with increasing acid concentration to a minimum $D \approx 1$ near 4 M, then increases

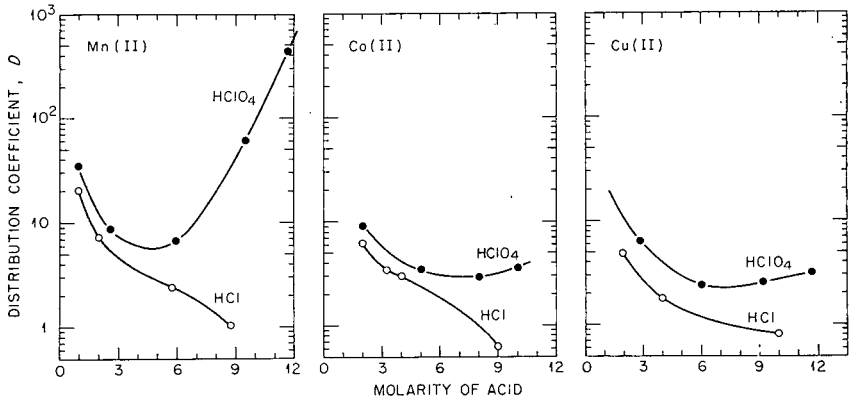


Fig. 18. Adsorption of Mn(II), Co(II) and Cu(II) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

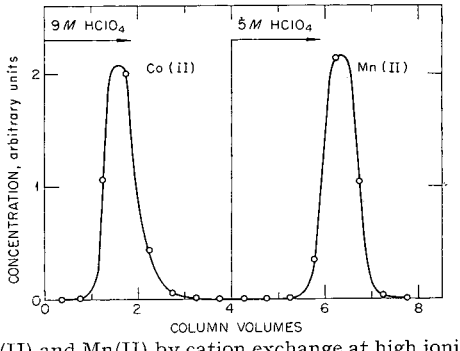


Fig. 19. Separation of Co(II) and Mn(II) by cation exchange at high ionic strength (Dowex 50-X4, 50°).

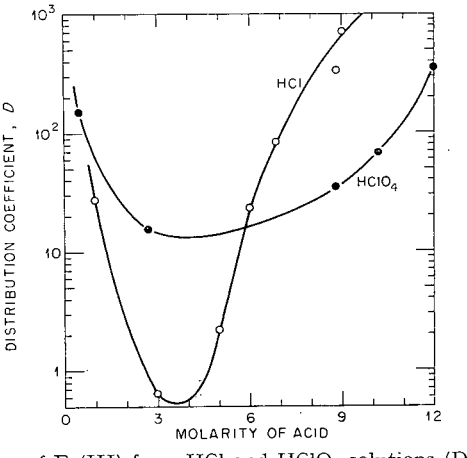


Fig. 20. Adsorption of Fe(III) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

rapidly to $D = ca.\ 10^3$ near 10 M. The present results with the 4 % crosslinked resin essentially confirm earlier measurements[6] with a more highly crosslinked (12 %) Dowex 50 resin. However, at low M HCl adsorbability of Fe(III) is lower with the 4 % resin while, in concentrated HCl, it is almost a factor of ten higher than with the 12 % resin. This unusual influence of cross linking on adsorbability at high HCl concentrations was also observed and discussed by TITZE AND SAMUELSON[32].

In $HClO_4$, the adsorption function of Fe(III) shows a shallow minimum near 4 M with $D = ca.\ 10$; D increases slowly to *ca.* 350 in 12 M $HClO_4$.

(*q*) *Technetium(VII) and rhenium(VII).* Adsorbability of Tc(VII) and Re(VII) is negligible from HCl solutions. In $HClO_4$, adsorbability is also small though slight adsorption with $D_v \approx 1$ occurs in relatively concentrated $HClO_4$ solutions (9 M). In these measurements, small amounts of chlorine gas were added to prevent reduction. In some experiments with Tc tracer (^{99}Tc) in which chlorine was not used, ^{99}Tc was strongly adsorbed from concentrated $HClO_4$ presumably in the form of a lower oxidation state.

(*r*) *Platinum elements.* It is rather well established that the chloride complexes of the platinum elements are not adsorbed from HCl by cation exchangers. For the present study, a series of confirmatory measurements was carried out with Ru(IV), Rh(III), Pd(II), Pd(IV), Os(IV), Ir(IV) and Pt(IV). Negligible adsorption was found for these ions in the range 0.2–9 M HCl.

Adsorbability measurements were not carried out in $HClO_4$ solutions because of uncertainties regarding species and oxidation states of these elements in these media. Significant adsorption of Pd, Rh and Ir in dilute $HClO_4$ has been reported[33]; presumably, the elements were in their higher oxidation states.

(*s*) *Silver(I) and gold(III).* Adsorbability of Ag(I) was negligible, $D_v < 1$, from 0.2–9 M HCl. In $HClO_4$, adsorbability decreases from $D = 11$ in 1 M $HClO_4$ to a shallow minimum, $D = 3$, near 7 M $HClO_4$; it then increases to $D = 9$ in 10 M $HClO_4$.

Au(III) is strongly adsorbed from moderately concentrated and concentrated HCl. The adsorption function found here is similar to that reported earlier[6] for a resin of higher crosslinking (12 % DVB). However, the values of D with the present 4 % resin are considerably larger than for the 12 % resin[6]. A similar inverse crosslinking effect was observed with Fe(III) (see Section 3 p).

Adsorption of Au(III) was not studied in $HClO_4$ media since reduction and precipitation occurs in the absence of stabilizing complexing agents.

(*t*) *Zinc(II), cadmium(II) and mercury(II)* (*Fig. 21*). In HCl adsorbabilities of Zn(II), Cd(II) and Hg(II) decrease rapidly with increasing M HCl and become negligible above *ca.* 2, 1 and 0.1 M HCl, respectively.

In dilute $HClO_4$, Zn(II), Cd(II) and Hg(II) are all significantly adsorbed. Adsorbabilities of Zn(II) and Cd(II) decrease to minima near 6 and 4 M $HClO_4$, respectively; D then increases with M $HClO_4$. Adsorption of Hg(II) only decreases with increasing M $HClO_4$.

(*u*) *Aluminum(III), gallium(III), indium(III) and thallium(III)* (*Fig. 22*). Adsorbability of Al(III) decreases with increasing M HCl to $D = ca.\ 4$ near 3 M and $D = ca.\ 1$ at 12 M HCl. In $HClO_4$, the adsorption function has a shallow minimum near 6 M where $D = ca.\ 5$; in 12 M $HClO_4$, $D = ca.\ 20$.

Adsorption of Ga(III) in HCl increases rapidly from its minimum $D = 1.5$ near 4 M to $D = ca.$ 800 near 10 M; D decreases slightly with further increase of M HCl.

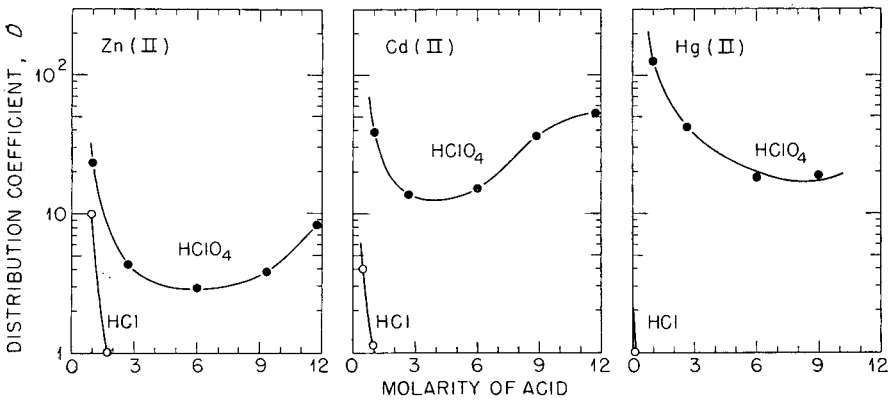


Fig. 21. Adsorption of Zn(II), Cd(II) and Hg(II) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

In $HClO_4$, adsorption is minimum near 4 M and increases rapidly to $D = ca.$ 10^3 at 12 M.

Adsorbability of In(III) decreases rapidly with increasing M HCl and becomes negligible above *ca.* 1 M HCl. In $HClO_4$, an adsorption minimum occurs near 5 M where $D = ca.$ 10. In 12 M $HClO_4$, D is larger than 10^2.

Tl(III) is weakly adsorbed near 1 M HCl; adsorption increases to a shallow maximum $D = ca.$ 10 near 9 M. The adsorption function in HCl is reminiscent of that for Au(III) but is an order of magnitude lower. In $HClO_4$, adsorbability decreases rapidly with M; there appears to be a shallow minimum near 5 M. The results at higher acid concentrations are in doubt because of the possibility of reduction (by the resin) of Tl(III) to Tl(I).

The results for In(III), Ga(III) and Tl(III) in concentrated HCl solutions are consistent with the earlier data[6] obtained with a 12 % crosslinked resin except that, as with Fe(III) and Au(III), adsorbabilities with the 4 % resin are larger at high μ.

(v) Germanium(IV) and tin(IV). Adsorbability of Ge(IV) and Sn(IV) is negligible from 9 M HCl. These elements have been reported[24] to be adsorbable from dilute HCl but adsorbability falls off rapidly since apparently non-adsorbable chloride complexes are formed.

Adsorbability of Ge(IV) and Sn(IV) in $HClO_4$ was not studied because of the tendency of these elements to hydrolyze and precipitate in these media.

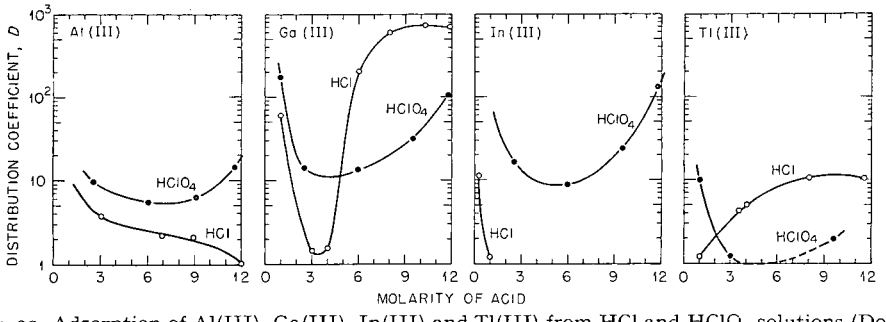


Fig. 22. Adsorption of Al(III), Ga(III), In(III) and Tl(III) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

(*w*) *Lead(II)* (*Fig. 23*). In HCl, adsorbability of Pb(II) decreases rapidly with increasing acid concentration to $D < 1$ above 1.5 M HCl.

In $HClO_4$, adsorbability of Pb(II) decreases less rapidly with acid concentration to a minimum $D = 13$ near 4 M, then slowly increases to $D =$ *ca.* 60 near 10 M.

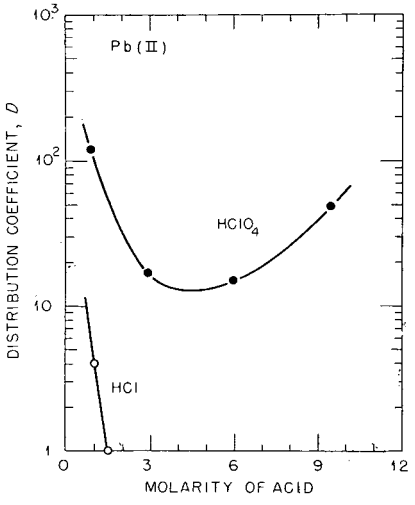


Fig. 23. Adsorption of Pb(II) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

(*x*) *Antimony(III) and (V) and bismuth(III)* (*Figs. 24 and 25*). Sb(III) and Bi(III) are adsorbable from dilute HCl but adsorbability decreases rapidly with increasing M HCl. For Sb(III), $D < 1$ above 3 M HCl while for Bi(III), $D < 1$ above *ca.* 0.5 M HCl.

In $HClO_4$, adsorbability of Sb(III) increases from a shallow minimum, $D = 1.5$ near 6 M to $D =$ *ca.* 50 near 11 M. Bi(III) is strongly adsorbed at all $HClO_4$ concentrations; its adsorption minimum lies near 4 M where $D = 40$.

In HCl, Sb(V) is strongly adsorbed in the range 9–12 M where $D_v =$ *ca.* 2,000. These values are markedly higher than the value reported earlier with a 12 % Dowex 1

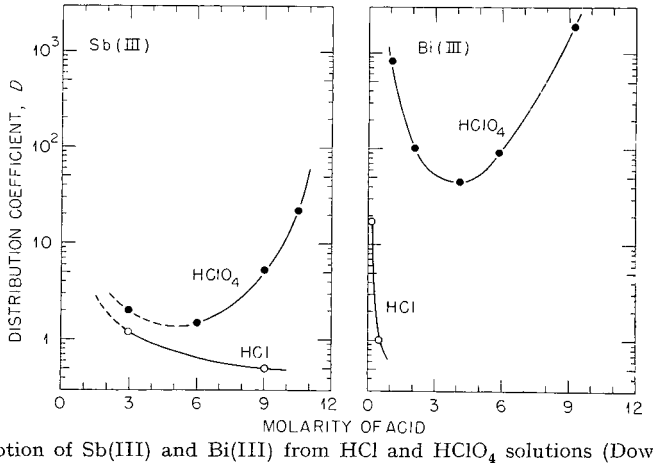


Fig. 24. Adsorption of Sb(III) and Bi(III) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

resin. The error in earlier measurements (by the batch distribution method) may have resulted from presence of the weakly adsorbable (tracer) Sb(III).

Adsorbability measurements of Sb(V) tend to be erratic for M HCl < 9. When Sb(V) was adsorbed from concentrated HCl, slow leak-through occurred on elution with 6–8 M HCl–Cl_2 solutions, although most of the Sb(V) remained strongly adsorbed. Presumably this partial elution results from relatively slow hydrolytic reactions of Sb(V) (see *e.g.*, ref. 34).

The HCl concentration at which Sb(V) adsorption is carried out also affects the rate of reduction of Sb(V) to Sb(III) with iodide. This reduction is useful for elution of antimony as illustrated in Fig. 25 with a separation of Sb from Te. If adsorption is

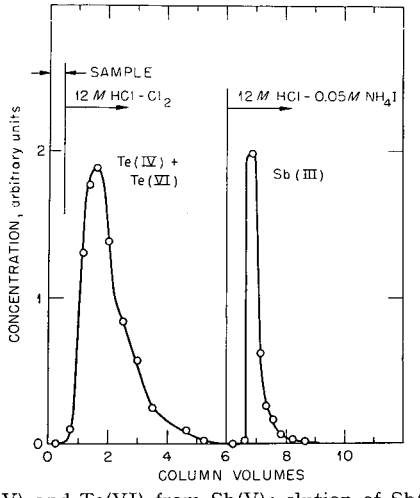


Fig. 25. Separation of Te(IV) and Te(VI) from Sb(V); elution of Sb(V) by reduction to Sb(III) (Dowex 50-X4, 25°).

carried out from 10–12 M HCl and reduction with 10–12 M HCl containing *ca.* 0.05 M NH_4I, immediate and quantitative elution of Sb (as Sb(III)) occurs. However, if adsorption is carried out at M HCl $\leqslant 8$ and elution with the same 10–12 M HCl–0.05 M NH_4I, incomplete removal and severe tailing occur.

(*y*) *Arsenic(III) and (V), selenium(IV), tellurium(IV) and polonium(IV)*. These elements are negligibly adsorbed from 9 M HCl and 9 M $HClO_4$ solutions. A cursory examination showed that adsorbabilities are also low ($D_v < 1.5$) from more dilute solutions (0.2–1 M HCl or $HClO_4$).

(*z*) *Other non-metals*. Adsorbability measurements were carried out with HCl and $HClO_4$ solutions containing a number of non-metallic elements as their common acids: H_3BO_3, HNO_3, HF, HCl, HBr, HI, H_2SO_4 and H_3PO_4. Negligible or only slight adsorbability was observed for these acids over a relatively wide range of HCl and $HClO_4$ concentrations (*ca.* 0.2 or 1 M to 9 M).

CONCLUSIONS

The cation exchange data for the elements in HCl and $HClO_4$ solutions are summarized in Figs. 26 and 27 as plots of log D_v *versus* molarity of acid. These adsorption

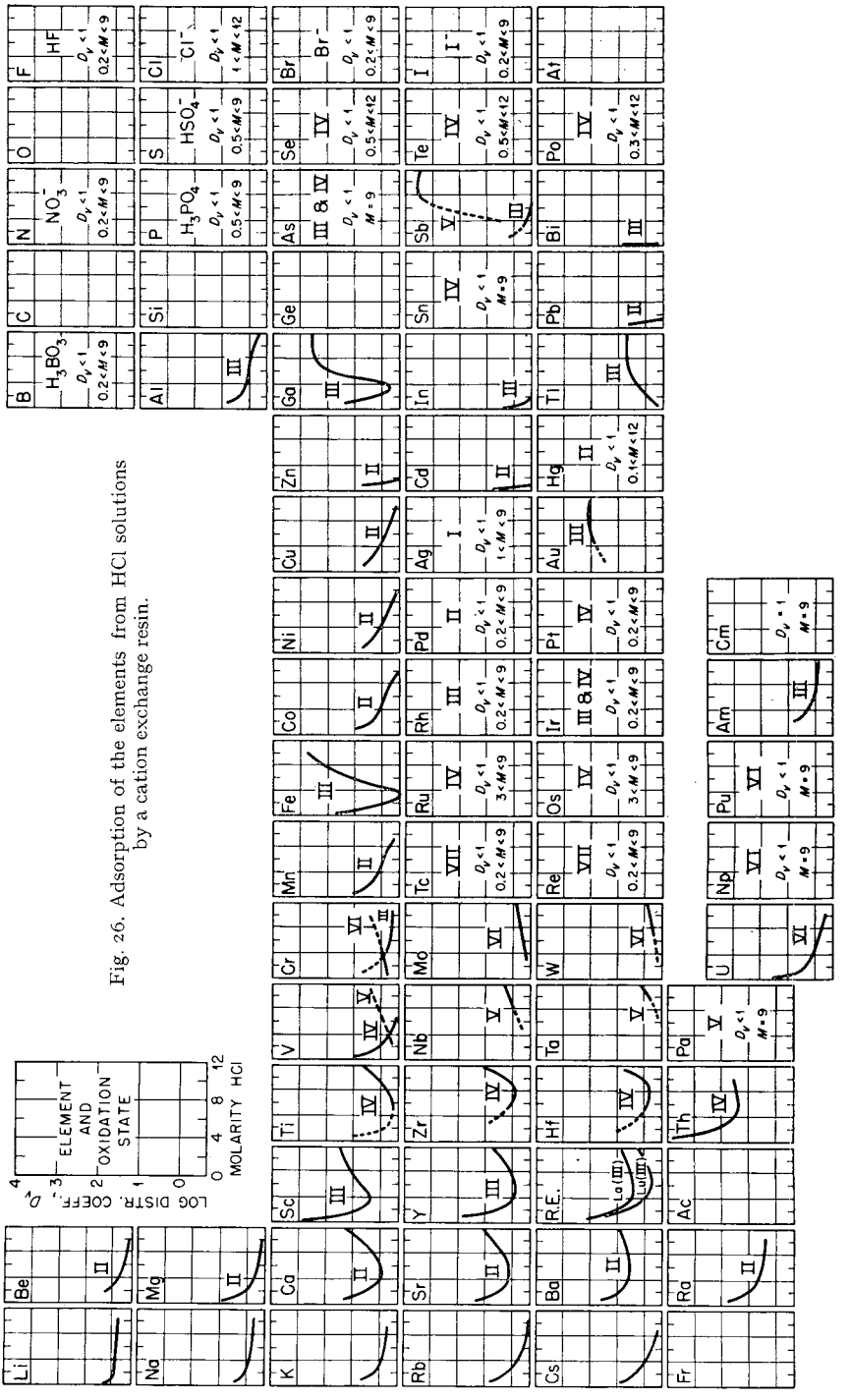


Fig. 26. Adsorption of the elements from HCl solutions by a cation exchange resin.

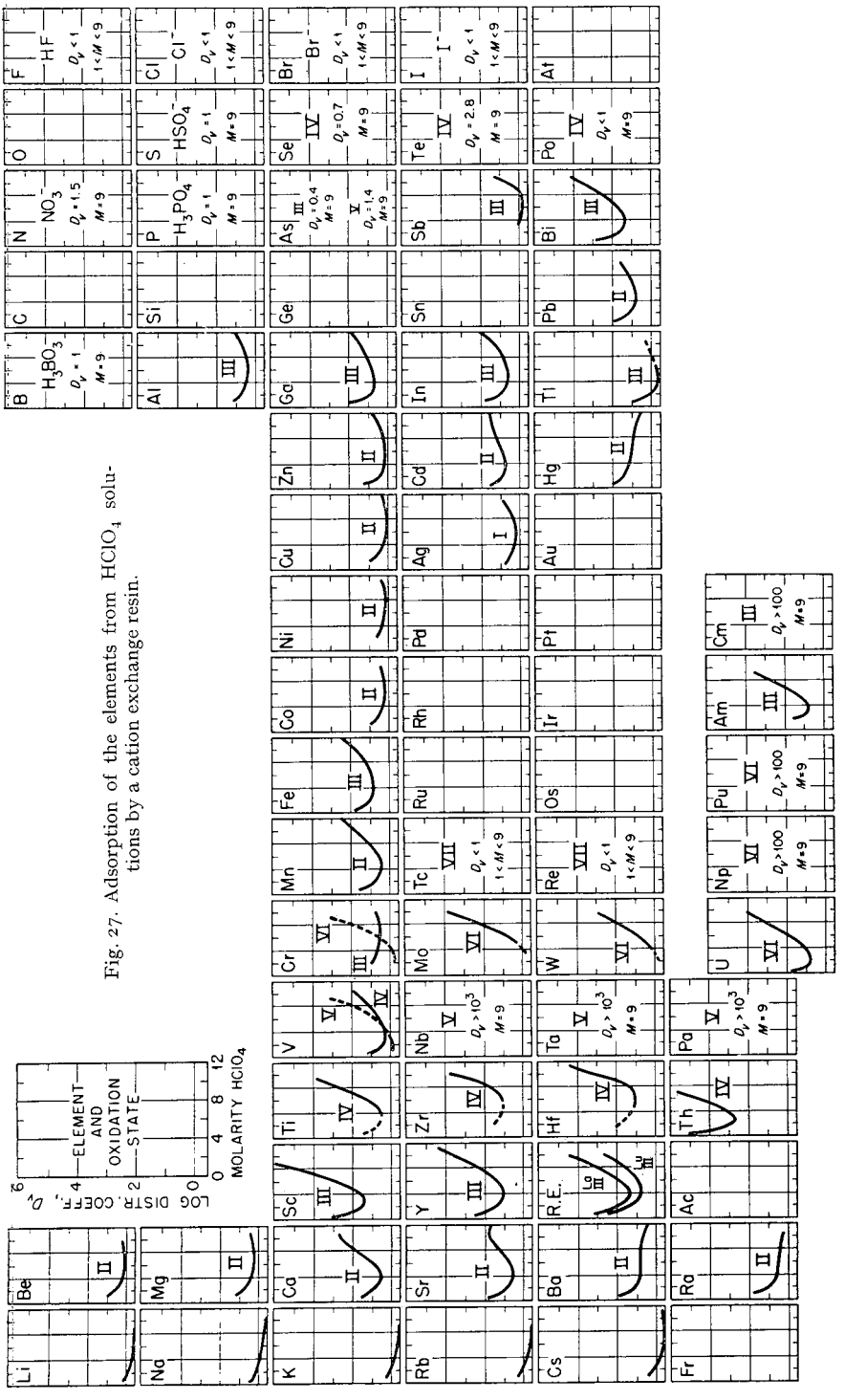


Fig. 27. Adsorption of the elements from $HClO_4$ solutions by a cation exchange resin.

functions are comparable; they refer to the same resin (Dowex 50-X4) and most of them were obtained at "tracer" loading ($L < 0.01$). Only for Bi(III), Ti(IV), Hf(IV), Zr(IV) and Mo(VI) in $HClO_4$ was it necessary to operate at higher concentrations; but even for these, loading was less than 10 % at the highest adsorbabilities measured. While adsorbabilities in HCl and $HClO_4$ often differ widely, the adsorption functions have a number of common features. Thus, at low ionic strength, most elements show a decrease of D with μ, as expected. However, several elements (*e.g.*, Zn(II), Cd(II), Hg(II), Pb(II), In(III) and Bi(III)) show a precipitous decrease in adsorbability in dilute HCl; for these there is no resurgence of adsorbabilities at high μ. These elements are strongly complexed by chloride ions and their complexes (as those of many other elements such as the platinum elements) are non-adsorbable. Conclusions regarding the charge of these complexes cannot be drawn from the ion exchange data alone since at least some negatively charged complexes can be adsorbed by cation exchangers[6].

Many elements show adsorption minima in HCl and $HClO_4$ solutions. In HCl the minima are usually shallower than in $HClO_4$ and the distribution coefficients at the minima are lower. This is a reflection of the slower increase of D with M in HCl compared with $HClO_4$. For some elements this slow increase of D in HCl, or the lack of such increase, results from formation of non-adsorbable chloride complexes at high μ. For others, *e.g.*, Ca(II) and Sr(II), the difference in adsorbabilities in HCl and $HClO_4$ solutions presumably is not due to formation of chloride complexes. Thus comparison of distribution coefficients in HCl and $HClO_4$ is often not sufficient to decide if complexing reactions occur. However, measurement of distribution coefficients in HCl and $HClO_4$ mixtures at constant μ often permits identification of complexing reactions and evaluation of the stability quotients.

The excellent adsorption which occurs at high ionic strength and the differences in adsorption functions between HCl and $HClO_4$ solutions may be utilized for the separations of the elements, both individually and in groups. For example, a number of elements can be adsorbed from concentrated $HClO_4$ solutions and thus be separated from the weakly adsorbed elements. The adsorbed elements may then be eluted, often sequentially, with concentrated HCl or more dilute HCl and $HClO_4$, or HCl–$HClO_4$ mixtures. However, for many purposes such group separations can be carried out more efficiently with HCl–$HClO_4$ mixtures containing small amounts of HF; our studies with such mixed acid systems will be discussed in subsequent publications.

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SUMMARY

A broad survey of the cation exchange behavior of the elements in HCl and $HClO_4$ solutions is presented and adsorption functions are given. The results are also summarized in the form of two "Periodic Tables" which give the distribution coefficients of the elements as a function of M HCl and M $HClO_4$.

Many elements are strongly adsorbed from concentrated $HClO_4$ solutions. There

are large differences in adsorbability between HCl and $HClO_4$ solutions, particularly at high ionic strength. Application of these data to high ionic strength cation exchange separations is illustrated.

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SEPARATION AND QUANTITATIVE DETERMINATION OF AMINO SUGAR ANTIBIOTICS AND THEIR DEGRADATION PRODUCTS BY MEANS OF AN IMPROVED METHOD OF CHROMATOGRAPHY ON RESIN

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Amino sugar antibiotics, such as kanamycin, neomycin, and paromomycin, are usually produced as a mixture of closely related antibiotics, whose separation and differentiation have always been difficult problems. In the present study, an ion-exchange method of chromatography, developed originally to separate kanamycins A and B[1], has been somewhat improved and applied successfully to the separation and quantitative determination of eight amino sugar antibiotics and their degradation products.

The technique consists of separation on strongly basic anion-exchange resins, preferably Dowex 1 X 2 in the hydroxyl form, the column being developed with distilled water. The modifications that have been introduced are: (1) the use of a shorter column (resin volume, 25–50 ml), (2) slower rates of elution (20–30 ml/h), (3) a finer particle size for the resin (200–400 mesh instead of 50–100 mesh), and (4) a ninhydrin method for the determination of the amino sugars.

The use of finer particles coupled with slower rates of elution resulted in a considerably improved separation and an increased sharpness of the elution peaks; these become primary advantages when more complex mixtures are analyzed. Introduction of the photometric ninhydrin method permits quantitative analysis of the amino sugar antibiotics on a milligram scale. The majority of the antibiotics examined gave 40 to 50% color yield (diketohydrindylidene-diketohydrindamine) when the ninhydrin procedure of MOORE AND STEIN for amino acids[2] was used. The modified system can be used either with a fraction collector or with an automatic recording equipment.

EXPERIMENTAL

Quantitative determination of a mixture of kanamycins A, B and C by means of an automatic amino acid analyzer

Chromatography on resin combined with an automatic amino acid analyzer provided an adequate method for the quantitative determination of kanamycins in a mixture. The procedure is essentially the same as that described for amino acid determination[3] except for the chromatographic column and developing solvent. The results obtained by this procedure when a synthetic mixture of kanamycins A[4], B[5] and C[6] was chroma-

tographed are superior to those previously published[1] and are illustrated in Fig. 1 by the satisfactorily sharp peak for kanamycin A together with complete separation of B and C. The size of a peak, which was integrated by the convenient height–time–width method[3], was linear with respect to the concentration of kanamycin A, B, or C within the respective ranges so far examined: 1–3.5 mg for A, 0.1–0.35 mg for

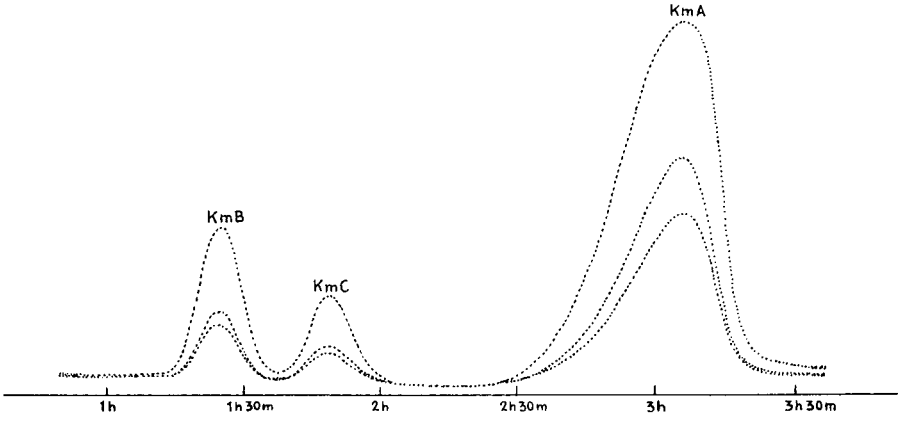

Fig. 1. Tracing of the chromatographic separation of a kanamycin mixture automatically recorded by Hitachi Amino Acid Analyzer (Type KLA-2). Column, Dowex 1 X 2 0.9 × 39 cm. Flow rate, 30 ml/h. KmA 2.08 mg; KmB 0.207 mg; KmC 0.143 mg.

B, and 0.05–0.25 mg for C. A sample may be analyzed within 3.5 h by this method, which is particularly suitable for the determination of kanamycin B in the presence of A and C, as it was indicated in the present investigation that the differential bioassay[7] of B from A gives an abnormally high estimate of B in samples which are contaminated with C.

Separation of a mixture of other amino sugar antibiotics and isolation of paromomycin II

The modified procedure, with its good resolving power, was then applied to the study of other amino sugar antibiotics and related compounds.

Fig. 2 illustrates a typical chromatogram obtained with a mixture of kanamycins A, B and C, neomycins A (neamine)[8], B[9] and C[9], and paromomycins I[10] and II[9],

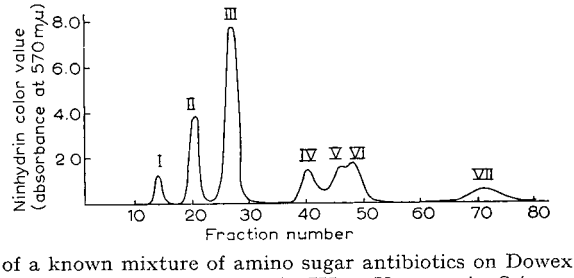

Fig. 2. Separation of a known mixture of amino sugar antibiotics on Dowex 1 X 2 (50 ml). I = Neamine (0.5 mg); II = Kanamycin B (2.2 mg); III = Kanamycin C (2.2 mg) and neomycin C (2.2 mg); IV = Paromomycin II (2.2 mg); V = Kanamycin A (2.2 mg); VI = Neomycin B (2.2 mg); VII = Paromomycin I (4.4 mg).

while their relative peak effluent volumes are shown in Table I*. With the exception of the overlapping of neomycin C and kanamycin C, separation of the other six amino sugar antibiotics is satisfactory. Since the antibiotics belonging to the neomycin group or paromomycin group are separated completely, it is possible, as in the case of

TABLE I

RELATIVE PEAK EFFLUENT VOLUMES (*Rv*) OF VARIOUS AMINO SUGARS AND RELATED COMPOUNDS ON DOWEX 1 X 2

Compound	*M/N*[a]	*Rv*[b]	
Tetrasaccharide			
Neomycin B	102	1.04	
Neomycin C	102	0.59	
Paromomycin I	123	1.54	
Paromomycin II	123	0.87	
Trisaccharide			
Kanamycin A	121	1.00	(1.00)[c]
Kanamycin B	97[d]	0.45	(0.33)
Kanamycin C	121	0.59	
Disaccharide			
Neamine	81	0.26	(0.22)
Paromamine	108	0.32	
6-O-α-D-3-Amino-3-deoxy-glucopyranosyl-2-deoxystreptamine	108	0.43	
Methyl (3-O-neosaminido-B)-α-D-ribofuranoside	162	0.50	
Methyl (3-O-neosaminido-B)-α-D-ribopyranoside	162	0.64	
Methyl (3-O-neosaminido-B)-β-D-ribofuranoside	162	0.96	
Methyl (3-O-neosaminido-B)-β-D-ribopyranoside	162	1.24	
Methyl (3-O-neosaminido-C)-α-D-ribofuranoside	162	0.38	(0.28)
Methyl (3-O-neosaminido-C)-α-D-ribopyranoside	162	0.44	(0.36)
Methyl (3-O-neosaminido-C)-β-D-ribofuranoside	162	0.64	(0.61)
Methyl (3-O-neosaminido-C)-β-D-ribopyranoside	162	0.88	(0.76)
Monosaccharide			
Methyl 3-amino-3-deoxy-α-D-glucopyranoside	193	0.48	(0.47)
Methyl 6-amino-6-deoxy-α-D-glucopyranoside	193	0.38	(0.32)
Methyl 2-amino-2-deoxy-α-D-glucopyranoside	193	0.38	
2-Deoxystreptamine	81	0.275	(0.22)
Streptamine	89	0.38	
Streptidine	—	0.13	
Amines			
Benzylamine	107	0.50	(0.40)
Histamine	56	0.38	(0.21)

[a] Molecular weight divided by the number of amino groups.
[b] Peak effluent volume of a substance divided by the peak effluent volume of kanamycin A.
[c] *Rv* on a column of Dowex 1 X 4 resin.
[d] *M/N* was calculated on the basis of the revised molecular formula $C_{18}H_{37}N_5O_{10}$ with five amino groups[16].

kanamycin, to estimate the amount of each antibiotic produced in a mixture by chromatography in combination with ninhydrin photometry. Such a chromatographic analysis when applied to a fradiomycin preparation obtained from the fer-

* For this procedure a fraction collector was employed.

mentation broth of *Streptomyces fradiae* No. 260, indicated that it consisted of 74% of neomycin B and 26% of neomycin C. Application of the procedure to a commercial paromomycin preparation revealed the presence of 91% paromomycin I, admixed with a minor amount of paromomycin II (9.0%).

In addition to analytical uses, resin chromatography can be used for preparative purposes and has the advantage of yielding pure antibiotics, as evaporation of the aqueous alkaline portions of the effluent usually affords the free bases. Thus, the free base of paromomycin II was easily isolated* when a column overloaded with a paromomycin mixture was used. The product had an $[\alpha]_D^{20}$ of + 99° (in water), and gave an almost identical ultraviolet absorption curve to that of neomycin C, but differed from that of neomycin B and paromomycin I when heated with 50% sulfuric acid. Its biological activity against many micro-organisms was in general weaker than that of neomycins B, C and paromomycin I. On methanolysis with 2 *N* HCl it yielded equimolar amounts of paromamine and methyl neobiosaminide C, the main component of the latter being methyl (neosaminido-C)-β-D-ribopyranoside. These results are consistent with the proposed structure for paromomycin II[9] and zygomycin A_2[11].

Separation of methanolysis products of amino sugar antibiotics and isolation of new ribofuranoside forms of neobiosaminides B and C

Methanolysis of neomycins B and C in dilute HCl gave neamine and the respective methyl neobiosaminides, the latter usually being isolated as a mixture of their anomeric α- and β-glycosides. A crude methyl neobiosaminide B, when subjected to chromatographic analysis, afforded five ninhydrin positive peaks (B_1, B_2, B_3, B_4 and B_5 in Fig. 3); B_1 was identified as neamine. The four remaining peaks showed a

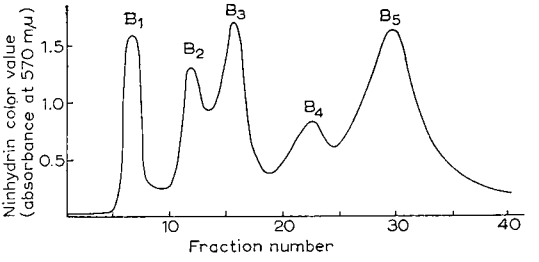

Fig. 3. Separation of crude methyl neobiosaminide B derived from mild methanolysis of neomycin B on Dowex 1 X 2 (25 ml).

positive furfural assay and must be anomeric glycoside isomers in the ribose moiety of the disaccharide methyl neobiosaminide B, since all the components could be hydrolyzed in *N* HCl, at 90°, to products with the same constant rotation ($[\alpha]_D^{20}$, + 30°) as that of neobiosamine B[12] and retreatment of the hydrolyzate of B_5 with 0.3 *N* methanolic HCl at 4° yielded the same four components (B_2, B_3, B_4 and B_5) as before. A ring structure was assigned to the ribose moiety in these components (furanoside for B_2 and B_4, pyranoside for B_3 and B_5) on the basis of the relative rates of formation

* After completion of this work, a paper on the isolation of zygomycin A_2 which was identical with paromomycin II was published[15].

and data from hydrolysis under various conditions[13]. Assignment of an anomeric configuration (α-form for B_2 and B_3, β-form for B_4 and B_5) was made from a comparison of their optical rotations with those of anomeric methyl D-ribosides as shown in Table II. Similar evidence allowed assignment of a ribose structure to the four isomers* of methyl neobiosaminide C which were isolated in the same manner (see Table II). Methyl α- and β-neobiosaminides B and C containing ribofuranoside moieties have not been isolated in previous studies[7,9,12].

TABLE II

OPTICAL ROTATIONS OF ANOMERIC RIBOGLYCOSIDE ISOMERS OF METHYL NEOBIOSAMINIDES AND METHYL RIBOSIDE

Compound	α-D-*Ribofuranoside*	α-D-*Ribopyranoside*	β-D-*Ribofuranoside*	β-D-*Ribopyranoside*
Methyl neobiosaminide B[a]	+109	+ 69	+ 25	— 10
Methyl neobiosaminide C[a]	+168	+124	+ 88	+ 41
Methyl D-riboside[b]	+147	+103	— 62	—107

[a] $[\alpha]_D^{20}$ in water (concn. 0.6–1.2 %).

[b] $[\alpha]_D^{20}$ in methanol (concn. 1.0 %)[14].

Paromomycin I, when treated in methanol with 0.3 *N* HCl, afforded five components, of which four were identified as the anomeric riboglycoside isomers of methyl neobiosaminide B as would be expected from the structure[9,10]. The first emerging peak was identified as paromamine, which is eluted more slowly than neamine. 6-O-α-D-3-amino-3-deoxy-glucopyranosyl-2-deoxystreptamine, a partial degradation product of kanamycin A[4], emerges immediately after paromamine with complete separation.

As for the monosaccharide amino sugars, chromatography on Dowex 1 X 2 separated a mixture of 2-deoxystreptamine, methyl 6-amino-6-deoxy-α-D-glucopyranoside and methyl 3-amino-3-deoxy-α-D-glucopyranoside, which are the main methanolysis products of kanamycin A[4]. Further application of the method indicated the resolution of streptidine, streptamine and 2-deoxystreptamine, the former two being the degradation products of streptomycin.

The above results together with data from studies still in progress, emphasise the usefulness of chromatography on resins for the fractionation and quantitative estimation of various types of amino sugar glycosides.

Scope of chromatography on resin

The strongly alkaline and adsorptive properties of Dowex 1 X 2 limit the number of substances which can be effectively used on such columns. Neobiosamines, 2-amino-2-deoxy-D-glucose, isonicotinic acid hydrazide, homosulfamine and lysine, when examined, were adsorbed strongly on the resin with decomposition in some cases. On the other hand, a mixture of histamine and benzylamine was successfully separated, the former being eluted faster than the latter.

The above limitation, however, offers a convenient purification procedure for an

* Thin-layer and paper chromatography using various solvent systems was unsuccessful in resolving these isomers.

appropriate basic substance when it is contaminated with substances which are highly adsorbed or have alkaline unstability. In fact, the separation of methyl 2-amino-2-deoxy-D-glucoside from unreacted 2-amino-2-deoxy-D-glucose was easily accomplished by passing a crude methylation product through a short column of the resin.

As indicated by ROTHROCK *et al.*[1], chromatography on Dowex 1 X 2 is a typical example of adsorption chromatography on ion-exchange resins. Although the data available are relatively few, consideration of the data shown in Table I reveals some correlation between structure and relative peak effluent volume (*Rv*): (1) Among the amino sugars having similar molecular weights, *Rv* in many cases increases with increasing *M/N* value (molecular weight divided by the number of amino groups); (2) with regard to the relationship of molecular size to pore size of resin, it is noted that, although Dowex 1 X 2 is preferable for oligosaccharide amino sugars, the 4% cross-linked resin gave a much improved resolution with monosaccharide amino sugars and the alkyl amines so far examined.

Full experimental will be published elsewhere.

SUMMARY

Chromatography on Dowex 1 X 2 resin was modified to improve the sharpness of the effluent peaks and applied to the separation and quantitative determination of amino sugar antibiotics and their degradation products. A photometric ninhydrin method was used to determine the amino sugars.

The usefulness of the method was further demonstrated by the isolation of paromomycin II and methyl neobiosaminides B and C containing a ribofuranoside moiety. Finally, limitations of the technique arising from the strong alkaline and adsorptive properties of the resin were indicated.

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ANORGANISCHE RADIODÜNNSCHICHTCHROMATOGRAPHIE

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Die Dünnschichtchromatographie bietet eine wertvolle Ergänzung zur Papierchromatographie. Auch anorganische Stoffe lassen sich auf diese Weise trennen, wobei bisher hauptsächlich eine Gruppentrennung im Sinne des klassischen Trennungsganges erfolgte[1-6]. Für viele Zwecke, wie etwa die Reinheitsprüfung bestimmter Ionen oder Gleichgewichtsbestimmungen radioaktiver Mutter–Tochter-Gemische, ist eine Trennung von 2- und 3-Stoffgemischen von Interesse. Die Dünnschichtchromatographie bietet hierfür gegenüber der Papierchromatographie — neben einer erheblichen Verkürzung der Trennzeit und grösserer Auswahl der Sorbentien — durch die Verringerung der aufgetragenen Menge eine bessere Übertragbarkeit von wägbaren auf unwägbare radioaktive Substanzmengen.

Die Methode zeigt allerdings Nachteile, wie etwa die Möglichkeit des Abblätterns der Schicht und somit eine Kontaminationsgefahr und die Notwendigkeit der Entaktivierung der Glasplatten.

Im folgenden werden Trennmethoden für einige radioaktive Zwei- und Mehrfachgemische beschrieben, wobei Versuche unternommen wurden, die genannten Nachteile weitgehend auszuschalten. Die in der Papierchromatographie üblichen Fliessmittel können in der Dünnschichtchromatographie oft mit entsprechenden Veränderungen verwendet werden. Hierbei können erhebliche Verbesserungen in der Trennung beobachtet werden, da die R_F-Werte grössere Unterschiede aufweisen als in der Papierchromatographie. Andererseits können auch säulenchromatische Verfahren mit entsprechenden Modifizierungen übertragen werden, wie das Beispiel der Trennung auf Lanthanoxyd im folgenden zeigt.

EXPERIMENTELLES

Es wurden Glasplatten von 38 × 200 mm Grösse verwendet. Diese Abmessung ist insofern günstig, als sich die Randeffekte hierbei nicht bemerkbar machen und die Glasplatten in einem modifizierten kommerziellen Radiopapierchromatographie-Gerät automatisch gemessen werden können.

Die Schicht bestand aus Kieselgel (Wölm) bzw. Kieselgur G (Merck) bzw. Lanthanoxyd. 60 g Kieselgel bzw. Kieselgur wurden entsprechend den Angaben der Herstellerfirmen mit 60 ml Wasser gut angerührt und mit einem Streichgerät auf 25 Platten aufgetragen. Lanthanoxyd (Auer Remy, Hamburg) wurde je nach Beschaffenheit mit wechselnden Mengen Stärke vermischt, wie oben beschrieben mit Wasser angerührt und auf die Platten aufgestrichen.

Bei den vorliegenden Versuchen wurde festgestellt, dass der Eisengehalt der Trägerschicht die Trennung wenig beeinflusst, jedoch den Nachweis der inaktiven Ionen insofern stört, als das Eisen mit den meisten Anfärbereagenzien eine Farbreaktion ergibt, die oft andere Farben überdeckt.

Die Trägerschicht wurde nach der Vorschrift von SEILER[1] von Eisen befreit, wobei im Falle von Kieselgel kein Gips zugesetzt wurde. Es sei erwähnt, dass dieser Prozess die Haftfestigkeit der Sorbentien etwas beeinträchtigt.

Die beschichteten Platten wurden in einem Plexiglaskasten aufbewahrt, damit sie vor Staub geschützt bleiben.

Die folgenden Trennungen konnten sowohl auf Kieselgur als auch auf Kieselgel durchgeführt werden. Wenn Unterschiede zu beobachten waren, wird es erwähnt. Aufgetragen wurden die Anionen als Natrium- bzw. Kaliumsalz und die Kationen meist als Chloride. Wo dies nicht der Fall war, wird es ebenfalls vermerkt.

Entwicklung der Chromatogramme

Die vorliegenden Versuche wurden mit inaktiven Ionen sowie mit trägerfreien radioaktiven Ionen durchgeführt. Die inaktiven Ionen wurden mit den üblichen Anfärbereagenzien entwickelt. Der Nachweis der radioaktiven Ionen wurde mit einem modifizierten kommerziellen Gerät automatisch vorgenommen. Fig. 1 zeigt die Skizze des

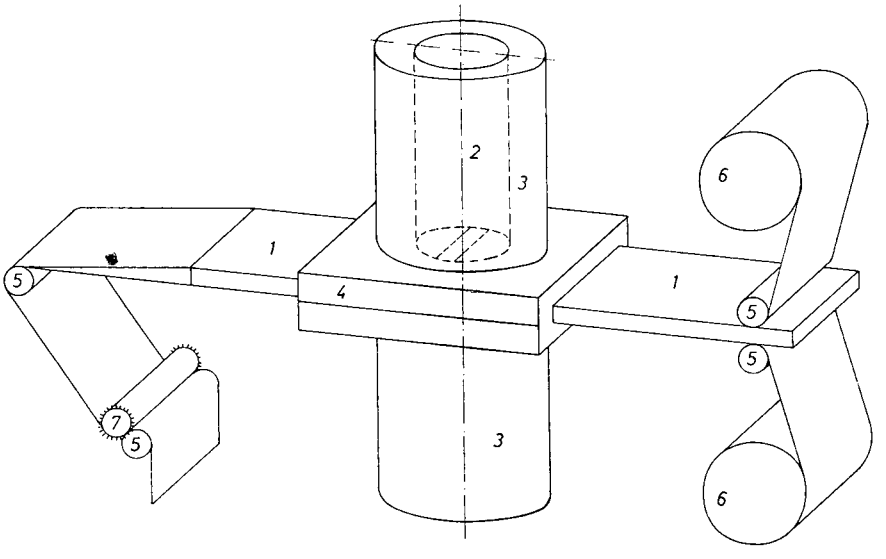

Fig. 1. Schema eines für die Auswertung der Dünnschichtchromatogramme modifizierten Radiopapierchromatographen der Firma Frieseke und Hoepfner. (1) Dünnschichtplatte; (2) Zählrohr; (3) Bleiabschirmung; (4) Metallplatte mit Führung und Schlitzblende; (5) Führungsrollen; (6) Rollen für Polyäthylenfolien mit Foliendicke 20 μ; (7) Transportrolle.

Gerätes, das sowohl für die Auswertung der Dünnschicht- als auch der Papierchromatogramme verwendet wurde.

Die Platte wird mit einer Lösung aus 2 Teilen Aceton und 1 Teil Kollodium (4%ig Merck) besprüht. Dabei ergibt sich eine dünne Schicht, die das Abfallen der

Trägerschicht und damit eine eventuelle Kontamination des Gerätes verhindert. Die besprühte Trägerschicht lässt sich aber auch mit Klebstreifen entsprechender Breite leicht abheben und — auf einem Streifen Papier angebracht — wie ein Papierchromatogramm behandeln. Der Verfasser zieht es jedoch vor, die Platten direkt zu messen, da bei Radionukliden mit geringer Strahlungsreichweite die Zählausbeute hierbei weniger beeinträchtigt wird.

TRENNUNGEN

Trennung des ionogenen vom kolloidalen Gold

Für diese Trennung eignet sich die Dünnschichtchromatographie besonders, da hierbei im Gegensatz zur Papierchromatographie keine Reduktionsgefahr des ionogenen Goldes durch die Trägerschicht besteht. Die folgenden Versuche wurden auf Platten mit Kieselgur bzw. Kieselgel vorgenommen. Da das kolloidale Gold nicht wandert, wurden Fliessmittel gesucht, die für ionogenes Gold einen hohen R_F-Wert ergeben. Auf Papier wurden Äthylacetat–Salpetersäure[7] und Aceton–Salzsäuregemische[8] verwendet. Da bei Verwendung von Salpetersäure eine Oxydationsgefahr für das kolloidale Gold besteht, wurde Salzsäure (12.5 N) eingesetzt. Die Abhängigkeit des R_F-Wertes von der Salzsäurekonzentration zeigt die Tabelle I.

TABELLE I

ABHÄNGIGKEIT DER R_F-WERTE VON DER SALZSÄUREKONZENTRATION

Salzsäuregehalt (Vol. %)	*R_F-Wert des ionogenen Goldes*
5	0.3
15	0.5
20	0.8
30 und höher	0.8

Das geeignetste Fliessmittel hat eine Zusammensetzung von Äthylacetat und Wasser (4:1) mit einer Salzsäurekonzentration von 2.5 Mol./l. Die Trennzeit beträgt 2 Stunden.

Eine gute Trennung erzielt man mit einer Mischung von Aceton und Wasser (7:3) mit einer Salzsäurekonzentration von 2 Mol./l. Der R_F-Wert des Au(III) beträgt etwa 0.9, Laufzeit 1 Stunde. Das kolloidale Gold bleibt — wie zu erwarten war — bei allen Versuchen am Startpunkt. Diese Methode wurde zur Reinheitsprüfung des kolloidalen ^{198}Au eingesetzt.

Barium–Lanthan- und Barium–Cäsium-Trennung

LEDERER[9] UND CARVALHO[10] haben Butanol–Salzsäure-Mischungen für Kationen-Trennung verwendet, wobei auf Papier Unterschiede im R_F-Wert von 0.2–0.3 für die Ionenpaare Ba–La bzw. Ba–Cs gefunden wurden. Auf Dünnschichtplatte mit Butanol und 6 N HCl (3:7) bleibt Barium am Start, während Lanthan mit der Fliessmittelfront wandert. Cäsium weist einen R_F-Wert von 0.8 auf.

Diese Trennungen wurden mit ^{140}Ba–^{140}La bzw. ^{133}Ba–^{133}Cs nachgeprüft. Anfärbemittel für Barium und Lanthan: Rhodizonsäure, für Cäsium: eine Mischung verschiedener Metallacetate nach MILLER[11].
Trennzeit: etwa 3 Stunden.

Calcium–Scandium-Trennung

Von der Neigung der Erdalkalien zur Komplexbildung mit Rhodanidionen[12] ausgehend, wurde mit einem Fliessmittel bestehend aus Wasser und Äthanol (5:3), das 0.8 *M* an Ammoniumrhodanid war, eine Trennung des radioaktiven Mutter–Tochter-Paares von ^{47}Ca–^{47}Sc durchgeführt. Hierbei wurden R_F-Werte von 0.8 für Calcium und 0.1 für Scandium gemessen.
Anfärbemittel: Alizarin oder Oxin.
Trennzeit: 1.5 Stunden.

Strontium–Yttrium-Trennung

Hier wurde von der unterschiedlichen Löslichkeit der Nitrate in Äther–Äthanol-Mischungen Gebrauch gemacht. Zu einem Äthanol–Äther-Gemisch (1:1) wurde etwa 4% Salpetersäure zugesetzt (36%ige d = 1.52). Mit diesem Fliessmittel und als Nitrate aufgetragen, wandert Yttrium mit der Fliessmittelfront, während Strontium am Startpunkt bleibt. Das Ergebnis einer solchen Trennung zeigt die Fig. 2.

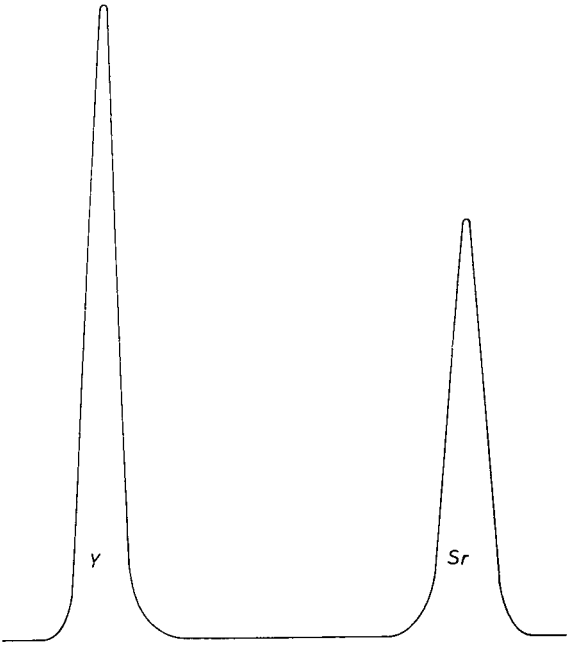

Fig. 2. Radiodünnschichtchromatogramm einer Strontium–Yttrium-Trennung.

Anfärbemittel für Strontium: Rhodizonsäure, für Yttrium: Alizarin.
Trennzeit: etwa 1 Stunde.

Zink–Gallium-Trennung

Interessant ist diese Trennung zur Reindarstellung bzw. Reinheitsprüfung von ^{72}Zn oder ^{72}Ga. Hierfür wurde ein Fliessmittel bestehend aus Butanol, das mit 1 *N* HCl gesättigt war[9,10] verwendet. Die R_F-Werte betragen für Gallium 0.1 und für Zink 0.6 auf Kieselgel und 0.9 auf Kieselgur.
Anfärbemittel für Zink: Dithizon, für Gallium: Oxin oder Quercitin.
Trennzeit: etwa 2 Stunden.

Niob–Tantal-Trennung

Auf Kieselgel oder besser auf Kieselgur wurde mit Aceton und Wasser (8:2), die eine Salzsäurekonzentration von 0.8 Mol/l und eine Oxalsäurekonzentration von 0.1 Mol./l aufwiesen[12], die Trennung auch mit trägerfreiem ^{95}Nb und ^{182}Ta vorgenommen. Als Oxalatkomplexe aufgetragen, betrugen die R_F-Werte für Niob 0–0.1 und für Tantal auf Kieselgur 1 und auf Kieselgel 0.8.
Anfärbemittel: Morin.
Trennzeit: etwa 1 Stunde.

Zirkon–Niob-Trennung

Auch hier wurde das aus der Papierchromatographie bekannte Fliessmittel zur Trennung von ^{95}Zr und dessen Folgeprodukt ^{95}Nb[13] eingesetzt. Mit einer 0.25 *M* oxalsauren und 0.1 *M* salzsauren Lösung von Methyläthylketon, Dioxan und Wasser (5:1:1) wurde für Niob ein R_F-Wert von 0.9 gemessen. Zirkon bleibt am Startpunkt. Aufgetragen wurden die Elemente auch hier als Oxalatkomplexe.
Anfärbemittel: Morin.
Trennzeit: etwa 2 Stunden.
Auf Papier beträgt der R_F-Wert-Unterschied 0.3 und die Trennzeit etwa 4 Stunden.

Trennung von Jodid, Jodat und Tellurit

Diese Trennung ist zur Reinheitsprüfung des ^{131}J von Bedeutung. Dieses Nuklid wird durch Bestrahlung von natürlichem Tellur mit Neutronen gewonnen. Als Verunreinigung kommen daher das Tellurit aber auch — durch Oxydation des Jods — Jodat in Frage. Hierfür wurden auf Papier Fliessmittel aus Aceton, Wasser und Ammoniak eingesetzt[14]. Mit einer Mischung von 6 *N* Ammoniaklösung und Aceton wurden auf Kieselgel die günstigsten R_F-Werte gesucht. Die Abhängigkeit der R_F-Werte von der Fliessmittelzusammensetzung zeigt die Tabelle II.

TABELLE II

ABHÄNGIGKEIT DER R_F-WERTE VON DER FLIESSMITTELZUSAMMENSETZUNG

Fliessmittel			*R_F-Werte*		
Aceton	:	*6 N NH_4OH*	*J^-*	*JO_3^-*	*TeO_3^{2-}*
2	:	6 und höher	0.8–1	0	0
2	:	5	0.9	0.1	0
2	:	4	0.9	0.2	0
2	:	3	0.9	0.3	0.05
2	:	2	0.9	0.4	0.1
2	:	1	0.9	0.6	0.5

Das günstigste Fliessmittel bestand somit aus Aceton und 6 *N* Ammoniak (1:1). Die R_F-Werte betrugen für Tellurit 0.1, für Jodat 0.4 und für Jodid 0.9.

Anfärbemittel für Jodid: Natriumjodat, für Jodat: Natriumjodid, für Tellurit: Thioharnstoff oder Zinnchlorid.

Trennzeit: 45 Minuten.

Phosphat–Sulfat- und andere Anionentrennungen

Bedingt durch die Herstellungsprozesse ist das mit ^{35}S markierte Sulfat möglicherweise mit [^{32}P]-Phosphat und umgekehrt das Phosphat mit Sulfat verunreinigt.

Die Adsorptionsfähigkeit von Lanthanoxyd ist für diese Anionen sehr verschieden, wie HAYEK UND SCHIMANN[15] feststellten. Daher wurde diese Trennung auf Lanthanoxyd vorgenommen. Mit einer 1 *N* Ammoniak-Lösung läuft Sulfat mit der Front, während Phosphat am Startpunkt bleibt.

Trennzeit: 1 Stunde.

Es wurden auch andere Anionen versuchsweise untersucht. Die R_F-Werte hierfür zeigt die Tabelle III.

TABELLE III

R_F-WERTE DER ANIONEN AUF LANTHANOXYD

Ion	*Fliessmittel*	
	1 N NH_4OH	*1 N NH_4OH–Aceton (1:1)*
SO_4^{2-}	1	0.6*
PO_4^{3-}	0	0
$Fe(CN)_6^{4-}$	1	0.45
$Fe(CN)_6^{3-}$	1	0.9
MnO_4^-	0	0
SCN^-	1*	0.9
$Cr_2O_7^{2-}$	0.8	0.35*
JO_3^-	0.85	0.45
J^-	0.95	0.9
Br^-	1	0.9
Cl^-	1	1
SO_3^{2-}	1	0.8
$S_2O_3^{2-}$	1	0.6
SeO_3^{2-}	0.75	0.3
TeO_3^{2-}	0	0
MoO_4^{2-}	0.9	0.6
NO_2^-	0.9	0.85
NO_3^-	0.9	0.9

* Schwanzbildung.

DANK

Herrn Doktor K. HOGREBE danke ich für die Unterstützung dieser Arbeit, Fräulein H. KUSTERER und H. GÖBELBECKER für die Ausführung der Laboratoriumsarbeiten.

ZUSAMMENFASSUNG

Es wurden einige radiochemisch interessante zwei- und mehrfach-Trennungen mit Hilfe der Dünnschichtchromatographie durchgeführt. Die Chromatogramme wurden

mit einem Strahlungsmessgerät automatisch ausgewertet. Als Trägerschicht konnte zusätzlich zu den üblichen Lanthanoxyd mit Erfolg verwendet werden.

SUMMARY

Some binary and multiple separations of radiochemical interest were carried out by means of thin-layer chromatography. The chromatograms were evaluated automatically by a radiation counter. Lanthanum oxide was used successfully besides the usual thin layers.

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THE SEPARATION OF PLUTONIUM FROM URANIUM AND FISSION PRODUCTS ON ZIRCONIUM PHOSPHATE COLUMNS

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In recent years special attention has been given to the ion-exchange properties of zirconium phosphate and similar compounds in aqueous solutions[1–12]. These inorganic cation-exchangers are stable in oxidizing media and at elevated temperatures. Their resistance to ionizing radiation[5] makes them particularly suitable for work with radioactive solutions.

On account of this we considered it worthwhile to investigate the separation of plutonium from uranium and fission products on zirconium phosphate columns. We were interested in nitric acid solutions containing macro-amounts of uranium (a few grams per litre), and micro-amounts of plutonium and long-lived fission products.

To obtain a better insight into the ion-exchange behaviour of the different ionic species towards zirconium phosphate, we first determined the dependence of the distribution coefficients of uranium, plutonium and fission product cations on the aqueous nitric acid concentration. Then, taking the distribution data as a guide, we separated plutonium on small glass columns filled with zirconium phosphate and calculated the decontamination factors.

EXPERIMENTAL

The exchanger characteristics

The term "zirconium phosphate" refers to the product whose stoichiometric composition is given in Table I. The exchanger was prepared by precipitating zirconyl chloride in 1 *M* HCl with an excess of phosphoric acid. After standing for 24 h, the precipitate was filtered and washed with distilled water until chloride ions disappeared in the filtrate, then dried at about 100°, crushed and ground. The granular product was conditioned by converting it to the Na^+ form with a NaCl solution, and then changing it back to the H^+ form with a HCl solution. Finally, it was washed with distilled water and dried at room temperature to a constant weight. The main properties of the exchanger are given in Table I.

The capacity of the exchanger was determined by the column method. A neutral salt solution (NaCl, $CaCl_2$) was percolated through a small exchanger bed (in H^+ form) until the effluent reached pH 5. The collected effluent was then titrated with a standard alkali solution and the capacity calculated. The anion-exchange capacity of zirconium phosphate seems to be negligible; the upper limit of Cl^- uptake at pH 1.65 is below 0.05 mequiv./g. No significant swelling of particles was observed in acidic and neutral solutions.

TABLE I

DATA FOR THE ZIRCONIUM PHOSPHATE CATION-EXCHANGER

Particle size	100–150 mesh
Capacity for Na^+	2.12 mequiv./g
Capacity for Ca^{2+}	2.30 mequiv./g
"Free H_2O" (by weight loss at 105°)	1.92%
Stoichiometric composition (by quantitative analysis)	$(ZrO_2)_{1.15}P_2O_5.2.7H_2O$

Tracers and solutions used

The following radioactive tracers were used: ^{90}Sr, ^{144}Ce, ^{95}Zr + Nb, ^{106}Ru, ^{137}Cs and ^{59}Fe. Except for ^{59}Fe, they all belong to the important long-lived fission products. The isotopes were practically "carrier free" as nitrate salts in nitric acid solutions. In addition, solutions of ^{239}Pu (0.1–0.5 mg/l) were prepared. The plutonium ions were held at the desired valency by means of the following oxidizing and reducing agents: hydrazine for Pu^{3+}, sodium nitrite for Pu^{4+} and potassium bromate for PuO_2^{2+}. In the nitrate solutions uranium was always in the form of uranyl ion.

Procedure

The distribution coefficients (q) were determined by equilibrating 25 ml of tracer solution with 1 g of the airdried exchanger. This was performed by shaking the mixtures in small flasks on a simple laboratory shaker for 17 h at 20°. The distribution coefficient (expressed as: amount or c.p.m. per 1 g exchanger divided by the amount or c.p.m. per 1 ml solution) was calculated from the difference between the concentration of the adsorbate before and after equilibrium.

Column separations were performed at constant temperature in small Pyrex columns jacketed with a heating mantle. The exchanger bed in the columns was usually 0.4 cm ⌀ × 15 cm. Constant flow-rates in the range 0.5–1 ml cm^{-2} min^{-1} were maintained. The free volume of the columns was about 55% of the geometrical volume of the bed. Usually, in a run 5 ml of the active solution was introduced on to the column and then eluted with 10 and 15 ml portions of nitric acid of appropriate concentration. Fractions of effluent were collected continuously. For each fission product separate runs were performed at 20 and 50°. The amount of uranium introduced on to the columns was the same in all runs.

Analysis

The uranium in the solutions was determined spectrophotometrically by the thioglycollate method[13]. The plutonium, on stainless steel disks, was counted with a ZnS alpha scintillation counter (Tracerlab, type P-12). In most cases the self-absorption could be neglected as thin samples (less than 0.1 mg/cm^2) were used. However, in some plutonium samples uranium and sodium nitrite were present, so that a correction for self-absorption had to be made. Counts due to uranium were then subtracted (these were determined by counting specially prepared uranium samples) from the total corrected alpha counts and the net alpha activity of plutonium in the sample was obtained. To check the correctness of the counting, a balance of the total counts between the input and the output in every run was made; this balance was always within 100 ± 2%. The activity of the fission products in the solutions was measured

by commercial GM tubes and gamma activity was determined in well type scintillation counters in the usual way. Since discrimination between the gamma rays of ^{95}Zr and ^{95}Nb is not possible, the sum of the activities of both isotopes in the effluent was recorded, and then the Zr was separated from Nb by extraction with TTA (thenoyltrifluoroacetone)[16].

RESULTS AND DISCUSSION

The dependence of the distribution coefficient of the uranyl ion on the exchanger load for different HNO_3 concentrations is shown in Fig. 1. The initial concentration of nitric acid is given for each curve in the figure, but for a low exchanger load this concentration is practically equal to the equilibrium concentration.

Fig. 2 shows the distribution coefficients of UO_2^{2+}, Pu^{3+}, Pu^{4+}, PuO_2^{2+}, Fe^{3+} and fission product cations as functions of the equilibrium HNO_3 concentration. For UO_2^{2+}, the extrapolated values from Fig. 1 were used. The valence state of each cation, except for ruthenium whose actual state was rather uncertain, is given.

The position of some of the curves in Fig. 2 is noteworthy. For instance, Cs^+ is adsorbed more strongly than many multivalent cations. Also, the PuO_2^{2+} curve does not coincide with the UO_2^{2+} curve; this can be attributed to the presence of $KBrO_3$ in the solutions of hexavalent plutonium. Finally, the position of the Ce^{3+} and Pu^{3+} curves relative to the UO_2^{2+} curve is unexpected if the charges of these cations are

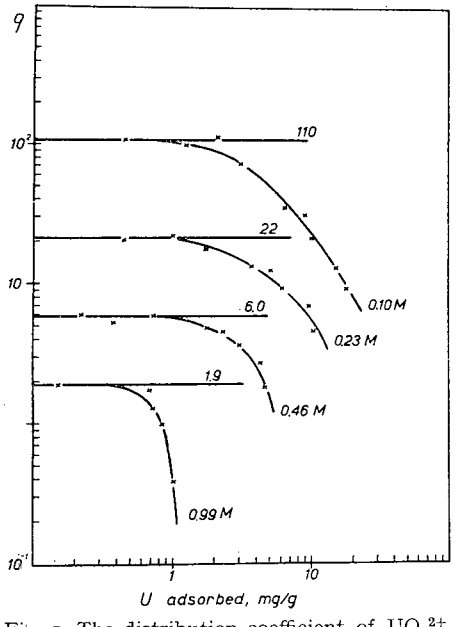

Fig. 1. The distribution coefficient of UO_2^{2+} plotted against the exchanger load. Initial aqueous HNO_3 molarity given on each curve.

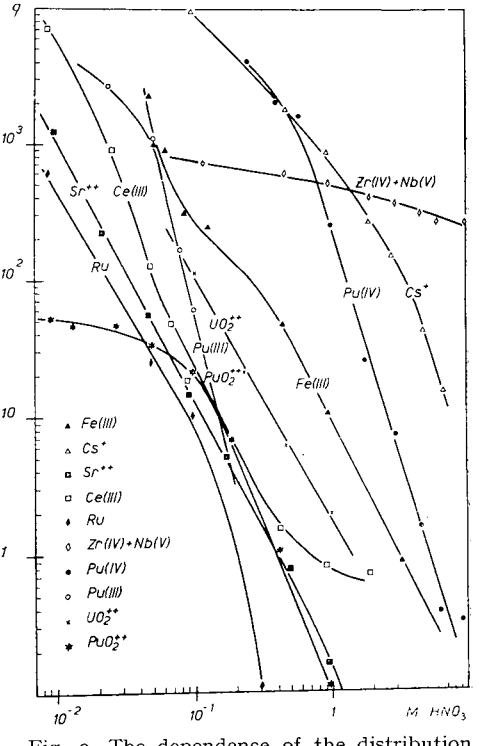

Fig. 2. The dependence of the distribution coefficients on the equilibrium HNO_3 concentration. All adsorbates present in tracer amounts. Pu (III) solutions 0.02 *M* in hydrazine, Pu (IV) 0.02 *M* in $NaNO_2$, and Pu (VI) 0.02 *M* in $KBrO_3$.

taken into account. However, the uranyl ion is not a true bivalent cation such as Sr^{2+}, for instance; its chemical behaviour is rather like that of a cation of higher charge. Similar behaviour was noted on silica gel[14], where UO_2^{2+} was adsorbed more strongly than Gd^{3+}.

The sorption of the cations is strongly influenced by the acidity of the aqueous solution; this would be expected for a true ion-exchange process. The only notable exception is the pair Zr–Nb, as is seen from Fig. 2. In this case, the sorption is apparently not much affected by the aqueous acidity, so that merely ion-exchange cannot be accepted.

In an earlier paper[12] we showed that the mass action law can be applied to the exchange of uni–univalent and uni–divalent ions on zirconium phosphate. This was also demonstrated by LARSEN AND VISSERS[10], and by BAETSLÉ AND HUYS[11]. In this respect, the very strong affinity of Cs^+ towards zirconium phosphate is noteworthy. We have found, for instance, that the standard free energy change for the substitution Cs^+–H^+ is $\Delta F^\circ_{293} = -3.7$ kcal/mol., compared to $\Delta F^\circ_{293} = +1.2$ kcal/mol. for $^1/_2$ Sr^{2+}–H^+. For more details about the equilibrium and kinetics of adsorption, the reader should refer to our earlier paper[12].

From the distribution data given in Fig. 2, it can be concluded that: (1) the separation of Pu^{4+} from UO_2^{2+} can be achieved by merely changing the HNO_3 concentration, (2) among the long-lived fission products, ^{90}Sr, ^{144}Ce and ^{106}Ru should follow the uranium fraction, while ^{137}Cs and ^{95}Zr + Nb will probably contaminate the plutonium fraction.

After some preliminary experiments, we chose the following procedure for practical separations: a 0.5 *M* HNO_3 + 0.02 *M* $NaNO_2$ solution containing uranium, plutonium and fission products is percolated through the column; uranium passes through practically unadsorbed, while Pu^{4+} remains firmly fixed. After washing the column with a 0.5 *M* HNO_3 solution, plutonium is removed with 8 *M* HNO_3. To check this separation quantitatively, some experiments with solutions containing only UO_2^{2+} and Pu^{4+} (without fission products) were made at 20 and 50°. Two typical results are shown in Table II.

TABLE II

SEPARATION OF UO_2^{2+} AND Pu^{4+}

Column 0.4 cm ø × 15 cm, flow-rate 0.73 ml cm^{-2} min^{-1}. Input: 5 ml 0.5 *M* HNO_3 + 0.02 *M* $NaNO_2$ with U and Pu, followed by 10 ml 0.5 *M* HNO_3 and 2 × 15 ml 8 *M* HNO_3. Effluent collected in 4 fractions: 5 + 10 + 15 + 15 ml.

Effluent fraction	*Uranium found*		*Plutonium found*	
	mg	*% of input*	*c.p.m.*	*% of input*
Temp. 20°. Input: 9.50 mg U, 48050 c.p.m. Pu				
(1) 5 ml (0.5 *M*)	7.85	82.7	685	1.4
(2) 10 ml (0.5 *M*)	1.64	17.2	310	0.6
(3) 15 ml (8.0 *M*)	—	—	46375	96.7
(4) 15 ml (8.0 *M*)	—	—	368	0.8
Temp. 50°. Input: 9.45 mg U, 38700 c.p.m. Pu				
(1) 5 ml (0.5 *M*)	7.30	77.2	660	1.7
(2) 10 ml (0.5 *M*)	2.00	21.0	225	0.6
(3) 15 ml (8.0 *M*)	—	—	37325	96.3
(4) 15 ml (8.0 *M*)	—	—	291	0.7

It can be seen from Table II, that no significant difference exists between the two runs at 20 and 50°. The recovery of plutonium (the last two fractions) is 97–98%; the rest is lost in the uranium fraction, probably as Pu^{3+} or colloidal plutonium. The recovered plutonium is essentially free from uranium. For instance, in the third fraction the uranium content (by the ferrocyanide spot test) is below 0.01 mg (less than 0.1% of the input), so that plutonium is decontaminated with respect to uranium by at least a factor of 1000. The clear-cut separation of Pu^{4+} from UO_2^{2+} was also confirmed by an elution curve which is reproduced in Fig. 3. This curve was obtained by collecting fractions of 0.3 ml on stainless steel disks, which were later dried and checked for total alpha counts.

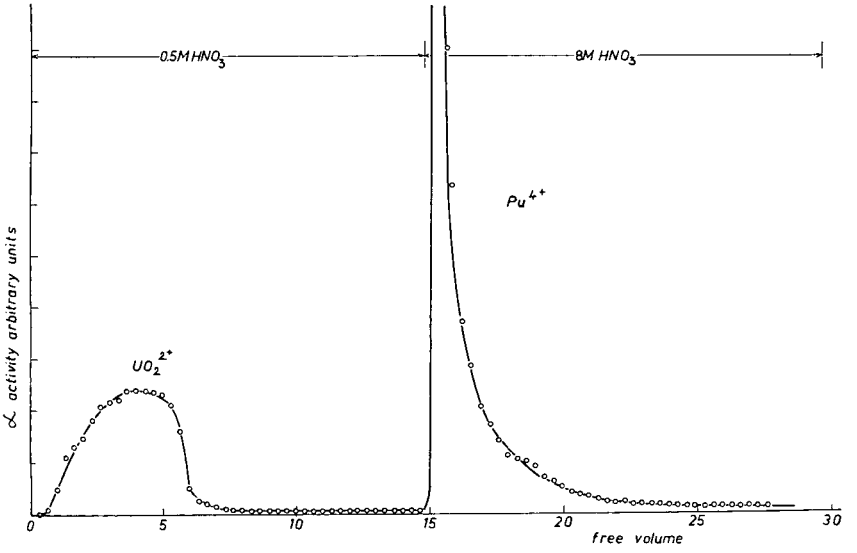

Fig. 3. Separation of Pu^{4+}–UO_2^{2+}. Column 0.6 cm ø × 11 cm, temp. 20°, flow-rate 0.5 ml min^{-1} cm^{-2}. Loading and elution as in Table II.

To determine how the fission products are distributed between the uranium and plutonium fractions of the effluent, a number of runs identical to those in Table II were made. In each run, one fission product was added to the 5 ml feed solution and the effluent was collected in two fractions of exactly 15 ml each. The first fraction contained the whole amount of uranium, the second 96–97% of plutonium (see Table II). Both fractions were analyzed, and the individual fission product decontamination factors of the plutonium product were thus easily calculated. The results are summarized in Table III.

The isotopes ^{95}Zr and ^{95}Nb were added together, in a known proportion, but in the effluent they were separated by TTA extraction, as mentioned above. This enabled us to follow the distribution of both isotopes individually in the effluent.

Table III shows that for Ru, Ce and Sr the sum of activities in both fractions is within 100 ± 2% of the input. However, this is not true for Cs, Zr and Nb which remain to a great extent fixed on the column. Zirconium, for instance, is not eluted at all. This is not surprising if we bear in mind the affinity of zirconium towards phosphate

TABLE III

DISTRIBUTION OF FISSION PRODUCTS IN THE EFFLUENT

For experimental conditions see Table II

Fission product	*Input c.p.m.*	*Found in U-fraction*		*Found in Pu-fraction*	
		c.p.m.	*% of input*	*c.p.m.*	*% of input*
Temperature 20°					
^{106}Ru	$4.40 \cdot 10^5$	$4.31 \cdot 10^5$	98.0	$6.42 \cdot 10^3$	1.5
^{144}Ce(III)	$2.51 \cdot 10^5$	$2.45 \cdot 10^5$	97.5	$1.42 \cdot 10^3$	0.57
^{90}Sr	$1.45 \cdot 10^5$	$1.47 \cdot 10^5$	101.3	n.d.*	—
^{137}Cs	$4.74 \cdot 10^5$	$3.15 \cdot 10^3$	0.7	$3.11 \cdot 10^5$	65.6
^{95}Zr	$1.95 \cdot 10^5$	n.d.	—	n.d.	—
^{95}Nb	$4.25 \cdot 10^5$	$1.45 \cdot 10^4$	3.4	$1.77 \cdot 10^4$	4.17
Temperature 50°					
^{106}Ru	$5.38 \cdot 10^5$	$5.31 \cdot 10^5$	98.6	$7.07 \cdot 10^3$	1.3
^{144}Ce(III)	$2.51 \cdot 10^5$	$2.43 \cdot 10^5$	97.0	$1.46 \cdot 10^3$	0.58
^{90}Sr	$6.28 \cdot 10^4$	$6.39 \cdot 10^4$	101.5	n.d.	—
^{137}Cs	$4.91 \cdot 10^5$	$2.40 \cdot 10^4$	4.9	$4.58 \cdot 10^5$	93.1
^{95}Zr	$2.01 \cdot 10^5$	n.d.	—	n.d.	—
^{95}Nb	$4.39 \cdot 10^5$	$1.01 \cdot 10^4$	2.3	$2.83 \cdot 10^4$	6.5

* n.d. = not detected.

ions. The effect of temperature upon the elution is significant only for Cs and Nb.

The behaviour of niobium depends, unfortunately, on many unpredictable factors. For example, the results for Nb in Table III were obtained with a feed solution (0.5 M HNO_3) to which a few drops of a Zr–Nb stock solution (3 M HNO_3) had previously been added. The solution thus prepared was aged for 2 days before use. When the same solution was allowed to age for 15 days, we found 20 and 46% of niobium in the uranium and plutonium fraction, respectively. It seems that slow processes such as hydrolysis, colloid formation, etc., which alter the niobium species in aqueous solution, interfere in the adsorption. This is probably the reason why the amount of niobium eluted depends so much on the mode of preparation of the feed solution.

In contrast to the peculiar behaviour of niobium, we found that zirconium is in all circumstances firmly fixed on the exchanger.

The decontamination factors (DF) for the plutonium product were obtained from the data in Table III and are shown in Table IV.

The DF values in Table IV can be compared favorably with the values obtained on synthetic organic cation-exchangers[15], except perhaps those for caesium. However, plutonium solutions obtained by a solvent-extraction process usually do not contain

TABLE IV

PLUTONIUM PRODUCT DECONTAMINATION FACTORS

	UO_2^{2+}	^{90}Sr	^{144}Ce(III)	^{106}Ru	^{95}Zr	^{95}Nb	^{137}Cs
DF(20°)	$\sim 10^3$	$\sim 10^3$	178	68	$> 10^3$	24*	1.5
DF(50°)	$\sim 10^3$	$\sim 10^3$	172	76	$> 10^3$	15*	1.1

* Feed solution aged for 2 days.

caesium, and therefore the low DF value is not a serious drawback. However, the DF value of Zr is very high, and the fact that zirconium remains fixed on the column may be inconvenient. (It can be accumulated up to a high radiation level.)

On the whole, zirconium phosphate columns have been found to be useful in the separation and isolation of plutonium, especially if a high radiation stability of the exchanger is essential. However, for a strict comparison between zirconium phosphate and other ion-exchangers regarding plutonium recovery, further experiments with high plutonium loading-rates are required.

SUMMARY

A procedure for the separation of plutonium from uranium and long-lived fission products on zirconium phosphate columns is described. Traces of plutonium are separated from nitric acid solutions containing a few grams per litre uranium. Data such as the distribution coefficients, plutonium recovery and decontamination factors are presented.

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Notes

Effect of buffer equilibration on paper electrophoresis

In paper chromatography, equilibration of the paper with the chamber atmosphere and solvents is an important variable (HEFTMANN[1]). The importance of this variable in paper electrophoresis was emphasized when different buffer systems were employed.

Methods

In the experiments to be described, paper electrophoresis was carried out in a Spinco Model R-paper electrophoresis cell, powered by a Duostat, Model RD-2, essentially as described by BIER[2].

Two common buffer systems were tested in this study: (a) The first buffer system was composed of diethylbarbituric acid (0.075 ionic strength) at pH = 8.6. (b) A second buffer according to ARRONSON AND GRONWALL[3], which has a pH of 8.9 was composed of tris-hydroxymethylaminomethane (TRIS), 60.5 g/l (0.5 mole); ethylenediaminetetraacetic acid (EDTA), 6.0 g/l (0.021 mole); and boric acid 4.6 g/l (0.075 mole).

Equilibration of the paper strips was achieved either by pouring the buffer directly over the paper or by capillary action. Saturation of the strips by capillary action was examined at two time intervals. We arbitrarily chose 60 minutes as the minimum time interval and 72 hours for the maximum saturation time interval.

After electrophoretic separation was achieved, the strips were removed from the cell, air dried, and the proteins fixed and stained in the usual manner with Lissamine Green[4].

Results

A summary of the electrophoretic procedures may be seen in Fig. 1. In both the veronal and TRIS buffer systems, we have observed superior excursion and separation of serum proteins on those strips which were saturated by pouring the buffer solution directly on the paper strips before sample application and electrophoresis. Equilibration by capillary action for 1 and 72 hours, tended to distort the migration of the plasma proteins to a much greater extent with the TRIS–borate–EDTA buffer system than with the veronal buffer system.

Discussion

Of the variables influencing the electrophoretic mobility of proteins on paper, the equilibration of the paper strips before sample application is most important. Little attention has been given to this factor in various books (BIER[2] and SMITH[4]) on electrophoresis and by various laboratory technicians (personal communications). A marked difference for the TRIS–borate–EDTA buffer was noticed between equilibration obtained by pouring this buffer over the paper strips and where equilibration was accomplished by capillary action. The chromatographic effects due to saturation by

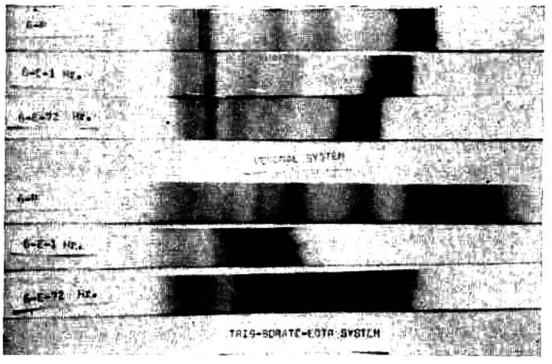

Fig. 1. Comparison was made between two buffer systems in regard to paper saturation before the application of human serum. Papers were saturated by pouring (6-P) and after 1 (6-E-1 h) and 72 hours (6-E-72h) of equilibration. The number 6 refers to the position of the strip in the Spinco Model R cell.

capillary action gave rise to changes in salt concentration in certain parts of the paper. Since conductivity is proportional to buffer concentration, the electrical field will differ along the length of paper. The distortion noted in the present experiments probably was due to these chromatographic effects. The effect differs for the different buffers, being more noticeable with the TRIS–borate–EDTA system (Fig. 1).

Therefore, it is recommended that the procedure of pouring buffer over the strips to attain equilibrium be rigidly followed before sample application. Where these conditions are not scrupulously followed, equilibration by capillary action requires more time for certain buffers and results in poor electrophoretic separations.

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Zur Chromatographie von 4-Dimethylamino-3,5-dinitrobenzoaten

Die Verwendung der intensiv gelb gefärbten Ester der 4-Dimethylamino-3,5-dinitrobenzoesäure zur Charakterisierung von Alkoholen wurde erstmals von JENSEN, LUNDBORG UND SOLSTAD[1] in Betracht gezogen. Aufgrund der wenig befriedigenden Ergebnisse bei der Adsorptionschromatographie an Al_2O_3 erschienen diese Verbindungen jedoch für eine chromatographische Trennung ungeeignet.

VAN DUIN[2] gelang dann eine Auftrennung von 4-Dimethylamino-3,5-dinitrobenzoaten mit Hilfe der Verteilungschromatographie unter Verwendung einer Nitromethan–Kieselgel-Säule[3]. Die Trennung homologer Reihen der Ester kann auch durch Verteilungschromatographie an einer Polyäthylensäule erfolgen.

Die Polyäthylensäule als Mittel zur Verteilungschromatographie mit umgekehrten Phasen wurde zuerst von NAUDET und Mitarb.[4] angewandt. Wir benutzten diese Methode in abgeänderter Form zur Auftrennung homologer Reihen von 2,4-Dinitrophenylhydrazonen[5]. Die Anwendung der Polyäthylensäule auf die Ester der 4-Dimethylamino-3,5-dinitrobenzoesäure erfolgte in völlig analoger Weise, so dass an dieser Stelle nur über das Ergebnis bei der Chromatographie der gesättigten aliphatischen Alkohole* C_1–C_{10} berichtet werden soll.

Mit Heptan als stationärer und Dioxan–Wasser als mobiler Phase wurden die in Tabelle I folgende scheinbare Retentionsvolumina beobachtet.

TABELLE I

SCHEINBARE RETENTIONSVOLUMINA VON 4-DIMETHYLAMINO-3,5-DINITROBENZOATEN
(ml/g Polyäthylen)

C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}
1.6	2.2	3.0	4.1	5.8	8.0	11	16	21	29

Bei der analytischen Anwendung dieses verteilungschromatographischen Verfahrens auf Gemische, die neben gesättigten auch andere Alkohole enthalten, kann es jedoch in bekannter Weise zum Auftreten kritischer Partner kommen. Eine solche nicht trennbare Gruppe wird z.B. durch Pentanol-1, Butanol-2 und *cis*-3-Hexenol-1 gebildet. Bei der Untersuchung des adsorptionschromatographischen Verhaltens der 4-Dimethylamino-3,5-dinitrobenzoate an Aluminiumoxydsäulen wurde festgestellt, dass eine Trennung solcher Gemische primärer, sekundärer und ungesättigter Alkohole mit Hilfe von Aluminiumoxyd mit 8% Wassergehalt und Heptan als Laufmittel möglich ist. Jedoch verläuft auch an dem partiell desaktivierten Absorbens die Chromatographie nicht ohne Nebenreaktionen.

So liess sich von einem auf die Säule gegebenen Ester stets nur ein Teil wieder eluieren. Wurde die Chromatographie für einige Zeit (über Nacht) unterbrochen, bildete sich an der Stelle, wo sich der Ester befunden hatte, eine nicht eluierbare gelbe Zone. Offenbar wurde der Ester an dem Adsorptionsmittel partiell verseift. Die dabei entstehende 4-Dimethylamino-3,5-dinitrobenzoesäure kann mit dem benutzten Lösungsmittel nicht eluiert werden.

* Auch wenn nicht ausdrücklich erwähnt, sind die Ester der 4-Dimethylamino-3,5-dinitrobenzoesäure gemeint.

Neben dem Verseifungseffekt wurde auch eine teilweise Zersetzung der Derivate beobachtet. Bei der papierchromatographischen Prüfung des Eluates zeigte jeder Ester einen braunen Begleitfleck, der etwa wie ein um ein C-Atom ärmerer Ester lief. Es wird angenommen, dass es sich dabei um Oxydationsprodukte handelt, da sich die Substanzen auch langsam bei Stehen von Lösungen der Ester an der Luft bilden.

Diese bei der Adsorptionschromatographie auftretenden Schwierigkeiten lassen sich jedoch umgehen. Es wurde festgestellt, dass bei Verwendung von *saurem* Aluminiumoxyd im Gegensatz zu dem zuerst benutzten neutralen Al_2O_3 eine Hydrolyse der Ester nicht auftritt. Auch bei längerer Chromatographierzeit wurde an diesem Adsorbens eine Gelbfärbung der Säule nicht beobachtet. Die Bildung der braunen Zersetzungsprodukte lässt sich vermeiden, wenn man ein Aluminiumoxyd benutzt, das man frisch aktiviert und unmittelbar danach mit 8% Wasser desaktiviert hat.

Das genannte Alkoholgemisch konnte auf diese Weise ohne Nebenreaktionen getrennt werden. Die Komponenten wurden in der Reihenfolge Butanol-2, Pentanol-1 und *cis*-3-Hexenol-1 eluiert. Durch Kombination der beiden Methoden, also Verteilungschromatographie mit anschliessender Adsorptionschromatographie der einzelnen Fraktionen, ist es somit möglich, auch komplizierter zusammengesetzte Gemische von 4-Dimethylamino-3,5-dinitrobenzoaten zu trennen.

Experimentelles

Darstellung der 4-Dimethylamino-3,5-dinitrobenzoate. Die Alkohole wurden in Pyridinlösung mit einem Überschuss an Säurechlorid während einer Stunde umgesetzt. Danach wurde mit wenig Wasser versetzt und nach einer weiteren Stunde aufgearbeitet.

Verteilungschromatographie. Die Verteilungschromatographie erfolgte an einer Polyäthylensäule mit Heptan als stationärer und Dioxan–Wasser (2:1) als mobiler Phase. Einzelheiten des Verfahrens sind bereits an anderer Stelle ausführlich beschrieben[5]. Mit Hilfe einer Säule, die 20 g Polyäthylen enthielt, wurde 15 mg eines Gemisches der gesättigten aliphatischen Alkohole C_1–C_{10} aufgetrennt.

Adsorptionschromatographie. Saures Aluminiumoxyd (Merck) wurde gesiebt und die Siebfraktion 0.10–0.12 mm 15 Stunden auf 200° erhitzt. Unmittelbar nach dem Abkühlen wirde es mit 8% Wasser desaktiviert und danach unter Heptan in einer Säule von 20 cm Länge und 0.6 cm innerem Durchmesser eingeschlämmt.

1 mg eines Gemisches gleicher Teile der 4-Dimethylamino-3,5-dinitrobenzoate von Butanol-2, Pentanol-1 und *cis*-3-Hexenol-1 gab man in Heptanlösung auf die Säule. Die Derivate wurden in der angegebenen Reihenfolge mit Heptan eluiert.

Papierchromatographie. Imprägnierung mit 20% Sulfolan (Deutsche Shell) in Aceton. Heptan diente als Laufmittel.

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Die Anwendung vom Isatin–Cd-Komplex zum papierchromatographischen Nachweis von Aminosäuren

Chromatographischer Nachweis von Aminosäuren kan bei Anwendung verschiedener Entwicklungsreagenzien durchgeführt werden. Die meisten Entwickler aber, färben die Aminosäurenflecke in ähnliche Farbe, wenn man nicht über Ausnahmefälle spricht in denen manche Aminosäuren in verschiedene Farben gefärbt werden.

Die Intensität der angefärbten Flecke ist verschieden und hängt von der Konzentration der Aminosäuren ab, dabei ist die Dauerhaftigkeit der Flecke, bei der sie noch beurteilt werden können, bei Anwendung von verschiedenen Entwicklern nicht gleich, und nach kürzerer oder längerer Zeit dienen die angefärbten Flecke nicht mehr zum qualitativen sowie quantitativen Nachweis von Aminosäuren.

Gewisse Möglichkeiten einiger Modifikationen der Anfärbemethoden bieten die Arbeiten von BARROLLIER[1] und BODE[2]. Diese Autoren haben zum genannten Zwecke Ninhydrin–Cd oder Ninhydrin–Cu-Komplexe angewendet. Die Entwicklung der Aminosäurenflecke mittels Ninhydrin gibt gute Resultate bei zweidimensionaler Trennung, jedoch bei chromatographischem Nachweis auf Rundfiltern bietet auch die Komplexfärbung Schwierigkeiten, die bei Untersuchung des biologischen Materials eine einwandfreie Identifizierung unmöglich machen.

Diese Chromatogramme können aber mit Erfolg mittels Isatins, 5-Bromisatins oder 5-Nitroisatins[3] entwickelt werden. Ebenso gute Resultate erhielt KNAUT[4] beim Anfärben der Aminosäurenflecke bei eindimensionaler Entwicklung, mittels eines Isatin–Cd-Komplexes. Das Entwicklungsreagens wurde als Acetonlösung hergestellt, die 0.2 % Isatin, 4 % Essigsäure und 0.06 % Cadmiumchlorid erhielt.

Die Aminosäurenflecke die nach dem Eintauchen des Bogens in das Reagens und nachfolgendem Austrocknen (bei 90° während 10 Min.) hervorgerufen wurden,

TABELLE I

Folge	*Aminosäure*	*Farbeton*	*Empfindlichkeit* ($\mu g/cm^2$)	R_F
1	Cystein	lila-hellbraun	0.4	0.17
2	Cystin	rosa-violett	0.8	0.17
3	Lysin	karmin-lila	0.2	0.18
4	Histidin	violett-braun	0.4	0.19
5	Arginin	rötlich-rosa	0.4	0.19
6	Asparaginsäure	rosa-lila	0.2	0.31
7	Serin	siena	0.2	0.32
8	Glycin	rosa	0.2	0.33
9	Threonin	rosa-rot	0.4	0.36
10	Glutaminsäure	lila-rosa	0.2	0.37
11	Alanin	lila	0.1	0.39
12	Prolin	dunkelblau	0.05	0.41
13	Tyrosin	bläulich-grün	0.4	0.53
14	Methionin	rötlich-lila	0.4	0.57
15	Valin	rosa	0.2	0.57
16	Tryptophan	grau-braun	0.4	0.59
17	Norvalin	bläulich-lila	0.2	0.59
18	Phenylalanin	grau-blau	0.4	0.66
19	Isoleucin	rosa-lila	0.4	0.68
20	Leucin	karmin	0.4	0.72

haben den Farbton mehr ins Rot verschoben, als bei Entwicklung mit reinem Isatinreagens. Es wurde die in Tabelle I folgende Reihe bei Butanol–Eisessig–Wasser (4:1:5) nachgewiesen.

Die Farben entsprechen den Konzentrationen von 20 $\mu g/cm^2$. Die entwickelten Bogen wurden mit Paraffin fixiert.

Gegenüber den mit reinem Isatin gefärbten Bogen wurde eine stärkere Intensität der mit Isatin–Cd-Komplex angefärbten Flecke beobachtet und eine grössere Farbendifferenz, besonders bei Mischungen von Aminosäuren mit naheliegenden R_F-Werten wie: Cys-SH, Cys-SS, Lys, Hist, Arg und Tyr, Met, Val, Try, Norval. Gewisse Schwierigkeiten bieten ähnliche Farben von Arginin, Glycin, Valin und Leucin, welche jedoch wegen ihrer grossen R_F-Unterschiede ziemlich leicht identifiziert werden können.

Die nebeneinander wandernden Aminosäuren weisen keinen gleichen Farbton in Konzentrationen von 0.8–4 $\mu g/cm^2$ auf, und die Empfindlichkeit (siehe Tabelle I) der Farbreaktion ist gegenüber dem Isatin höher, und der Empfindlichkeit der Ninhydrinreaktion ähnlich[5–7]. Ausser obengenannten Eigenschaften, die dem besprochenen Isatin-Reagens einen Vorteil gegenüber reiner Isatin-Färbung geben, konnte festgestellt werden, dass die mit Isatin–Cd-Komplex gefärbten Bogen eine grössere Haltbarkeit aufweisen. Während die mit reinem Isatin gefärbten Flecke schon nach dreiwochiger Aufbewahrung keine qualitative Auswertung gestatten, ist die mit Isatin–Cd-Komplex hervorgerufene Farbe noch nach derselben Zeit deutlich und die Flecke noch leicht erkennbar.

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The exchange capacity of papers impregnated with zirconium phosphate

Papers impregnated with zirconium phosphate (ZP) have found extensive application in numerous problems[1]; however, so far only two methods of preparation have been used.

ALBERTI AND GRASSINI[2] dipped papers into a 30 % solution of zirconyl chloride in 4 *N* HCl, while CABRAL[3] used a 7 % solution; subsequent precipitation with excess H_3PO_4 and extensive washing with acid and water is practised in both methods.

No information was available so far concerning the relative exchange capacity of these papers. For such a study we prepared papers containing varying amounts of zirconium phosphate and studied the chromatographic behaviour of UO_2(II) and Pb(II). These two ions were selected because they have a suitable range of R_F values on all papers.

While carrying out this work we also wanted to obtain some data on the temperature variation of R_F values on zirconium phosphate papers. The chromatograms were thus performed at four different temperatures: 5°, 20°, 35° and 50°.

Strips (5 × 46 cm) of Whatman No. 1 filter paper were drawn as uniformly as possible through a solution of zirconyl chloride octahydrate (Merck) at different concentrations (5, 10, 15, 20, 25, 30%) in 4 *N* HCl and air dried for 12 h. The dry strips were then dipped into a 60 % solution of H_3PO_4 in 4 *N* HCl and after drying in air for 6 h, washed first in 2 *N* HCl for 30 min and then twice with distilled water for 30 min.

It is very important that the amounts of the various solutions for washing the paper and times of contact are strictly adhered to. R_F values were found to vary considerably with the number of washes of the paper. For example UO_2^{2+} developed with 1 *N* $HClO_4$ had an R_F value of 0.6 on a 5 % paper after three washes and of 0.8 after seven washes. On a 30 % paper the R_F value was 0.2 after three washes and 0.4 after seven washes.

All chromatograms were carried out with 1 % solutions of uranyl and lead nitrates in aqueous $HClO_4$ on paper strips 21 cm long (starting point 2 cm from one end). In order to obtain a convenient range of R_F values for measurements in the whole range of papers the uranyl ion was developed with 3 *N* $HClO_4$ and the lead ion with 0.4 *N* $HClO_4$.

Table I shows the R_F values obtained with a development of 1–2 h at the temperatures indicated.

A mathematical correlation between R_F values and the degree of impregnation may be obtained[4] using the equation:

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

where A_L/A_S is the ratio of the amounts of the mobile phase and the zirconium phosphate, the amount of cellulose remaining constant.

Here it can be assumed that the distribution coefficient α does not change with the degree of impregnation. Then $(1/R_F - 1)$ should vary linearly with an increase in A_S providing that A_L does not change appreciably due to the varying amounts of zirconium phosphate in the pores of the paper.

As shown in Fig. 1 this is indeed the case, good linear relationships between $(1/R_F - 1)$ and the degree of impregnation being obtained for the whole range of papers. Equation (1) may thus be used for calculating the separation effects which may be obtained with various degrees of impregnation if data on one kind of paper are available.

The temperature variation between 5° and 50° is not very considerable, somewhat higher R_F values being observed in all cases at higher temperatures. However there seems to be no definite advantage in working at any specific or at a rigidly controlled temperature.

TABLE I

R_F VALUES OF UO_2^{2+} AND Pb^{2+} ON PAPERS IMPREGNATED WITH VARIOUS AMOUNTS OF ZIRCONIUM PHOSPHATE AND DEVELOPED AT VARIOUS TEMPERATURES

UO_2^{2+} ions developed with 3 N $HClO_4$							*Pb^{2+} ions developed with 0.4 N $HClO_4$*						
Paper impregnated with % Zr OCl_2	*5%*	*10%*	*15%*	*20%*	*25%*	*30%*	*Paper impregnated with % Zr OCl_2*	*5%*	*10%*	*15%*	*20%*	*25%*	*30%*
Temp. 5°	0.70	0.62	0.43	0.38	0.33	0.29	Temp. 5°	0.67	0.66	0.52	0.51	0.43	0.40
Temp. 20°	0.71	0.63	0.46	0.44	0.38	0.30	Temp. 20°	0.74	0.68	0.58	0.57	0.52	0.47
Temp. 35°	0.78	0.58	0.46	0.45	0.36	0.33	Temp. 35°	0.79	0.73	0.62	0.55	0.52	0.45
Temp. 50°	0.80	0.62	0.52	0.40	0.37	0.34	Temp. 50°	0.86	0.85	0.69	0.66	0.65	0.48

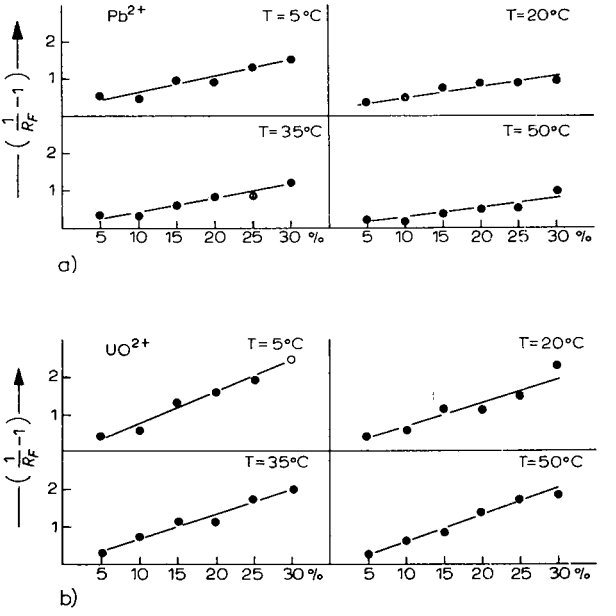

Fig. 1. $(1/R_F - 1)$ plotted against the amount of zirconium phosphate held on the paper. The zirconium phosphate content is here expressed in % $ZrOCl_2$ in the solution used to impregnate the papers. (a) Pb^{2+} ions developed at various temperatures with 0.4 N $HClO_4$. (b) UO_2^{2+} ions developed at various temperatures with 3 N $HClO_4$.

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Gas chromatographic determination of amines in aqueous solution

The gas chromatographic separation and identification of organic compounds in aqueous solution presents some problems owing to excessive tailing of the water peak. Various ways of treating the aqueous solutions before injection into the column have been described[1,2]. One method[1] proposed diluting the sample with an inert solvent followed by dehydration with Na_2SO_4. Another method[2] involved non-aqueous extraction of the solutes by organic solvents.

The use of highly polar stationary phases, which act as water retardants and thus increase the time available for chromatographic separation of low boiling compounds, was also suggested[3–6]. These methods, however, gave only qualitative results. ZAREMBO AND LYSYJ[7] found a column packing suitable for separating alcohols in aqueous solution which gave very satisfactory results.

SMITH AND RADFORD[8] suggested a column packing suitable for separation of aliphatic diamines; however, no quantitative results were reported and the method was not applied to aqueous solutions of amines.

In the present work, this column[8] was used for the quantitative separation of a variety of amines in aqueous solution.

Experimental

Equipment. An Aerograph Model A-350 Gas Chromatograph with a thermal conductivity detector was used in the present work. A Honeywell 1 mV revorder was used with the chromatograph and helium was the carrier gas. The column was a copper spiral 2½ m long, 5 mm I.D., packed with 20 % carbowax 20 M coated with 5 % KOH on Chromosorb W, 30–60 mesh (Johns Manville).

Procedure. Aqueous solutions of allyl-, propyl-, butyl-, di-*n*-butyl- and benzylamines were prepared. Five microliters of the solution were injected into the column, which was kept at 70°.

After the elution of the three lower amines, allyl-, propyl-, and butylamine (Fig. 1), programming was started and the column temperature was raised to 180° at a rate of 10 degrees per min. The other peaks were eluted in the following order: di-*n*-butylamine, water, benzylamine (Fig. 1).

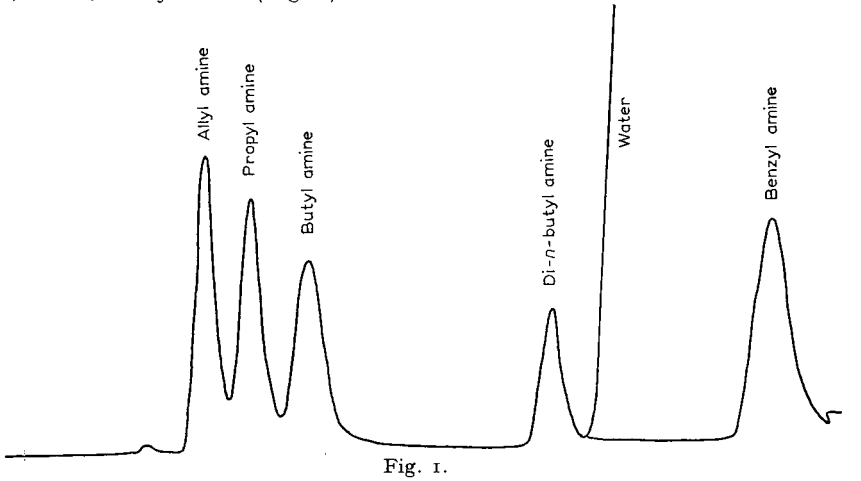

Fig. 1.

TABLE I

Run No.*	*Allylamine*			*Propylamine*			*Butylamine*			*Di-n-butylamine*			*Benzylamine*		
	Weight %	*Area* %	*Deviation* %	*Weight* %	*Area* %	*Deviation* %	*Weight* %	*Area* %	*Deviation* %	*Weight* %	*Area* %	*Deviation* %	*Weight* %	*Area* %	*Deviation* %
1	20.0	20.2	+1.0	20.0	19.0	—5.0	20.0	19.9	—0.5	20.0	20.4	+2.0	20.0	20.4	+2.0
2	20.0	19.6	—2.0	20.0	20.2	+1.0	20.0	20.7	+3.5	10.0	11.2	+12.0	30.0	28.2	—6.0
3	22.4	24.0	+7.1	22.4	22.0	—1.8	22.4	23.8	+6.2	5.5	5.4	—2.0	27.3	24.8	—9.1
4	15.0	15.3	+2.0	25.0	25.4	+1.6	30.0	29.7	—1.0	10.0	10.3	+3.0	20.0	19.3	—3.5
5	15.0	15.2	+1.3	15.0	14.0	—6.6	35.0	35.4	+1.1	10.0	19.9	—1.0	25.0	23.6	—5.6
6	22.2	22.7	+2.2	22.2	22.7	+2.2	22.2	22.4	+0.9	11.1	10.8	—2.7	22.2	21.6	—2.7
7	33.3	35.0	+5.1	11.1	10.8	—2.7	22.2	21.2	—4.5	11.1	11.6	+4.5	22.2	21.6	—2.7
8	10.0	10.5	+5.0	15.0	15.0	0.0	35.0	33.2	—5.1	15.0	13.3	—11.0	25.0	25.8	+3.2
9	22.2	21.7	—2.3	22.2	21.3	—4.0	33.3	33.4	+0.3	11.1	12.3	+10.8	11.1	11.3	+1.8
	Average dev.: ±2.15			Average dev.: ±1.7			Average dev.: ±0.1			Average dev.: ±1.7			Average dev.: ±2.5		

* Run No. 1: mixture of dry amines; runs 2-5: 20% amines and 80% water; runs 6–9: 90% amines and 10% water.

Results and Discussion

No attempts were made to determine quantitatively the water concentration under the described conditions. The calculation of the individual amine concentrations was made assuming the total area under the amine peaks as 100 %.

The results are presented in Table I.

It will be seen that the average error does not exceed ± 2.5 % although in some cases maximum errors of about 10 % were recorded.

Application

This method was applied to the analysis of propylamine in a complex mixture containing in addition to acrylonitrile, propionitrile, adiponitrile and potassium chloride, in acid solution. Butylamine was added as a marker.

On neutralization with NaOH, two phases appeared. The mixture was vigorously stirred with a magnetic stirrer and samples were withdrawn for chromatographic analysis during the stirring.

The determination of propylamine by the present method gave reproducible results, within the limits of ± 2.5 %.

The nitriles were determined on a separate column, as described earlier[1].

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Color detection of bile acids using thin-layer chromatography

There is common agreement that the usefulness of R_F values in thin-layer chromatography (TLC) is limited. To solve this difficulty relative mobility values are often suggested. However, use of these values, as well as of R_F, is at times unsatisfactory because of the problems of concave solvent fronts and/or incomplete separation of some acids having similar mobilities[1,2].

Recently KRITCHEVSKY, MARTAK AND ROTHBLAT[3] have demonstrated the usefulness of color detection in bile acid identification.

The present study expands on the use of color detection with particular stress

on advantages of specific detecting reagents in resolving the two problems stated above.

Materials

Solvent systems for TLC. Preliminary investigations, using several solvent systems, disclosed that two systems consistently gave satisfactory results in TLC of bile acids:

Solvent I, used for free acids: ethyl acetate (freshly distilled)–glacial acetic acid (96:4, v/v).

Solvent II, used for conjugated acids: amyl acetate–glacial acetic acid–*n*-propanol–water (40:30:20:10, v/v).

Detecting reagents for bile acids. The following detecting reagents should be freshly prepared in an ice bath:

A = 15 ml concentrated sulfuric acid in 85 ml of anhydrous *n*-butanol.

B = 20 g antimony trichloride (anhydrous) dissolved in 50 ml anhydrous *n*-butanol and mixed with 10 ml concentrated sulfuric acid and 20 ml glacial acetic acid.

C = 5 ml concentrated sulfuric acid in 95 ml of acetic anhydride[4].

D = 2 g ferric chloride dissolved in 83 ml anhydrous *n*-butanol and mixed with 15 ml concentrated sulfuric acid.

Methods

A suspension of Silica Gel G* was prepared by adding 30 g of dry gel to 90 cc of distilled water. Acidification of the gel to pH 3.0 with acetic acid improved the sharpness of bile acid spots, especially those of hyodeoxycholic acid (HDCA) and deoxycholic acid (DCA). Glass plates, 200 × 200 mm and/or 25 × 75 mm, were prepared by soaking in a chromic–sulfuric acid mixture. The acidic gel suspension was applied to the plates at a thickness of 75–100 μ, according to the method of STAHL[5]. They were next air dried at room temperature for about 5 min and then activated at 100° for 2 h. When not in use, the plates were stored in a desiccator over silica gel.

The bile acids were made up in concentrations of 20 μg/μl in *n*-butanol. Five to twenty micrograms of each acid was sufficient for color detection. In order to prevent a concave solvent front—a condition particularly common with some of the highly mobile solvent systems—linear grooves, on each side of the bile acid origin, were made with a small hypodermic needle.

Rectangular chromatographic chambers, 29 × 28 × 10 cm, were lined with Whatman No. 3 filter paper and saturated with appropriate solvents. The chromatoplates were allowed to develop in the chambers at room temperature from 1 to 3 h, depending on whether free acids were being separated with Solvent I or conjugated acids with Solvent II. In either case, the chromatoplates were removed from the chambers just before the solvent front reached the top of the plate, were air dried for several minutes, and then thoroughly dried at 110° for a minimum of 3 h. After cooling, the plates were sprayed with freshly prepared detecting reagents, air dried for 15 min and placed in an air-circulating oven at 110°. Since color development is dependent upon careful control of heat exposure, the exposure time should not exceed 25–30 min for conjugated acids, or 45–50 min for free acids. During the development

* Available from E. Merck, A. G., Darmstadt, Germany.

of the spots, the background remains white. It is advisable to record the pertinent color information as soon as the plates have cooled, preferably within an hour or two, although some of the free bile acids, particularly deoxycholic and 3α,12α-dihydroxy-7-ketocholanic acids, retain their initial color for several days after spraying if the chromatoplates are covered with clear glass plates.

Discussion

Color reactions of the free and conjugated bile acids are shown in Table I. In those instances where two colors are noted the first color predominates. The use of color charts for standardization would seem in order, but it is sugggested that each investigator, when feasible, prepare his own color standards. It is to be noted that the bile acids having a hydroxyl at 12 carbon position gave a yellow color, except when detecting reagent D (containing ferric chloride) was used. The conjugated acids gave colors similar to their free-acid counterparts. Current studies in this laboratory, using biological materials, indicate that color detection reagents facilitate bile acid identification.

TABLE I

COLOR REACTIONS OF FREE AND CONJUGATED BILE ACIDS

*Free bile acids, solvent system I**	*Detecting reagents*			
	A	*B*	*C*	*D*
Cholic	Y	Y-Gr	Y	Gr-Bk
Hyocholic	Gy	Br	Br	Gr
Hyodeoxycholic	Bk	Bk	Gy-Gr	Bk-Bl
Chenodeoxycholic	Gy-Gr	Gr-Y	Gy-Gr	P-Bk
Ursodeoxycholic	Gy-Gr	Gr-Y	Gy-Gr	P-Bk
Deoxycholic	Y	Y	Y-Br	Br
Apocholic	Y	Y-Gr	Y-Br	P-Bk
Lithocholic	P	Pk-P	P	P-Bk
3α,7α-Dihydroxy-12-ketocholanic	Y	Pk	Y	Y-Gr
3α,12α-Dihydroxy-7α-ketocholanic	Ys	Ys	Ys	Ys
Dehydrocholic	Pk	Pk	Pk	O-Br
Dehydrodeoxycholic	Pk	O	Pk	O-Br
*Conjugated bile acids, solvent system II**	*Detecting reagents*			
	A	*B*	*C*	*D*
Taurocholic	Y-Gr	Y	Y	Gr-Bk
Taurohyodeoxycholic	Bk	Br	Br	Bk-Bl
Taurodeoxycholic	Y-Gr	Ys	Ys	Br
Glycocholic	Y	Y	Y	Gr-Bk
Glycohyodeoxycholic	Bk-Bl	Br	Br	Bk-Bl
Glycodeoxycholic	Y-Gr	Ys	Ys	Br
*Sterol, solvent system I**	*Detecting reagents*			
	A	*B*	*C*	*D*
Cholesterol	P	P	P	P-Bk

Color code: Bl = blue; Bk = black; Br = brown; Gr = Green; Gy = Grey; O = Orange; P = Purple; Pk = Pink; Y = Yellow; Ys = Sun Yellow.

* For composition of solvent systems and detecting reagents, see text: "Materials".

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Separation of equol from oestrogens by thin-layer chromatography

Equol may be present in hen's urine in relatively large amounts[1,2]. It can be separated from steroid oestrogens by thin-layer chromatography (TLC). The procedure involves (i) separation by TLC of the phenols into (a) oestrone, (b) oestradiol-17β plus equol and (c) oestriol; (ii) methylation of (b); and (iii) subsequent TLC of the methyl ethers of (b), which affords excellent separation of these two compounds.

A mixture of oestrone, oestradiol-17β, oestriol and equol was chromatographed on silica gel G (Merck) in benzene–methanol (85:15)[3]. The two terminal strips of the chromatoplates were sprayed with 1 % (w/v) *p*-nitrobenzenediazonium fluoborate in acetic acid–water (1:1 v/v) while the middle section was protected by a plastic plate. The respective R_F values were 0.63, 0.41, 0.24 and 0.41. The blank area corresponding to the oestradiol-17β plus equol spot was removed, eluted with ethanol and methylated[4]. The methyl ethers were chromatographed in benzene–methanol (95:5) and the spots detected by spraying with 2 % (v/v) sulphuric acid in aqueous ethanol[3]. Methylation renders equol less "polar" than oestradiol in consequence of the formation of the dimethyl ether[5], which moves far ahead of the 3-methyl ether of oestradiol-17β in this solvent system (R_F:0.78 and 0.37 respectively).

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Separation of some aromatic amines by thin-layer chromatography

This work deals with the application of thin-layer chromatography to the separation of some isomeric ring-substituted anilines of the general formula:

where R is: $-CH_3$; $-OH$; $-OCH_3$; $-COOH$; $-NO_2$; $-NH_2$; $-Br$; $-Cl$.

Some of these compounds have already been reported in the literature[1-3], but a more systematic investigation was necessary for our purposes.

Experimental

The chromatoplates were prepared according to the method described by STAHL[4]. Silicagel G* was applied on 20 × 20 cm glass plates** to a thickness of 250 μ and activated at 105° for 1 h. 2 μl of a 5 % ethyl ether solution of each component were applied along a line 2.5 cm from the lower end of the plate and developed by the ascending method.

Five different solvent systems were chosen as mobile phase. They are reported in Table I with the corresponding R_F values. The aromatic amines were revealed by the following techniques:

(1) Direct observation (for coloured products).

(2) Irradiation with U.V. light (365 mμ).

TABLE I

R_F VALUES OF SOME AROMATIC AMINES OBTAINED BY MEANS OF THIN-LAYER CHROMATOGRAPHY ON SILICAGEL G

Solvent system	*ml*				
Dibutyl ether	100	150	50	200	—
Ethyl acetate	100	50	150	—	—
Acetic acid	10	10	10	10	20
n-Hexane	—	—	—	40	—
n-Butanol	—	—	—	—	80
Water	—	—	—	—	100
Aromatic amines	*R_F values*				
o-Toluidine	0.62	0.42	0.64	0.17	0.84
m-Toluidine	0.54	0.29	0.63	0.10	0.83
p-Toluidine	0.40	0.20	0.59	0.05	0.80
o-Aminophenol	0.34	0.24	0.58	0.00	0.80
m-Aminophenol	0.29	0.13	0.53	0.00	0.75
p-Aminophenol	0.06	0.01	0.12	0.00	0.62

(continued on p. 572)

* E. Merck, A. G. Darmstadt.

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TABLE I (*continued*)

Aromatic amines	R_F *values*				
o-Aminobenzoic acid	0.62	0.47	0.74	0.44	0.98
m-Aminobenzoic acid	0.50	0.28	0.61	0.12	0.95
p-Aminobenzoic acid	0.59	0.37	0.68	0.29	0.97
o-Anisidine	0.60	0.42	0.70	0.15	0.81
m-Anisidine	0.51	0.30	0.62	0.09	0.80
p-Anisidine	0.11	0.02	0.17	0.02	0.58
o-Nitroaniline	0.69	0.55	0.77	0.52	0.93
m-Nitroaniline	0.64	0.44	0.71	0.36	0.92
p-Nitroaniline	0.58	0.37	0.67	0.29	0.91
o-Phenylenediamine	0.00	0.00	0.00	0.00	0.63
m-Phenylenediamine	0.00	0.00	0.00	0.00	0.53
p-Phenylenediamine	0.00	0.00	0.00	0.00	0.40
o-Bromoaniline	0.81	0.78	0.85	0.69	0.95
m-Bromoaniline	0.70	0.58	0.75	0.44	0.93
p-Bromoaniline	0.61	0.47	0.67	0.27	0.89
o-Chloroaniline	0.78	0.75	0.82	0.66	0.96
m-Chloroaniline	0.68	0.51	0.75	0.40	0.94
p-Chloroaniline	0.60	0.41	0.64	0.22	0.89

(3) Spraying with 5 % $NaNO_2$ in 0.2 *N* HCl solution. After drying at 50° the plate was sprayed with 5 % α-naphthol in methyl alcohol. The amines appear as coloured spots.

(4) Spraying with a 1:1 (v/v) aqueous mixtures of 0.1 *M* $FeCl_3$ and 0.1 *M* $K_3[Fe(CN)_6]$. The amines appear as blue spots.

Conclusions

By determining the R_F values in the solvent systems mentioned in Table I, the components of an unknown mixture of aromatic amines can in most cases be identified.

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Dünnschichtchromatographie von Zuckerphenylosazonen

Reduzierende Zucker werden zur Identifizierung oder zur Abscheidung aus Gemischen häufig in Osazone übergeführt. Die I.R.-Spektren der Phenylosazone sind so deutlich verschieden, dass eine einwandfreie Zuordnung möglich ist.[1]

FISCHER JØRGENSEN[2] konnte die Phenylosazone von Monosacchariden durch Säulenchromatographie an Calciumcarbonat trennen. Auch die papierchromatographische Trennung ist beschrieben worden[3].

Wir haben nun die Dünnschichtchromatographie von Osazonen an Polyamid* untersucht. Die Platten wurden mit einem üblichen Streichgerät** in bekannter Weise[4,5] beschichtet. Die Chromatographie erfolgte aufsteigend im System Dimethylformamid–Benzol (7:93), in dem die Phenylosazone der Triosen, Tetrosen, Pentosen, Methylpentosen und Hexosen wandern, während diejenigen der Oligosaccharide am Start bleiben (Fig. 1). Um gute Trennungen zu erhalten, ist es unbedingt erforderlich,

Fig. 1. Dünnschichtchromatogramm an Polyamid im System Dimethylformamid–Benzol (3:97) von Phenylosazonen von (1) Glycolaldehyd, (2) Glycerinaldehyd, (3) D-Erythrose, (4) D-Xylose, (5) D-Glucose, (6) D-Lactose. Es wurden 2 cm vom unteren Rand der Platte 1 %ige Lösungen in Pyridin in Abständen von jeweils 1 cm aufgesetzt. Laufstrecke: 14 cm.

mit Kammerübersättigung zu arbeiten. Aber auch dann schwanken die R_F-Werte mehr als gewöhnlich, wenn man die gebräuchlichen Chromatographierkästen** ver-

* Polyamid Woelm zur Dünnschichtchromatographie von Firma M. Woelm, Eschwege.
** Firma Desaga, Heidelberg.

wendet, da sich beim Öffnen der Kästen die Zusammensetzung des Laufmittels ändert. Es ist deshalb ratsam, stets frisches Lösungsmittelgemisch zu verwenden und authentische Osazone mitlaufen zu lassen. Die gelben Flecke auf den entwickelten Chromatogrammen werden beim Besprühen mit einer frisch bereiteten Lösung von diazotierter Sulfanilsäure in $2N$ Na_2CO_3 braun bis rotbraun.

Die beiden Pentosazone laufen fast gleich schnell. Ebenso haben die vier Hexosazone fast gleiche R_F-Werte. Auch die Phenylosazone von Rhamnose und Fucose sind im angegebenen Laufmittel nicht zu trennen. Eine geringe Auftrennung ist aber in Äthanol–Chloroform (3:97) möglich, in welchem Sorbose-Phenylosazon etwas schneller läuft als die anderen Hexosazone. Auch die Pentosazone sind in diesem Gemisch verschieden schnell. Da zudem die Phenylosazone von Arabinose, Altrose und Galaktose beim Besprühen mit diazotierter Sulfanilsäure rotbraune, diejenigen von Xylose, Glucose und Sorbose braune Färbungen ergeben, dürfte eine Identifizierung in vielen Fällen möglich sein.

Die Trennung der Phenylosazone von Oligosacchariden von denen der Monosaccharide ist auch möglich in Pyridin–Wasser (15:85), in dem die letzteren am Start bleiben.

Wir danken Fräulein Dr. A. GAUHE und Herrn Dr. W. OTTING für die Überlassung von Osazon-Präparaten.

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Chemistry To-day, a Guide for Teachers, published by the Organisation for economic co-operation and development, Paris, 1963, 365 pp., price $ 3.50.

Selected topics for a modern approach to the teaching of school chemistry.

Analysis Instrumentation 1963, edited by L. FOWLER, R. D. EANES AND T. J. KEHOE, Plenum Press, New York, 1963, 261 pp., price $ 12.50.

Proceedings of the Ninth National Analysis Instrumentation Symposium, held April 29–May 1, 1963, at Houston, Texas.

Separation Methods in Inorganic Chemistry, Vol. II (Postgraduate Summer School, Rome, 17–27 Sept., 1962, published by the Consiglio Nazionale delle Ricerche, Rome, 1963, 145 pp., price $ 8.00.

This volume contains the following lectures: Estrazione in controcorrente (in Italian), by G. C. CASINOVI; Techniques and principles of partition chromatography of metal ions and anions (in English), by F. H. POLLARD; Rassegna dei metodi fondamentali di elettroforesi su carta a bassa tensione e loro applicazione alla separazione di sostanze inorganiche (in Italian), by G. DE ANGELIS; Radiocromatografia quantitativa su carta (in Italian), by F. POCCHIARI AND C. ROSSI; and seminars by G. CETINI, R. BARBIERI and A. BRECCIA.

CEC, Orgão Oficial do Centro de Estudos Cientificos, a new journal published by the Centro de Estudos Cientificos, P.O. Box 11585, São Paulo, Brasil.

The first issue appeared in September 1963 and contains 15 pages which feature short review articles on chemical topics.

Cronache di Chimica, rassegna di Chimica Analitica e Applicata, Editor Dr. LUIGI ROSNATI, published by Carlo Erba S.p.A., Milano, Via Carlo Imbonati 24, and distributed free of charge on request.

This journal features longer review articles on chemical topics, some abstracts from the literature and book reviews. The first issue (June, 1963) and the second (October, 1963) contain 36 pp. each.

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Thin-layer Chromatography

3. TECHNIQUES I

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PFEFFERELE, W. C.: Trace gas analyzer. *U.S. Pat.* 3,087,112, April 23, 1963 — 1 p.p.m. sensitivity with tetrode ionization electron tube detector; purified helium or hydrogen as carrier gas.

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SMITH, E. D. AND JOHNSON, J. L.: Measurement and use of substrate and partition liquid selectivities in gas chromatography. *Anal. Chem.*, 35 (1963) 1204–1207 — relative volatility of 2- and 3-pentanone on 16 stationary phases at 50° is examined and given.

SUPINA, W. R.: Cyclohexanedimethanol polyesters as stationary phases for gas chromatographic analysis of ketosteroids. *Anal. Chem.*, 35 (1963) 1304–1304 — used up to 250°.

VILLALOBOS, R. AND TURNER, G. S.: The role of column backflushing in gas chromatography. II. Multiple column systems. *4th Intern. Symp. Gas Chromatography, East Lansing, Michigan State Univ., June 17–21, 1963* — multiple-column system is constructed so that the backflushed compounds from one column give one symmetrical peak in the middle of an analysis.

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ABEL, K. AND DABNEY, W. B.: An automatic, continuously variable attenuator for gas chromatography. *Anal. Chem.*, 35 (1963) 1335–1336 — a step-function and non-linear continuous attenuator is fully described; linearity up to 40% of deflection; accuracy less than 0.85 up to 40% and less than 3.2 for 94% of deflection.

COLSON, E. R.: A partition sampler for vapor analysis by gas chromatography. *Anal. Chem.*, 35 (1963) 1111–1112 — a small GLC-column is used for collecting a mixture and sampling is made by short heating in the stream of carrier gas.

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JENCKEL, L.: Combined mass spectrometer and gas chromatograph recording apparatus. *U.S. Pat.*, 3,084,012, April 2, 1963 — two signal records related to mass (MS) and partial pressure (GC) are used for the identification.

MARTIN, J. L.: Elimination of the water effect on argon ionization detectors fitted to Pye chromatography. *Analyst*, 88 (1963) 326–327 — an adaptor for venting of water vapour outside the argon detector during water fraction elution.

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SELF, R., LAND, D. G. AND CASEY, J. C.: Gas chromatography using capillary column units for flavour investigation. *J. Sci. Food Agr.*, 14 (1963) 209–220 — nylon capillaries with silicone oil, dinonyl phthalate and tritolyl phosphate; construction details.

WALKER, T. R. AND KARMEN, A.: An electrical integrator for gas chromatography. *J. Gas Chromatog.*, 1, No. 7 (1963) 24–26 — two records, a differential and an integral one, are made for distinguishing base-line drift and peak overlapping.

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TAKEDA, I.: Some factors affecting the efficiency of preparative scale gas chromatography. *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 442–446 (Engl. summary, A 33).

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BRADLEY, B. J. AND TILEY, P. F.: Differential vapour absorption and moving bed chromatography. *Chem. Ind. (London)*, (1963) 743–744 — separation of azeotropic mixtures on a moving bed with a flow of non-volatile solvent.

HOFF, J. E. AND FEIT, E. D.: Functional group analysis in gas chromatography. Treatment of vapors with selective reagents. *Anal. Chem.*, 35 (1963) 1298–1299 — step-wise treatment and chromatogram development (*e.g.* water, NaOH, $NaBH_4$, $KMnO_4$, ozone, used).

LEVY, E. J., MILLER, E. D. AND BEGGS, W. S.: Application of time of flight mass spectrometry and gas chromatography to reaction studies. *Anal. Chem.*, 35 (1963) 946–949 — small-scale reactor coupled with MS and GC.

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PFEIFFER, G.: (Kinetic studies on the hydrogenation of *cis*-butene in a microreactor). *Magy. Kem. Folyoirat*, 69 (1963) 203–206.

VILLALOBOS, R. AND TURNER, G. S.: Group analysis in process chromatography. *ISA J.*, 10, No. 5 (1963) 67–70 — regrouping of different compounds in group-type single peaks is made by means of backflushing programme on multiple parallel columns.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

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SMITH, B. AND ERWIK, M.: Determination of hydrocarbon constituents in the benzene pre-run. *Acta Chem. Scand.*, 17 (1963) 283–295 — benzene and 25 saturated and 35 unsaturated hydrocarbons on 5 stationary phases were identified.

SOJÁK, L., MASARYK, Š., GÁLFY, K. AND MOZOLA, A.: (Separation of products from cracking of higher linear *n*-alkanes by means of temperature-programmed gas chromatography). *Ropa Uhlie*, 5 (13) (1963) 195–201 — C_5–C_{23} hydrocarbons on PEGA at 75–225°, or on Apiezon L at 100–300°.

STAIRS, R. A., DIAPER, D. G. M. AND GATZKE, A. L.: Reaction of chromyl chloride with some olefins. I. The products from cyclohexene, cyclopentene, 1-hexene, and 2-methyl-1-pentene. *Can. J. Chem.*, 41 (1963) 1059–1064 — $AgNO_3$ in ethylene glycol for purity of olefins, Apiezon T at 115° or neopentyl glycol sebacate at 125° for products.

5c. Halogen derivatives of hydrocarbons

BUCK, K. W. AND FOSTER, A. B.: Reactions of some alkyl chloroformates. *J. Chem. Soc.*, (1963) 2217–2221 — chloro- and dichlorocyclohexanes and -hexenes on silicone E-301 at 180°.

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